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**Mediating role of corticotropin-releasing hormone (CRH) in anxiety and ischemia:
behavioural & physiological correlates**

Samir Khan

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

**School of Psychology
Faculty of Social Sciences
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Dedicated to my parents, Laila and Rafiq Khan

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Abstract

Corticotropin-releasing hormone (CRH) has been considered the quintessential 'stress' neuropeptide, as it mediates stress-induced activation of the hypothalamic-pituitary-adrenal (HPA) axis and produces behavioural and autonomic responses analogous to natural stressors. However, the role of CRH in mediating the behavioural response to psychological stressors, also termed anxiety, is unclear due to inconsistent anxiolytic effects for CRH antagonists in processive stress models, and the central activation of CRH by non-anxiogenic stimuli. The first half of the current thesis examined the role of CRH, and amygdala CRH in particular, in the behavioural response to anxiety. The first experiment standardized the use of a neophobia-based feeding model for use as a behavioural index of anxiety. Using this model, the second experiment demonstrated that while exposure to an unfamiliar environment activates stress-relevant neural circuitry, activation of CRH receptors is not necessary for the expression of anxiety-like behavioral responses. In addition, amygdala CRH activation by novel stimuli is unaffected by changes in the organism's anxiety state. The second half of the current thesis focused on the role of CRH in neurotoxicity and behavioural deficits produced by global ischemic insults. It was demonstrated that global ischemic insults of 10-15 min duration produced short-term CRH increases in hypothalamic and amygdalar regions as well as the piriform cortex, perhaps mediating stress, inflammatory and/or epileptiform activity. This was followed by widespread CRH depletions throughout the brain at 24 h post-ischemia. However, by 72 h post-ischemia, just prior to the onset of cell death, CRH concentrations had normalized throughout the brain. Furthermore, there were no short or long term CRH changes at the CA1 hippocampal

region, the area most vulnerable to cell death. Both selective and non-selective CRH antagonists failed to confer neuroprotection when administered before or shortly after an ischemic insult, and failed to protect against ischemia-induced spatial memory deficits. However, CRH1 antagonists did significantly attenuate ischemia-induced hyperactivity in the open field and in the elevated plus maze. Thus while ischemia-induced CRH activation does not appear to mediate subsequent neuronal degeneration, it may play a role in anxiety-related behavioural changes.

Summary

Corticotropin-releasing hormone (CRH) is a 41 amino acid polypeptide, first characterized in 1981 and widely distributed within the central and peripheral nervous system. Since its characterization, CRH (primarily of hypothalamic origin) has gained the reputation of being the quintessential neuropeptide involved in the mediation and/or modulation of the stress response. This reputation stems from its mediating role in stress-induced activation of the hypothalamic-pituitary-adrenal (HPA) axis, the principal neuroendocrine response to most physical and psychological stressors, and its ability to produce behavioural, autonomic and endocrine responses analogous to natural stressors when administered centrally.

In the last decade, CRH has also been implicated in the behavioural response to anxiety. Anxiety refers to the state produced by a psychological or 'processive' stressor, which does not directly threaten the organism's physical integrity but is evaluated as stressful in comparison to previous experiences. Evidence has suggested that central CRH activation, particularly within the amygdala (a limbic region involved in emotional processing of sensory stimuli), mediates the behavioural response to processive stressors. However, 'processive stress' remains poorly defined, and in a number of models believed to assess this stressor-type, a high degree of inconsistency has been observed in the effectiveness of CRH antagonists in modulating behavioural responses. Furthermore, although amygdaloid CRH has been strongly associated with anxiety, recent evidence suggests a more comprehensive set of functions, as non-anxiogenic and even rewarding stimuli can activate this system.

Thus, the first section of the present thesis focused on elucidating the role of CRH, and amygdala CRH in particular, in the behavioural response to anxiety.

1. Experiment 1 attempted to refine existing testing procedures through development of a neophobia-based feeding paradigm with features highly characteristic of a processive stress model, possessing strong ethological validity. In this paradigm, it was shown that rats placed in an unfamiliar environment (a novel cage) were reluctant to consume a palatable snack, as consumption declined by over 50% as compared to intake in a familiar (home cage) environment. This suppression was dose-dependently reversed by the prototypical anxiolytic drug diazepam (a benzodiazepine). At anxiolytic doses, diazepam failed to alter consumption of the palatable snack in the non-stressful environment of the home cage, although it increased consumption of other diets, namely powdered sugar. This suggested that snack consumption in an unfamiliar environment is a sensitive and reliable index of anxiety.

2. Based on these findings, Experiment 2 utilized this testing paradigm to investigate the mediating role of CRH in the behavioural response to anxious stimuli. It was found that under such circumstances, this type of stressor stimulated the release of immunoreactive-CRH (ir-CRH) at the central nucleus of the amygdala (CeA). Of interest, however, both selective and non-selective CRH antagonists failed to alter the behavioral effects elicited by the stressor. As well, despite the potent anxiolytic effect of diazepam in this paradigm, this compound failed to prevent the stressor-associated release of ir-CRH at the CeA.

Thus, although exposure to an unfamiliar environment may be interpreted as being “stressful” by neural circuits involving CRH receptors at the CeA, the data for the current thesis do not support the view that activation of CRH receptors is necessary for the expression of anxiety-like behavioural responses. Rather than provoking anxiety, it is suggested that these systems might serve to draw attention to events or cues of biological significance, including those posing a potential threat to survival.

In addition to a role in anxiety, CRH has also recently been implicated in neurotoxicity produced by global ischemia. Models of global ischemia attempt to reproduce physiological conditions analogous to cardiac arrest or stroke in humans that produce a temporary reduction in cerebral blood flow. Forebrain ischemia produces selective neurodegeneration, particularly effecting areas of the hippocampus such as the CA1, as well as a number of behavioural deficits. A direct neurotoxic role for CRH has been inferred from studies showing effective neuroprotection by CRH antagonists in rodent models of global and focal ischemia.

However, a number of questions remained unaddressed by existing studies. For instance, findings on the endogenous expression of CRH in response to ischemia remains fragmented and mainly focussed on hippocampal expression. Moreover, the CRH antagonists thus far demonstrating neuroprotection involve non-selective blockade of CRH receptors, leaving unclear the relative contribution of the different CRH receptor subtypes in these effects. The ability of CRH antagonists to attenuate ischemia-induced behavioural impairments concomitant to inhibition of neuronal damage also remains unexamined. At present, a restricted number of validated behavioural tasks are available for assessing neuroprotective compounds, and the precise nature and the physiological

(anatomical and/or biochemical) basis of certain established deficits remain poorly understood.

Thus, the second section of the current thesis investigated the physiological response of CRH neurons in various brain regions to global ischemic insults, and its mediating role in subsequent neurotoxicity and behavioural impairments. An initial step of this research sought to expand and further characterize the available testing paradigms to try to better capture ischemia-induced functional deficits and/or behavioural changes.

3. Experiment 3 established that, although robust, ischemia-induced hyperactivity in a novel open field may represent a transient phenomenon that stabilizes within a relatively short period of time. Increased locomotor activity in a novel environment in ischemic animals is also accompanied by anxiolytic behaviours in the elevated plus maze, suggesting that this behaviour may partly be attributable to altered anxiety levels following ischemia. Our findings also suggest that long-term habituation deficits in ischemic animals, eluded by prior research, are absent in ischemic animals exposed to highly motivating stimuli such as palatable food, suggesting that this impairment might be context-dependent.

4. Experiment 4 characterized region and time-dependent changes in endogenous CRH peptide following global ischemia. CRH concentrations were assessed in 10 brain regions at 3 post-ischemic intervals: 4, 24 and 72 h. In vivo microdialysis was also used to assess dynamic fluctuations in CRH release at the CA1 region of the hippocampus and at the amygdalar CeA. Significant time-dependent post-mortem alterations in CRH

expression following global ischemia were present in extra-hippocampal structures such as the piriform cortex and amygdala as well as hypothalamic nuclei, frontal cortex and dentate gyrus. In vivo, a significant increase in CRH release was found at the CeA, following global ischemia, although no changes in release were observed at the CA1.

5. Experiment 5 evaluated the effectiveness of selective and non-selective CRH antagonists in reversing global ischemia-induced neurodegeneration and behavioural deficits. In these studies, a highly selective CRH1 antagonist failed to reverse ischemia induced cell death at the CA1 and spatial memory impairments as assessed in the radial arm maze. A non-selective CRH antagonist showed comparable effects against ischemic damage. Interestingly, CRH1 antagonism did attenuate ischemia-induced hyperactivity and increased open-arm activity in the elevated plus maze, suggesting that these behavioural impairments can be attenuated without concomitant neuroprotection.

Together, these findings assessing the contribution of CRH in global ischemia do not support a neurotoxic role for this peptide. Our findings however demonstrate that this peptide is widely activated across different brain nuclei and regions in response to ischemia, suggesting its participation in the initiated stress response as well as potential indirect contribution to endogenous processes associated with ischemia-induced neuronal degeneration. In addition, findings from behavioural assessment suggest that ischemia-induced hyperactivity may be attributable to changes in anxiety levels, rather than memory and/or habituation deficits attributable to CA1 neuronal damage.

General Introduction

The investigation into corticotropin-releasing hormone (CRH) began over 50 years ago, as scientists attempted to characterize a mystery substance in the hypothalamus that presumably stimulated the pituitary gland. The primary function of the pituitary is to influence hormone release from other endocrine glands, such as the adrenals and gonads, and research into CRH represented one of the first attempts to determine the brain's role in regulating this system [94]. Originally, the substance was referred to as corticotropin-releasing factor (CRF) because some of its physiological effects were known, including its ability to stimulate corticotropin from the anterior pituitary, but its molecular structure was unclear [173,464]. In 1981, exhaustive attempts to isolate and purify this factor from ovine hypothalamus culminated with the identification of a 41 amino acid peptide, which classified the factor as a hormone [541].

Subsequent research has identified CRH cell bodies and receptors throughout the brain and body. In addition to stimulating the pituitary, CRH has been implicated in a wide array of behavioural and physiological phenomena. In recent years, it has also become one of the first neuropeptides targeted as a potential treatment for psychological and neurological disorders. However, at this point much still remains unknown regarding the functions and mediating role of CRH.

The overall objective of the current thesis is to further characterize two of the most promising CRH research areas. Specifically, the focus of investigation will be on the mediating role of CRH on the behavioural and physiological correlates of anxiety-related states and global ischemia. Anxiety is the term generally used to designate the emotional state produced by a psychological stressor. In contrast to physiological stressors,

psychological stressors do not directly threaten the organism's homeostasis or survival. Rather, they are evaluated as stressful in comparison to previous experience. CRH activation following both physical (eg. ether, hypoxia) and psychological stressors (e.g. loud noises, elevated surfaces) has been well established. However, as will be elaborated upon later, the particular role of CRH activation in mediating the behavioural response to psychological stressors remains in debate. This is in part due to inconsistent effects of CRH antagonists in behavioural models of anxiety, questions regarding the validity of the behavioural models themselves, as well as the ability of non-anxious stimuli to activate CRH.

More recently, CRH has been implicated in neurotoxicity, caused by events such as kainic acid administration or traumatic brain injury, which produce excessive CRH activation. The excitatory effects of CRH have also been linked to ischemia-induced brain damage. In studying the role of CRH in neuronal death, its role in ischemia has perhaps received the most attention. Cerebral ischemia represents a condition characterized by a temporary restriction of blood flow to the brain, perturbing homeostatic, energetic and metabolic processes. This condition results from a stroke, cardiac arrest or other major disruption in blood circulation to the brain and ultimately leads to widespread neuronal damage and consequent behavioural impairments.

Although anxiety and ischemia represent different levels of physiological activation, both states are associated with CRH activation and appear to be mediated by its excitatory effects. As well, in both these research areas there is a growing interest towards developing pharmacological treatments involving CRH antagonism [320,478,516]. Given the widespread presence of CRH throughout the CNS and the

strong interest in pursuing CRH-modulating therapies, efforts to better understand the scope of its effects and its multiple roles in mediating behavioural and physiological events are warranted.

The following paragraphs will attempt to characterize the pharmacological, physiological and neuroanatomical characteristics of CRH and its role in mediating behaviour. It will then proceed to describe the study of anxiety and global ischemia and the relationship between CRH and these two phenomena. This will render possible the identification of specific areas and issues that require further investigation.

1. Corticotropin-releasing hormone (CRH)

1.1 Gene expression and synthesis

Soon after the isolation of the CRH peptide by Vale and colleagues in 1981, investigation into the nature of CRH synthesis and gene structure began. The direct precursor of CRH is a 196 amino-acid structure called pre-pro CRH, and CRH itself emerges following cleavage of the C- terminus (the last 41 amino acids in the amino acid chain) from the larger peptide [542]. Human and rat CRH are identical to one another but differ from ovine CRH by seven amino acids [108]. The gene coding for the CRH precursor in the human genome is located on the long arm of chromosome 8 [13] and at the analogous position in rodents on chromosome 3 [260,361]. The complete coding for the CRH precursor resides on exon 2 of the gene [590].

The 41 amino acid polypeptide belongs to a family of CRH-like peptides which include two non-mammalian peptides, sauvagine and urotensin I, each sharing a 50% sequence identity with human or rat CRH [108]. In vertebrates, an important additional

member of the CRH-peptide family is urocortin, which has a 43-45% homology with CRH and is highly conserved across species. CRH and urocortin share the same precursor gene [590] and, like CRH, urocortin has been isolated from human, rat, mouse and sheep [75,590].

CRH gene expression and subsequent secretion are regulated via multiple pathways in a site-specific manner. Transcription of the CRH gene is initiated at one major site (TSS + 1) and approximately 10 minor sites [2] and the binding sites are particularly sensitive to stimulation by protein kinase A (PKA) [3,142,520] and protein kinase C (PKC) [207,208]. Thus, not surprisingly, numerous substances capable of stimulating CRH gene and peptide expression operate through these second messengers. Several endogenous ligands are involved in regulation of CRH mRNA, including catecholamines, serotonin, acetylcholine, cytokines, glucocorticoids and GABA (see [420] for review). In particular, CRH gene expression is stimulated by PKC via activation at α_2 , 5-HT₂ and muscarinic receptors from norepinephrine, serotonin and acetylcholine respectively. Similarly, PKA stimulation by activation of noradrenergic β and α_2 receptors and serotonergic 5-HT_{1A} also stimulates CRH gene expression. It should be added, however, that while these pathways are among the most widely studied and characterized, it is almost certain that numerous other pathways and/or effector ligands remain to be identified.

1.2 CRH distribution

CRH mRNA and protein are abundantly distributed throughout the CNS, with major sites of expression in the paraventricular nucleus of the hypothalamus (PVN), cerebral

cortex, cerebellum and the amygdala-hippocampal complex (an important area for stress adaptation, learning and memory) [44]. Immunocytochemical studies have also identified CRH fibers at the hypothalamus as well as the bed nucleus of the stria terminalis (BNST) and in brain stem nuclei, particularly the locus ceruleus (LC) and nucleus of the solitary tract (NST) [472]. Outside the CNS, CRH is expressed in the adrenal gland, testis, placenta, gut, spleen, thymus and skin [542].

The paraventricular nucleus of the hypothalamus (PVN) contains most of the CRH cell bodies that stimulate anterior pituitary secretion of adrenocorticotropin hormone (ACTH). These neurons originate in the parvocellular portion of the PVN and send axon terminals to the capillaries of the median eminence. CRH is also present in a small group of PVN neurons that project to the lower brain stem and spinal cord. These latter neurons are involved in regulating autonomic nervous system functions [77,549].

CRH cell bodies are also present in the central nucleus of the amygdala (CeA) with projections to the parvocellular region of the PVN and the brain stem parabrachial nucleus. The former projection, as will be described in detail later, is involved in coordination of the behavioural and neuroendocrine responses to psychological stressors. The latter projection is believed to mediate autonomic nervous system effects. Also coordinating autonomic activity are CRH neurons originating in the bed nucleus of the stria terminalis (BNST), which send axon terminals to the parabrachial nuclei and dorsal vagal complex in the brain stem. CRH fibers also connect the CeA to the BNST [472].

In the brain stem, the LC - norepinephrine system, which is strongly implicated in arousal systems, also contains CRH interneurons. These neurons, either through their own release or through activation of glutamate and GABA systems, play an important

role in noradrenergic adaptation and sensitization to acute and chronic stressors [544,545]. CRH cell bodies in the caudal medial raphe nuclei have also been implicated in severe depression [17]. Elsewhere in the brainstem, CRH mRNA expression has been detected in Barrington's nucleus, a pontine cell group which regulates excretion [215]. CRH cell bodies in the pons and in midbrain regions also project to higher regions of the brain or towards the spinal chord [472].

Finally, CRH neurons are present in the cerebral cortex, although their behavioural and physiological functions in this region are less characterized. The highest density is observed in prefrontal, insular and cingulate areas, with CRH interneurons having a particularly dense concentration in the second and third layers of the cerebral cortex, projecting to layers I and IV [527].

Thus, although initially discovered in the hypothalamus and the most intense investigations into its functions have originated from this region, it is clear that localization of CRH neurons throughout the CNS and the periphery strategically position this peptide to play important mediating roles in regulatory, emotional and cognitive processes.

1.3 CRH Receptors

In the central nervous system and periphery, CRH has three distinct receptor targets, each with its own unique DNA sequences and encoded by three different genes. These are the CRH1 and CRH2 receptors and CRH-binding protein (CRH-BP). A CRH3 receptor has been found in catfish pituitary, but a mammalian equivalent has yet to be identified [11].

Both CRH1 and CRH2 belong to a class of receptors referred to as “gut-brain” neuropeptide receptors because of their dense concentration in both regions. These receptors share other important molecular characteristics, such as the fact that their transmembranes contain seven domains, and they all signal by coupling to specific G-proteins [541]. This family of receptors, often referred to as the class B subfamily of seven-transmembrane receptors, also include receptors for calcitonin, vasoactive intestinal peptide (VIP), parathyroid hormone, secretin, pituitary adenylate cyclase activating peptide, glucagon and growth-hormone-releasing factor [479].

Briefly, the mechanism by which CRH stimulates CRH1 and CRH2 receptors involves binding to the extracellular domain of the receptor, which transforms the membrane in a manner that increases its affinity for the Gs protein. Through increased G protein receptor kinase (GRK), a sequence or cascade is initiated which involves adenylyl cyclase, cyclic AMP and protein kinase A stimulation. This ultimately leads to protein phosphorylation and gene transcription [109,190]. Another second messenger pathway has recently been characterized involving coupling with Gq proteins and activation of protein kinase C [167,236] although this mechanism is less characterized.

1.4 CRH receptor distribution

The CRH1 receptor is a 415-420 amino acid polypeptide [406] that shares a 70% homology with the most common CRH2 receptor splice variant CRH2 α [309]. CRH1 receptor mRNA is widely expressed in mammalian brain and pituitary with the highest densities being found in the anterior pituitary, cerebral cortex, cerebellum, amygdala, hippocampus and olfactory bulbs [76,468,547]. Significant concentrations are also found

in the periphery at the testis, ovaries and adrenal gland [372,391]. The mRNA distribution of CRH1 receptors matches closely the distribution of CRH binding sites in that expression is highest in known high-binding areas such as the pituitary, cerebral cortex and cerebellum [44].

The CRH2 receptor is a 397-438 amino acid protein for which four known splice variants or subtypes (CRH2 α , 2 β , 2 δ , 2 α -tr) have been identified [309]. Despite this variety, the current evidence supports similar pharmacological actions for these splice variants [110,542]. In humans, CRH2 is widely distributed in the periphery. CRH2 receptor mRNA has been observed particularly in skeletal muscle, gastrointestinal tract, lungs and ovaries [308,375]. However, CRH2 α mRNA is expressed almost exclusively in the CNS, with high densities at the paraventricular nucleus of the hypothalamus, lateral septum, amygdala and hippocampus [308,468]. CRH2 β receptors are primarily localized in the heart, skeletal muscle, and in the choroid plexus of CNS [76] while CRH2 δ has only recently been identified in human brain and has not yet been identified in the rat. When identified in humans, CRH2 δ mRNA expression appeared localized at the amygdala and hippocampus [264]. Finally, the distribution of 2 α -tr, a shorter variant of CRH2 α , has not been fully characterized and its function remains largely unknown [348].

Thus, as with CRH itself, CRH receptors show a wide distribution in several critical central and peripheral areas suggesting that much of the endogenous effects of the peptide are mediated by its own receptors and not through secondary systems.

1.5 CRH-binding protein

In addition to binding to CRH receptors, CRH binds with a high affinity to CRH binding protein (CRH-BP). CRH-BP was discovered incidentally when it was observed that substantial increases in plasma CRH during the third trimester of pregnancy was not accompanied by a simultaneous increase in plasma ACTH levels. It turned out that the majority of plasma CRH was being bound, and rendered inactive by CRH-BP, thus neutralizing the ability of CRH to stimulate the pituitary [298,409].

CRH-BP is a secreted glycoprotein that contains 322 amino acids and is structurally different from CRH receptors [426]. Research has also established that human CRH binds to CRH-BP with an equal or greater affinity than the CRH1 receptor [523]. In humans, CRH-BP is found in plasma, amniotic fluid, placenta, the pituitary and in the brain. Upon binding with CRH, the complex appears to activate CRH receptors in a manner distinct from CRH agonists, although most of its physiological effects, at present, remain unknown [78]. Although it has not been found in rodent plasma, immunocytochemical and in situ hybridization studies have demonstrated that CRH-BP is expressed in many regions of the rat brain including the cerebral cortex, numerous hippocampal subregions (including the CA1, CA3 and the dentate gyrus) as well as the BNST and olfactory bulbs [427]. The predominant tissue expressing CRH-BP is the pituitary where it is believed to modulate CRH action in response to stress exposure or changes in endogenous glucocorticoid levels. CRH is co-expressed with CRH receptors and CRH-BP in a number of regions including, the pituitary, amygdala, and the preoptic nucleus [245,427]. In the preoptic nucleus, it is estimated that up to 60% of CRH is bound to CRH-BP [35], providing an indication of the binding potency of CRH-BP to

CRH. The functional importance of CRH-BP is related to its ability to reduce the bioavailability of CRH in any region in which the two are co-expressed. It thus represents a potential factor in modulating the endogenous effects of CRH.

1.6 CRH: physiological and behavioural effects

Given the widespread distribution of CRH and CRH receptors throughout the CNS, particularly in the brain stem, hypothalamus, amygdala and cerebral cortex, it is not surprising that CRH is implicated in numerous regulatory, behavioural and cognitive functions. Thus, among its physiological effects, CRH plays an important role in thermogenesis and energy balance. Central CRH administration stimulates sympathetic outflow, metabolic rate and brown adipose tissue thermogenesis in rodents [59-61,285]. This peptide also plays an important mediating role in the effects of endogenous pyrogens, such as interleukin-1beta (IL-1 β), which regulate the thermogenic responses to diet, injury and stress. For instance, IL-1 β –induced increases in body temperature, metabolic rate and white cell count activity are prevented by pretreatment with CRH antagonists or antibodies in rodents [460].

CRH also appears as a key player in several immune system functions. CRH indirectly suppresses the immune response through stimulation of glucocorticoid release. In turn, glucocorticoids suppress the pro-inflammatory cytokine IL-1 in the brain and periphery [165]. Centrally administered CRH also produces several anti-inflammatory effects on its own, including the suppression of natural killer (NK) cell activity [220]. Moreover, the anti-inflammatory effect of certain stressors, such as footshock, appear to suppress NK activity via hypothalamic CRH [219]. Interestingly, IL-1 has been shown to

stimulate CRH synthesis and release at the hypothalamus [378,379,495] and hypothalamic CRH mediates IL-1-induced stimulation of the HPA axis [129,450,469].

Among the well-established behavioural effects of centrally administered CRH are dose-dependent increases in locomotor activity [522]. Interestingly, although CRH administration increases locomotor activity, grooming and rearing behaviours in familiar environments [230], it can decrease these same behaviours in an unfamiliar, and presumably stressful, environment [130]. The increase in locomotor activity is reliably blocked by administration of CRH antagonists [56]. Interestingly, there are as yet no demonstrations of CRH antagonists reversing the inhibition of locomotion produced in unfamiliar environments. The effect on locomotor behaviour appears mediated at the central nervous system level, since peripherally administered CRH is ineffective in this regard [549]. Other behavioural effects of centrally administered CRH include an inhibition of feeding [195] and sexual behaviour [497] and an increase in defensive burying behaviour [123]. CRH also has effects on memory, which appear site-specific. Thus, CRH injection into the dorsal hippocampus appears to enhance associative memory processes in rodents, whereas when injected into the lateral septum, it impedes these same processes [437].

2. CRH, stress and anxiety

Among its numerous behavioural and physiological effects, CRH has been most closely associated with the stress response. CRH is the primary effector of the hypothalamic-pituitary-adrenal (HPA) axis. A wide range of physical and psychological stressors provoke HPA activation, through stimulation of hypothalamic CRH, as part of

the organism's adaptive or coping response [198]. In addition, central administration of CRH reliably produces behavioural, neuroendocrine and autonomic effects akin to natural stressors [58,59,139,452,453]. Notwithstanding the acknowledgement of the role of CRH as a critical mediator of certain components of the stress response, the full scope of its function remains unclear. Thus, the participation of CRH in the regulation of most stressor types and its involvement in all components of the stress response is uncertain. Indeed, although CRH-deficient mice demonstrate a full behavioural response to certain types of stressors, their neuroendocrine response remains blunted [360]. This suggests that under certain conditions, the behavioural component of the stress response is not directly mediated by CRH. This is further supported by recent findings showing the ineffectiveness of CRH antagonists in reversing the behavioural response to psychological or 'processive'-type stressors [170,380,510].

Stressor types in general have been classified as being either systemic (physical) or processive [198]. Systemic stressors present immediate challenges to the physiological makeup or homeostasis of an organism. Examples include ether exposure or oxygen deprivation, which induce a rapid stress response, including immediate HPA activation. A prolonged assessment or appraisal of the stimuli would delay coping responses and work to the organism's disadvantage. In terms of neurocircuitry, these stressors produce a rapid stimulation of hypothalamic CRH, which then directly activates the pituitary. By contrast, processive stressors do not directly alter or threaten physiological homeostasis but rather are *perceived* to be stressful in comparison to previous experiences. Such stressors thus involve a degree of cognitive processing or evaluation before they are deemed threatening. In general, and with regards to the current thesis, the term

processive stressor is synonymous with psychological stressor. The state produced by processive stressors (be it behavioural, emotional or physiological) is referred to as anxiety or the anxiety response.

In contrast to systemic stressors, brain regions that mediate anxiety appear to reside outside the hypothalamus, which itself is largely a regulatory region. Limbic structures, and specifically the amygdala, have demonstrated a well-established role in regulating the emotional response to fear and have generally been thought to mediate the physiological and behavioral response to processive stressors [168,198]. Subregions of the amygdala also contain dense concentrations of CRH and CRH receptors, and intra-amygdala injections of CRH produce behavioural and physiological changes characteristic of natural stressors [174,565,566]. Given this confluence, many have proposed that amygdala CRH mediates the anxiety response to processive stressors. However, it is now acknowledged that this system responds to a wider range of stimuli than originally assumed, and thus may not be specific to anxiety. Indeed, studies have shown that non-anxious stimuli can also provoke amygdala CRH activation at a level comparable to stress exposure [317,338].

Thus at present, two key questions regarding the scope of the role of CRH in mediating stress remains: 1) Is CRH activation involved in mediating the behavioural response to processive stressors and, 2) Is CRH activation at the amygdala reflective of an anxious state. The ensuing paragraphs will characterize in greater detail the relationship between CRH and stress and the role of CRH and amygdala CRH in anxiety. Then, we will try to clarify what constitutes a processive stressor and which behavioural models appear particularly relevant for addressing the impact of such stressors.

2.1 Characterization of stress and the stress response

The concept of stress originated from physics and engineering as a term meant to denote "pressure or strain." However, in the early part of the 20th century, the Canadian endocrinologist Hans Selye began to apply this term to biological phenomena. He thus redefined stress as the "mutual actions of forces that take place across any section of the body, physical or psychological." He would later provide a more generic definition "the nonspecific response of the body to any demand made upon it" [482]. Selye's own perspectives on stress were heavily influenced by the work of the neurologist Walter Cannon. Cannon had introduced the concept of "homeostasis" while observing that organisms appeared to maintain steady levels of water, salt, sugars, proteins, fats, body temperature and oxygen levels in the blood. This homeostatic mechanism appeared designed to maintain equilibrium and counteract influences that could disrupt or threaten the body's regulatory processes. In this context, Selye saw the prototypical stress response as being an alarm signal to real or perceived threats against homeostasis.

In addition to the concept of homeostasis, Cannon also introduced the first conceptualization of a stress response by organisms, the so-called 'fight or flight' response. Cannon described this response as the way the body prepares itself when confronted with a threat by either standing one's ground and fighting or by running away [482]. Cannon also thought it was a good metaphor to represent the way people respond to stressful circumstances at both the physiological and psychological levels..

Physiologically, it involves a sympathetic activation (including increased heart rate and blood pressure) that aids in mobilizing the organism to either confront the threat or to

flee. Psychologically, it refers to the tendency to cope actively with a stress or to withdraw from it [482].

To complement Cannon's short-acting 'fight or flight' response, Selye also introduced a longer term, multi-phase response to stress that he called the General Adaptation Syndrome (GAS). A wide range of physiological and environmental stressors were found to produce consistent endogenous changes including a significant enlargement of the adrenal cortex, atrophy of the thymus, spleen and lymph nodes and the appearance of ulcers at various levels of the gastrointestinal tract [481]. These changes were reduced in animals that had been adrenalectomized, implicating the adrenal glands in the GAS. In fact, subsequent findings established that much of the GAS was mediated by activation of cortisol, norepinephrine and epinephrine from the adrenals glands, which was under the control of the pituitary. This led to one of Selye's most famous proposals that this hypothalamic-pituitary-adrenal (HPA) axis was the central mediator of the GAS response to stress. Although initially serving an adaptive purpose, excessive or prolonged HPA activation would eventually lead to illness, damage to the body and ultimately death.

The empirical and theoretical advances provided by Cannon and Selye were highly influential and served as a framework to stress research for several decades. Stress itself has become a complex concept and its study has spanned numerous disciplines including sociology, anthropology, psychology and physiology. A unanimous definition of stress remains elusive, as the concept incorporates many factors, including the stressful stimuli, individual responses (behavioural and physiological) and numerous mediating factors such as cognitive processing, social context, personal history and/or physical integrity.

However, a survey of numerous definitions suggests the common element of a threat or a perceived threat to the organism. In this context, a well-accepted definition of stress has been “a state that occurs when people [or organisms] encounter events that they perceive as endangering their physical or psychological well-being” [15]. The scientific study of stress has further differentiated between the *stressor*, i.e. the threatening stimulus or situation, and the *stress response*, which is the physiological and behavioural reaction produced [71].

2.1.1 The stress response

Within moments of a threat or perceived threat (either from internal or external sources), two physiological systems are activated which are designed to enhance the organism's ability to cope with the impending danger and to activate the so-called ‘fight or flight’ response [65]. The first system is the autonomic nervous system (ANS) which itself is subdivided into counterbalancing parasympathetic and sympathetic systems. Upon stress exposure, visceral signals and those from various cortical and subcortical regions are relayed to the hypothalamus which in turn activate neurons in the brain stem, including those at the nucleus of the solitary tract (NTS), medulla and parabrachial nucleus [551]. These in turn shift the emphasis of the ANS in favor of the sympathetic nervous system, in part by directly or indirectly activating sympathetic preganglionic neurons located at the spinal cord [101,386].

The efferent neurons of the sympathetic nervous system (SNS) innervate a wide range of tissues and organs throughout the body. Some of the immediate effects of SNS activation following stress exposure include the mobilization of energy stores towards

muscle groups, modulating immune functions, increasing cardiovascular output and glucose utilization, inhibiting reproductive physiology, appetite and feeding, and producing a sharpened and focused attention [551]. In both humans and animals, additional SNS effects include pupillary dilation, piloerection, heart rate acceleration, enhanced breathing and increased blood pressure. Most of these latter effects are mediated by epinephrine release from the adrenal glands [551].

The second system activated following stress is the HPA axis. The HPA axis is a complex system that functions under both normal and stressful conditions. In normal, basal conditions, its activity fluctuates in a circadian fashion. Under stressful conditions, it becomes activated and sets into motion a cascade of events, which maximize the body's ability to respond effectively and efficiently to particular homeostatic threats. Within seconds of stress exposure, neurons of the parvocellular division of the PVN (at the hypothalamus), projecting to the external zone of the median eminence, become activated and secrete a number of ACTH stimulators, the most important of which are CRH and vasopressin (AVP). These secreted peptides are then transported by the portal blood system to the anterior portion of the pituitary gland where they synergistically stimulate the release of ACTH [198,228,342].

The glucocorticoids represent the final step in the chain of HPA activation and are released upon ACTH stimulation of the adrenal glands. Glucocorticoids, including cortisol or the rodent equivalent corticosterone, exert many different effects which are aimed at redirecting energy stores and providing a negative feedback signal to prevent stressor-activated defense mechanisms from overshooting or overcompensating (to the detriment of the organism) [342]. More specifically, the effects of glucocorticoids

include 1) elevation of blood glucose levels by stimulating gluconeogenesis in the liver and lipolysis, as well as by inhibiting insulin secretion, 2) suppression of immune and inflammatory activity and, 3) termination of the stress response through inhibition of CRH and ACTH via multiple feedback loops at sites at the pituitary, hypothalamus and hippocampus [342].

2.2 CRH and the stress response

The central role of CRH in mediating stress-induced HPA activation initiated a tremendous amount of research into the role of CRH in other components of the stress response. This has led to the discovery that central administration of CRH produces behavioural, endocrine and autonomic effects similar to those produced by natural stressors. These include activation of the autonomic nervous system [58,59], an increase in ACTH [452,453] and β -endorphin release from the pituitary [6], increased corticosterone release from the adrenals [452] and an inhibition of growth hormone release [385]. CRH also activates sympathetic-mediated increases in norepinephrine, epinephrine, glucose and glucagon [101]. It has also been demonstrated that mice genetically deficient in the CRH1 receptor gene have a markedly reduced ability to mount an effective physiological stress response [503].

As indicated earlier, CRH appears to produce different behavioural effects depending on the environmental context. For instance, CRH administered in a familiar (and presumably non-stressful) environment increases behaviors such as grooming, exploration and rearing [230]. However, in an unfamiliar (and presumably stressful) environment, a similar dose enhances freezing and decreases these same behaviours

[130]. It has thus been suggested that the role of CRH in stress may depend to large extent on the particular context of the stress exposure.

Other effects of centrally-administered CRH indicative of a state of stress include inhibition of feeding and sexual behaviour and increased defensive burying and aggressive behaviours [139]. These CRH-induced behavioural effects remain unaffected by peripherally-administered CRH antiserum or glucocorticoid antagonists, suggesting that they are likely mediated at the level of the brain [55,133,413]. In support of this contention, central administration of CRH antiserum or CRH antagonists does attenuate or block the autonomic, neuroendocrine and behavioural effects of exogenously administered CRH [56,58,61,454].

Despite a growing body of evidence, caution is required in assuming that endogenous CRH mediates the full range of stress responses. As mentioned earlier, it was recently demonstrated that CRH-deficient mice (i.e. transgenic animals without a CRH-producing gene) still mount a behavioural response to stress exposure indistinguishable from that of normal mice [360]. The neuroendocrine response (ACTH and glucocorticoid release) was however blunted in these animals, suggesting dissociation between HPA activation and components of the behavioural stress response.

In addition, other findings have shown inconsistencies regarding the mediating role of CRH in the behavioural response to processive stressors. In this context, Herman and Cullinan [198] have suggested that different neurocircuits mediate or modulate the effects of different types of stressors. Processive or psychological stressors are perceived to be stressful in comparison to previous experience and are thought to influence homeostatic processes secondary to stimuli appraisal. Included in this category are models involving

immobilization, restraint, neophobia and exposure to noise, bright lights or elevated surfaces.

The ability of CRH antagonists to produce anti-anxiety effects has been particularly inconsistent in behavioural models involving processive stressors. For instance, in the elevated plus maze, which tests the willingness of animals to walk on an elevated plane, some studies have demonstrated an anxiolytic effect for CRH antagonists while others have not [22,43,170,510]. Interestingly, the anxiolytic effects of CRH antagonists in this test were only observed if testing was preceded by either central CRH infusion or ethanol withdrawal, both of which represent anxiogenic conditions or stimuli [22,510]. Greibel et al. [170] also showed that systemic administration of a CRH1 antagonist effectively attenuated defensive behaviours, but did not influence the anxiety state caused by ‘exploratory’ stressors (elevated plus maze, free exploration). Similarly, Okuyama et al. [380] reported that recently developed, highly selective CRH1 antagonists failed to antagonize anxiogenic-like behaviours expressed in the light/dark exploration task or the elevated plus maze. These findings question the degree to which the behavioural response to processive stressors is mediated by CRH activation.

2.3 Amygdala CRH and processive stressors

In trying to understand the neural mechanisms mediating processive stressors and anxiety, attention has been focussed on brain regions involved in the regulation of emotional processes such as the amygdala. The amygdala lies in the lateral pole of the temporal lobe and, despite its relatively small size, contains at least 17 different nuclei, each with unique afferent and efferent projections and serving different functions

[131,278,526,550]. The four most widely investigated amygdaloid nuclei are the medial (MeA), basolateral (BLA), basal (BA), and central (CeA) nuclei. A preponderance of research findings, in both animal and human subjects, have supported a central role for this structure in the emotional response to fear and anxiety [368,405]. Thus, electrical stimulation of the amygdala produces subjective feelings of fear in humans [71]. Neuroimaging studies also demonstrate enhanced neuronal activity at the amygdala in people who are undergoing conditioned fear acquisition, recalling violent events, in patients with post-traumatic stress disorder and/or people exposed to fearful visual or audiovisual stimuli [68,272,293,441,474]. Conversely, damage to the amygdala induces deficits in normal emotional reactions following the presentation of aversive stimuli [405]. These people also lose the ability to form associations between unconditioned and conditioned stimuli and to acquire conditioned emotional responses [405].

The amygdala, in particular the CeA, appears important in processes by which sensory stimuli gain motivational and emotional significance, be they positive or negative. This includes stimulus-reward associations and fear memories [156,229,277]. Animal research has further demonstrated that the CeA is central in the acquisition and expression of conditioned fear and anxiety, tasks which involve forming associations between conditioned and unconditioned stimuli and between conditioned stimuli and behaviour [69,112,114,552]. Electrical and/or chemical stimulation of the CeA evokes behavioral, endocrine and autonomic changes characteristic of stressor exposure [33,69,131,514]. Conversely, lesioning of the CeA attenuates or prevents stressors from eliciting many of these same responses [32,149,330,587]. Research has also demonstrated that lesions to the CeA reliably impair or block the HPA response to

processive stressors, such as restraint or non-harmful acoustic or photic stimuli, while having no effect on the response to systemic stressors such as ether exposure [145,198,429]. Current theory has thus contended that the CeA plays an important role in mediating the response to processive stressors.

Importantly, CRH protein, receptors (CRH1 and CRH2) and binding protein are all prominent at the CeA [317,335,427,468,528]. Centrally administered CRH provokes c-fos activation at the CeA [44] and CeA administered CRH dose-dependently elicits behavioral, endocrine and autonomic responses characteristic of natural stressors [174,565,566]. The view has thus been expressed that CRH within the amygdala, and the CeA in particular, contributes to the promotion and/or expression of anxiety [113,277,501]. This contention has been supported by data showing that processive-type stressors, such as restraint, increase amygdala CRH mRNA expression [232], while administration of CRH antagonists at the CeA modulate anxiety-like behaviours [130,528].

It should be noted however that the data involving behavioural models has often been inconsistent. For instance, while some have shown a robust increase in CRH mRNA expression at the CeA in response to restraint stress [206,232], Pacák et al. [387] failed to show elevated levels following immobilization stress. Interestingly, recent findings have suggested that CRH response at the CeA may not be restricted to stressful stimuli. Indeed, Makino et al, [317] found that stimuli which were not stressful, but merely arousing, also activated CRH mRNA at the CeA. Moreover, seemingly pleasurable events, such as intake of palatable food, also stimulate CRH release at the CeA, at a magnitude similar to that produced by restraint stress [338].

Thus, in examining the role of CRH in processive-type stressors and anxiety, two questions have emerged: 1) Is CRH activation at the CeA reflective of an anxious state and, 2) Does CRH activation following exposure to processive stressors mediate the behavioural component of the stress response. To answer these questions, appropriate models for evaluating processive stressors need to be selected as inconsistencies in the literature may result from the assumption that different stress models characterize the same phenomena. It is likely, however, that the reality is more complex.

2.4 Processive stress models

Overt or explicit threats to physical integrity, such as toxic chemicals, temperature change or strong pain are detected by sensory and visceral systems and initiate a rapid stress response with minimal conscious evaluation. In cases such as predator exposure, a rapid recognition of a threat is also necessary for an effective 'fight or flight' response. This ability may benefit from genetic factors since rodents appear to demonstrate a natural aversion to certain predator odours without having ever confronted the predator itself [356]. This 'pre-programmed' aversion suggests an inborn neurocircuitry designed to quickly recognize certain threats and initiate a coping response.

In contrast to these situations, there are numerous stimuli or conditions in the environment that are more ambiguous regarding the particular nature of the threat. Processive stress models, by definition, involve stimuli that require a degree of processing before they are interpreted as threatening. A number of behavioural models have been developed to try to characterize the mechanisms underlying responses to these situations.

Conditioning models, for instance, use stimuli that have acquired the properties of a systemic stressor through association. This usually involves repeatedly pairing a neutral stimulus (for instance a light) with an unconditioned aversive stimulus, such as a shock or noxious drug. Through association, the conditioned stimulus (CS) is then able to elicit or potentiate a stress response on its own. Since the behavioural response is mediated by the strength of the association between the conditioned and unconditioned stimuli, a degree of processing is required. However, because the CS is associated with a systemic stressor, such as those involving pain or toxicity, it is uncertain whether the elicited response in such tests may partly be compromised through direct association with a systemic stressor. In addition, conditioning models tend to use relatively basic or 'hard-wired' behaviours (such as startle reflexes or avoidance) as the dependent variable, which do not allow for easy cognitive modulation. Due to these limitations, conditioned stress models may provide limited insight into mechanisms underlying responses to processive stressors.

In addition to conditioning models, a number of anxiety models involve the use of unconditioned stimuli. In general these models vary according to the overt nature of the threatening stimulus. Models such as prolonged restraint, immobilization or noise stress, although not intrinsically harmful, nonetheless involve strong sensory inputs (in this case touch or sound) which themselves are aversive and initiate physiological changes independent of cognitive processing. Somewhat less overt are stimuli involving a lit chamber (as in the light/dark exploration task) or an unpleasant odour (e.g. mouse odor to a rat), which are neither harmful nor dangerous but are innately unpleasant to the senses. Other models involve conditions such as the elevated plus maze, which expose animals to

cues that suggest the possibility of danger (i.e. an elevated ledge). These models all represent potential processive stress paradigms, as no physically harmful stimuli are present. They, however, vary in terms of the degree of processing that may be required, depending on the intensity of the sensory stimuli, the innate aversion to certain cues, or the explicit nature of the presented danger.

These models also tend to measure the willingness of subjects to perform anxiogenic or unpleasant tasks (e.g. light/dark exploration or the elevated plus maze), or their passive response to an aversive stimulus (e.g. restraint or noise). This might limit their ethological validity, since it may not accurately characterize anxiety problems or dysfunctions in human populations. For instance, the extent to which anxiety is a behavioural problem does not mainly depend on whether individuals are willing to accomplish a fearful task, but rather on whether an anxious state or situation impairs the ability to perform normal activities. Thus, animal models in which anxiety may inhibit reinforcing or biologically necessary functions might better reflect conditions in human populations in which an anxious states limits or interferes with behaviour.

In this context, animal models involving neophobia appear to possess few overt or explicit stimuli suggesting a threat or producing an aversion. In addition, they tend to examine the effects of an anxiogenic environmental context on behavioural performance. As such, they may offer a valid and ethologically relevant processive stress model. Neophobia models involve merely changing the subjects' environment from a familiar to an unfamiliar one (i.e. moving from its home cage to a novel cage). In such situations, the stressor stimuli are not confounded by pain or physiological threats, nor are the subjects presented with stimuli that are harmful, dangerous or overtly unpleasant.

Nonetheless, such a change in conditions induces behavioural and physiological alterations similar to those elicited by natural stressors, such as a predator scent, including the inhibition of non-defensive behaviours [45]. Other effects include spontaneous defensive withdrawal [597], increased grooming [128,143], increased defecation [107,146,182] and an inhibition of feeding behaviour [28,425,485,508]. In addition, pronounced increases in blood pressure and heart rate via ANS activation [546] and increased HPA activation [148,403,578] are produced upon exposure to novel environments. These effects mimic those observed following natural stressors or central injections of stress peptides.

The inhibitory effect of novelty on feeding is perhaps the most widely replicated of the behavioural changes and is reliably reversed by a number of anxiolytic compounds, with various pharmacological mechanisms, including benzodiazepines, serotonergic and noradrenergic anxiolytics [49,50,446,515,534]. Assessing food intake in an unfamiliar environment is a simple and practical behavioural paradigm, although its use as a processive stress model has been limited by certain methodological problems. For instance, some studies have used food or water deprivation (concurrent systemic stressors) or the presentation of a novel food (which may produce neophobia) that can confound the anxiogenic attributes of the unfamiliar environment. As well, the presentation of regular chow in some studies has yielded low basal feeding levels making further reductions of food intake less discernible (a floor effect). However, we observed that offering non food-deprived animals a highly palatable, familiar snack in a novel environment circumvented these problems, allowing for an unaltered appraisal of anxiety [247,362].

2.5 Specific research objectives

Using a novelty-induced feeding suppression paradigm, the objectives of the first part of the present thesis aims to examine whether 1) anxiety-like behavioural and neurochemical responses are elicited by a novel environment and attenuated by benzodiazepine pre-treatment, 2) antagonism of CRH action(s) attenuates the effects of the novel environment on behavioural signs of anxiety (i.e. snack consumption), 3) endogenous fluctuations in CRH release at the CeA can be elicited by a novel environment and, 4) attenuation of the behavioural signs of anxiety by benzodiazepine treatment does simultaneously influence stress-elicited CRH release. Together, it is hoped that these investigations can clarify the involvement of CRH and amygdala CRH in anxiety and/or the peptide role in the behavioural response to processive stressors.

3. CRH and Ischemia

The ability of exogenously administered CRH to mimic the effects of natural stressors not only supports a behavioural role for this peptide, but also reveals important physiological effects. Excitatory effects of CRH have subsequently been shown to extend to numerous brain regions outside the HPA axis, including the locus coeruleus [543], amygdala [565,567] and hippocampus [5]. Thus, at high doses, centrally administered CRH was shown to induce pronounced seizures and electrophysiological activity in the amygdala and hippocampus, indistinguishable from seizures produced by electrical kindling [27,88,137,203,561]. Neuroanatomically, these seizures appear

mediated at the level of the amygdala and hippocampus, as well as interconnecting regions such as the piriform and perirhinal cortices.

Experimentally induced seizures, in many circumstances, can also produce neurotoxic effects. A number of studies have demonstrated that seizures produced by kainic acid, which stimulates a subtype of ionotropic glutamate receptors, are characterized by cell death in a number of limbic structures, including the hippocampus. Interestingly, in brain regions undergoing cellular degeneration, there is a conspicuous increase in CRH mRNA expression [416,417] indicating that this peptide may play a contributing role.

Evidence suggesting neurotoxic effects for CRH has been obtained in studies investigating global and focal models of ischemia. In both models, a centrally administered CRH antagonist conferred significant neuroprotection when administered shortly before or after the ischemic insult [311,516]. Global models of ischemia have long been used to test the efficacy of neuroprotective compounds in the treatment of brain damage produced by stroke, cardiac arrest or other sources of temporary blood flow restriction. Transient ischemic insults reliably produce cell death, particularly in the CA1 and CA3 pyramidal cell layers of the hippocampus. This is interesting with regards to a potential mediating role for CRH, as studies have shown that exogenously administered CRH can produce profound excitatory effects on the large hippocampal pyramidal neurons of the CA1 and CA3 [5,203]. Based on the established excitatory effects of this peptide and the neuroprotective effects of CRH antagonists, a CRH theory of neurotoxicity has emerged.

The following sections will provide a characterization of the ischemic process and its physiological, anatomical and functional consequences. It is however likely that

numerous other physiological signals and reactions will emerge as mediators of the ischemic process in the future. Furthermore, a wide range of ischemia-induced biochemical signals and processes have not yet been reliably linked to actual cell death or functional impairments. The following paragraphs will thus attempt to focus on those effects most widely recognized as being involved in ischemia-induced neuronal degeneration and/or functional deficits. It will then become easier to examine the possibility of a participation of CRH in some of the neurophysiological and behavioural effects of ischemia.

3.1 Animal models of Cerebral Ischemia

Rodent models of cerebral ischemia generally fall into one of two subgroups: global or focal. Focal models usually involve proximal or distal occlusion of one middle cerebral artery, whereas global ischemic insults are most commonly produced by complete cessation of blood flow to the brain for limited time periods. Thus, focal differs from global ischemia in two important ways: 1) There is more systemic blood flow to the brain in focal, so longer duration insults are required to provide significant neuronal damage, 2) There is also gradation in damage from the core areas of affect (the cortex and caudoputamen) to outer boundaries and metabolic changes can vary greatly in the different areas. As a result, the ischemic process is experimentally more complex in focal models.

The four-vessel occlusion (4-VO) model of global ischemia involves permanent electro-coagulation of the vertebral arteries (which in itself has little impact on neuronal damage due to compensatory blood circulation via the circle of Willis) followed by

temporary ligation of the common carotid arteries. In rodents, this procedure gives rise to prompt unresponsiveness of the animal and loss of the righting reflex approximately 15 s after ischemia onset. Transient global ischemia affects widespread brain areas but is typically characterized by neuronal alterations within selectively vulnerable brain regions. Blood flow is reduced to below 3% in areas such as the hippocampus, striatum and neocortex [433] and spontaneous cortical activity, as assessed using EEG, is abolished within one minute [577]. The pyramidal neurons of the CA1 layer of the hippocampus represent the most vulnerable neuronal population to forebrain ischemic damage.

The 2-vessel occlusion (2-VO) model involves bilateral common carotid artery occlusions combined with systemic hypotension in the rat. The success of this model to induce a global ischemia depends on the production of adequate hypotension as bilateral carotid occlusions in normotensive rats are insufficient to lower cerebral blood flow into the ischemic range or to perturb tissue energy state and a longer duration insult is required to produce damage similar to the 4-VO model. Thus, brain protection against neuronal damage can be easier to obtain. These and other factors have limited its use as a model. In gerbils, however, bilateral common carotid occlusions are commonly used to induce global ischemia as brain blood circulation is mediated via an incomplete circle of Willis in these animals.

3.2 Nature of cell death

3.2.1 Necrosis vs Apoptosis

One of the end consequences of transient global ischemia is neuronal damage.

Although there is no accepted definition to formally identify the moment when a cell dies as yet, Lipton [299] formulated a practical definition which has considerable merit: “The point at which the cell becomes unable to recover its normal morphology and function even if all processes leading to its dissolution are stopped pharmacologically (the point of no return)”.

Focal and global ischemic insults induce two types of cellular death: necrotic and apoptotic (general features of each are summarized in Table 1). In general, necrosis only occurs following severe cellular injury or as the result of an exogenous insult. Contrary to apoptosis, this type of cell death is not genetically programmed or part of normal physiological processes such as seen during development. The main morphological features of necrosis include early cell and organelle swelling, with the eventual loss of cytoplasmic membrane integrity and cellular disintegration [246]. Cellular and mitochondrial swelling, enlargement of the endoplasmic reticulum, and chromatin clumping into poorly defined masses are followed by a progressive dissolution of the nucleus, organelles, plasma membranes and ribosomes, which causes cellular components to be released into extracellular space. Inflammation and damage to the surrounding tissues are also observed, with spillage of cellular debris. The pathways involved in instigating necrosis will be described below, but involve significant ATP reductions, rapid membrane depolarization, glutamate receptor over-activation and an overload of intracellular calcium.

Apoptosis, on the other hand, represents an adaptive mechanism that controls cell death without sacrificing the organism's survival [553]. It is in fact the result of a genetically regulated process that permits cells to die with a minimal release of genetic material [197,227]. Apoptosis appears to be the normal process by which cell death occurs during developmental stages or in processes involved in maintaining the cell numbers of particular regions [197,223,227,553]. In the early stages, apoptosis is characterized by a condensing of the chromatin within the cell nucleus, as they congregate in large masses. The cytoplasm also condenses and there is an overall loss of cell volume. This is followed usually by an expulsion of membrane-bound cytoplasmic fragments (also called apoptotic bodies or apoptosomes). Eventually, the cell nucleus (and its constituent DNA) become fragmented and break up into discrete units. Contrary to what is seen in necrosis, considerable organelle preservation remains in apoptosis and, although nuclear and plasma membranes become convoluted, the overall cellular integrity is maintained to a certain degree.

Apoptosis is generally mediated by one of two pathways, both of which increase intracellular levels of proteolytic caspase enzymes. Activation of caspases, such as caspase-3, stimulates DNases (specifically caspase-activated DNase or CAD) that enter the nucleus and cleave DNA to produce DNA fragmentation or 'laddering', a characteristic feature of apoptotic cells. A number of other effects, including nuclear shrinking and cleavage of cytoskeletal proteins, are also initiated.

One pathway triggering apoptosis involves stimulating the so-called 'death-receptor' complexes, which in turn stimulate the activation and metabolism of the caspase precursor pro-caspase-8. This process appears triggered by stimulation of a number of

receptors for ligands of the tumor necrosis factor (TNF) family of proteins. These include the TNF receptor (TNFR), the receptor for FAS, a type II membrane protein that belongs to the TNF family of proteins, as well as receptors for TNF-related apoptosis inducing ligand (TRAIL-R1 and TRAIL-R2). Binding by their respective ligands promotes the recruitment of multiple procaspase-8 molecules through interactions with various adaptor proteins such as the FAS associated death domain protein (FADD) and the TNFR1 associated death domain protein (TRADD). This results in caspase-8 activation, which has intrinsic apoptotic actions and stimulates caspase-3 activity. A second apoptotic pathway involves activation of several proteins transcribed from the BCL family of genes, which has three groups. Although some members of this gene family (i.e. BCL-2) exert anti-apoptotic effects, a second group of genes, which include *Bax* and *Bak*, are pro-apoptotic. These target the mitochondria to release cytochrome *c*, which interacts with the apoptotic-activating factor (Apaf-1) leading to the eventual activation of caspase-9. When activated, caspase-9 activation triggers proteolytic activation of caspase 3 [598].

In determining the nature of cell death in neuronal regions, the distinction between necrotic and apoptotic death largely relies on morphology. On this basis, the morphological features of cell death following global ischemia appear to be mostly necrotic [322,583]. Evidence favoring necrosis following global ischemia is also provided by studies using electron microscopy [540].

Thus, micro-structural studies of post-ischemic CA1 neurons appear to show important disintegration of ribosomes, mitochondria and plasma and nuclear membranes which are indicative of necrosis, not apoptosis [92,120,255,256,580,583]. As well,

apoptosis typically occurs in ATP-supplied cells, while necrosis occurs in ATP depleted cells [136]. Thus, energy failure and a loss of membrane integrity for the mitochondria usually lead to necrosis, whereas apoptosis tends to require intact and functioning mitochondrias [10].

However, it should be added that while necrosis is considered the predominant type of cell death following global ischemia (particularly with respect to the CA1 hippocampal neurons), it is also believed that considerable overlap exists between necrotic and apoptotic processes and that, indeed, on some occasions, the same cell can undergo both processes [487,488]. In addition, a number of cells do show apoptotic characteristics following ischemia, such as caspase activation [82,374], DNA fragmentation [313] (although this has also been attributed to DNA breakings caused by necrosis [408]) and sensitivity of these cells to caspase inhibition as a physiological mean to enhance neuroprotection [82].

Table 1: Characteristics of Necrosis and Apoptosis

	Necrosis	Apoptosis
Early Changes	• Margination of chromatin • Pyknosis (uniformly compacted chromatin) • Dilatation of the endoplasmic reticulum • Dispersal of ribosomes	• Chromatin congregates in large masses, convoluted nuclear outline • Cytoplasmic condensation • Blunt protuberances appear on the cell surface • Some cytoplasmic vacuoles may appear
Late Changes	• Swelling of mitochondrial matrix • Rupture of nuclear, organelle, and plasma membrane • Cytoplasmic boundaries indistinct • Flocculent, granular matrix densities in mitochondria • Ribosomes and lysosomes dissolve • Karyolysis – nucleus loses basophilia, leaving a nuclear ghost	• Nucleus breaks up into discrete fragments • Cell surface protuberances seal off to produce apoptotic bodies

3.2.2 Delayed Neuronal Degeneration

In addition to features of necrosis and apoptosis, ischemia-induced cell death is characterized by delayed neuronal degeneration. This phenomenon was first observed by Pulsinelli and Brierly [431,433] and has been most widely studied in the CA1 pyramidal neurons of the hippocampus, but is also observed at the hippocampal CA3 and CA4 pyramidal layers as well as the striatum and cerebral cortex. It is characterized by the observation that neurons do not immediately die following a global ischemia, but rather the normal state of the cell is disrupted to such an extent that the chain reaction initiated is beyond rescuing by normal homeostatic functions. Striatal neurons degenerate more rapidly than the vulnerable CA1 neurons, although it requires a longer ischemic duration to induce cell death [473]. The time interval between the ischemic insult and initiation of cellular death varies in global ischemia, but usually ranges between 48-96 hours, depending on the severity of the insult and the cell density of the region under investigation. In general, longer duration insults lead to a shorter delay for the initiation of neuronal damage. Of all the regions affected, the CA1 region of the hippocampus has reliably been shown to be the first and most significantly damaged region in response to global ischemia. In rats, a 2 minute global ischemia (4-VO occlusion) produces minor CA1 damage [505]. In contrast, a 5 minute global ischemia can produce upto 50% cell loss at the CA1 within 7 days [231], while 6-10 minute ischemias can induce near complete CA1 pyramidal cell loss within 3 days [291,301]. Other estimates have been more conservative and demonstrate that ischemic intervals of 10-20 minutes produce 40-85% cell death at the hippocampus [408,592]. In contrast, for the striatum to be consistently injured at least 15-30 minutes of ischemia is required [377,424,455,564] and

these pathologic changes are manifest within 24 hours [455]. More variable alterations have also been observed within the neocortex following similar ischemic duration.

3.3 Principal mediators of the ischemic cascade.

Neuronal tissue requires both oxygen and glucose and depends almost exclusively on oxidative phosphorylation for energy production [124]. Disruption of the electron chain by ischemia leads to inhibition of oxidative phosphorylation, followed by depletion of high-energy phosphates, which compromise adenosine triphosphate (ATP) formation. Thus, within 1 to 2 minutes of global ischemia, ATP levels in the brain fall by approximately 90 % [240,300,323]. Among the numerous cerebral functions dependent on ATP is the regulation of Na^+ - K^+ ion channels in cell membranes. The inhibition of ATP causes a specific inhibition of the Na^+ - K^+ ATPase, resulting in a large accumulation of Na^+ inside the cell. A compensatory efflux of K^+ ions is initiated but being insufficient, the end consequence is a profound membrane depolarization. Membrane depolarization rapidly occurs within 60 to 180 s after the onset of ischemia [238,274,575] and is characterized by a 10-30 mV extracellular negative current shift in cell body regions of ischemic tissue [266,274,323].

The important Na^+ influx is accompanied by similar migrations of Ca^{2+} and Cl^- ions inside the cell and an important K^+ efflux [266,274,323]. The depolarization of pyramidal cells at the hippocampus is particularly evident, as membrane potentials have been shown to decrease to -20 mV in this area within 3 minutes of the ischemia onset [436,577]. The massive Na^+ entry into the cells is thought closely linked to subsequent damage as numerous blockers of Na^+ influx reliably enhance neuronal protection

following a global ischemic insult [102,287,312]. Such neuroprotection, however, is only possible when these compounds are rapidly administered (from 15min - 2h) after the onset of the insult [102,287], limiting their usefulness as therapeutic molecules.

3.3.1 Calcium (Ca^{2+})

Simultaneous to membrane depolarization (60-90 s after the onset of ischemia), a 25% increase in intracellular/cytoplasmic Ca^{2+} is observed [40,237]. In particular, intracellular cortical and hippocampal Ca^{2+} concentrations have been shown to dramatically increase within the first few minutes of ischemic onset [1,303,491]. Ca^{2+} influx appears concomitant to large increases in extracellular glutamate [39,347], two associated phenomena as these early ischemia-induced increases in intracellular Ca^{2+} can be inhibited by glutamate antagonists [560]. However, within approximately 2.5 min following reperfusion, Ca^{2+} influx (assessed *in vitro* in hippocampal cells) then appears mediated by passage through L-type calcium channels (25%) or $\text{Na}^+/\text{Ca}^{2+}$ exchange (about 35%) or via other unknown pathways [303]. *In vitro* research has also suggested that Na^+ entering the cells significantly enhances cytosolic Ca^{2+} through activation of the mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ exchange pump. Thus, higher intracellular Na^+ causes greater mitochondrial exchange, the net effect being to increase cytosolic Ca^{2+} [589].

The profound effect of intracellular Ca^{2+} entry on subsequent neural degeneration stems largely from the fact that Ca^{2+} regulates a great number of enzymatic activities within the cell. Thus, a large upsurge in this regulating factor initiates a series of physiological events in the cytoplasm and cell nucleus that facilitate the development of

tissue damage. There are, in general, three main mechanisms by which Ca^{2+} ions induce neurotoxic effects.

1) Activation of proteolytic enzymes. Numerous proteases such as calpain, actin, spectrin and laminin are hyperactivated by elevated Ca^{2+} levels, which leads to metabolization of proteins that form the matrix of extracellular membranes [284,579]. Another family of proteolytic enzymes, the caspases, exists in 12 different forms within the cell. Two of the family members (caspase 1 and 3) are protein-cleaving enzymes, which exert particularly deleterious roles as they cleave DNA and cytoskeletal proteins, significantly impairing the functioning of other proteins involved in crucial homeostatic and repair functions [204].

2) Activation of nitrous oxide synthase and free radicals. The gaseous radical nitric oxide (NO) is catalyzed in the brain via the neuronal enzyme nitrous oxide synthase (nNOS). nNOS itself is regulated by cytosolic concentrations of free Ca^{2+} [52,212,213,394,395] as well as being activated through glutamate stimulation of NMDA receptors [310]. At high concentrations, nNOS produces a substance called peroxynitrite that promotes tissue damage by interfering with protein synthesis [115,116,151,529,530]. Furthermore, the NOS enzyme promotes the presence of other harmful molecules called free radicals. Free radicals are atoms or molecules with an odd or unpaired number of electrons that can be formed upon oxygen interaction with discrete molecules. Once formed, these molecules are highly reactive, which is of particular concern when they interact with vital cellular components such as DNA or the cell membrane. For instance,

free radicals strongly interact with unsaturated bonds in lipids, which may be a factor contributing to loss of cell membrane integrity [83]. As well, ischemia-induced free radical activation inhibits several critical intracellular enzymes [141,480,588] and promotes DNA breakdown [475]. Examples of free radicals generated from the ischemic process include xanthine oxidase and cyclooxygenase, which have the net effect of overwhelming endogenous scavenging mechanisms designed to protect the cell. They also can induce lipid peroxidation, which breaks down the cell membrane.

3) Activation of phospholipid metabolism. There is a considerable decrease (approximately 60%) in the concentration of phospholipids within 3 min of an ischemic insult [214]. This is mainly attributable to the rise in intracellular Ca^{2+} that hyperactivates phospholipases, leading to the breakdown of phospholipids (through hydrolysis). Such breakdown leads to alterations of the cell membrane and accumulation of free fatty acids such as arachidonic acid [533]. This particular acid triggers a series of biochemical reactions upon recirculation following ischemia, most of which are harmful. Thus, the accumulated arachidonic acid is then converted to oxygen free radicals, which oxidize critical cell proteins involved in cytoskeletal functions.

Phospholipases A2 (PLA2) appear particularly dependent on intracellular Ca^{2+} levels [119], although other enzymes of the same family, including phospholipases C (PLC), respond to intracellular Ca^{2+} but require activation of additional mechanisms including stimulation of platelet activating factor (PAF) [73]. Within neurons, phospholipid synthesis also appears significantly impaired due to profound ischemia-induced ATP reduction [521], a phenomenon further contributing to cell membrane dissolution.

In summary, a significant intracellular rise of Ca^{2+} , such as seen following ischemia, has the cumulative effect degrading phospholipids, proteins and DNA through hyperactivation of numerous enzymes and their by-products. The end result is a significant breakdown in cell integrity, structure and function. The importance of preventing Ca^{2+} entry following ischemia is attested by different studies demonstrating that blockade of voltage-dependent Ca^{2+} channels is neuroprotective in global ischemia under numerous conditions [121,357,376]. Optimal protection appears to occur when such blockers are administered just before or after the ischemia, although protection has been demonstrated with injection up to 24 hours post-ischemia.

3.3.2 Glutamate

In addition to the importance of intracellular Ca^{2+} in the ischemic cascade, is a central role for the excitatory amino acid glutamate. Using *in vivo* microdialysis, Benveniste et al. [39] first discovered a seven-to-ten fold increase in extracellular glutamate concentration in the hippocampus in the initial 15 minutes following onset of a global ischemia. The rapid and profound depolarization induced by ischemia leads to spontaneous release of glutamate into the extracellular space. The influx of Na^+ has been specifically linked to glutamate release, as Na^+ channel blockers produce up to 50-75% inhibition of the ischemia induced glutamate release *in vivo* [154,166,287]. The glutamate uptake system is also highly dependent on Na^+ concentrations [306,458], which are now drastically increasing inside the cell. The consequence of this sodium inflow is to reverse the glutamate uptake transporter, which provides a further source for

extracellular glutamate release. The subsequent activation of the glutamate receptors NMDA and AMPA appear to represent critical enablers in various steps that ultimately lead to neuronal degeneration.

The NMDA receptor specifically controls ion channels permeable to Ca^{2+} , Na^{+} and K^{+} . This process is somewhat aided by activation of the AMPA receptor. Under normal conditions, NMDA channels are blocked by a magnesium ion (Mg^{2+}). However, activation of AMPA by glutamate has a depolarizing effect, the consequence of which is to remove this Mg^{2+} ion, permitting NMDA receptor activation by glutamate and Ca^{2+} influx. In addition to directly influencing neuronal ionic imbalance, glutamate serves as an excitatory amino acid. Thus, large glutamate increases into synaptic areas have the additional effect of magnifying and prolonging the massive depolarizations initiated by ischemia. There may also be a delayed role for glutamate as AMPA receptors become increasingly permeable to Ca^{2+} ions 1-3 days following the ischemic insult [164]. Further investigations into long-term effects of glutamate are thus continuing.

The critical role of glutamate in mediating these cellular functions has been validated by studies showing that ischemia-induced membrane depolarizations, ionic imbalances and increased cytosolic Ca^{2+} concentrations can be significantly inhibited by administration of NMDA or other glutamate antagonists [30,471]. Indeed, NMDA antagonists count among the most acknowledged pharmacological neuroprotectants in animal models of global ischemia [241,294,358]. Unfortunately, NMDA inhibition is accompanied by numerous unwanted side effects including severe dyskinesia, cardiovascular irregularities and respiratory dysfunction, which have prevented its use in clinical settings. Nonetheless, from the point of view of understanding the

pharmacological basis for ischemia-induced cell death, glutamate remains one of the most closely examined targets.

3.3.3 Zinc (Zn^{2+})

A role for the Zinc ion (Zn^{2+}) has emerged in the last decade. Co-localized with glutamate in presynaptic vesicles of excitatory nerve terminals [324], elevated intracellular concentrations of this ion appears neurotoxic [87,539]. Thus, Zn^{2+} accumulation in the CA3 pyramidal neurons that undergo degeneration have been observed following kainite-induced seizures [153]. A significant accumulation of Zn^{2+} in vulnerable neurons (particularly the CA1) has also been observed at 30 min and 24 h intervals following a 5 or 10 min global ischemia in gerbils [539] and rats [262] respectively. Furthermore, Zn^{2+} translocation has been observed in hippocampal CA1, CA2 and dentate hilus 72 h following a 20 min global ischemia, a phenomenon significantly attenuated by hypothermia, a highly effective neuroprotective strategy [538].

Evidence suggests that following neuronal injury, Zn^{2+} is released into synaptic clefts and enters hippocampal neurons through NMDA channels, voltage-sensitive Ca^{2+} channels, and Ca^{2+} permeable AMPA/kainate channels [87,559,586]. Although the exact mechanism for inducing neuronal death remains unclear, one method may be to increase zinc-dependent proteases [558]. Evidence also suggests that accumulated Zn^{2+} in postsynaptic neurons may activate free radical generation and/or apoptotic cascades [253,302,399]. Interestingly, compounds that selectively inhibit Zn^{2+} expression, but spare Ca^{2+} , confer significant protection against ischemic damage. This effect however appears transient as neuroprotection noticeable 3 days following ischemia had

considerably diminished by 14 days [262]. The relationship between Zn^{2+} and ischemia does not appear as critical as that for Na^+ , Ca^{2+} or glutamate. Nonetheless, its co-localization with glutamate, its selective increase in vulnerable neurons following global ischemia, and its relationship to free radicals and protease functioning suggest that it may be an important contributor to neurodegeneration.

3.3.4 Cytokines

Cytokines represent a group of proteins that function as hormones within the immune system. Although wide arrays of cytokines have been identified, most can be classified as being either pro- or anti-inflammatory. Pro-inflammatory cytokines, such as interleukin-1 (IL-1), IL-6 and tumor necrosis factor- α (TNF α) enhance the immune response to physiological challenges, in part by facilitating the elimination of pathogens.

Of these, IL-1 and TNF α have been specifically linked to cerebral ischemia. Thus, Saito et al. [465] reported an increase in TNF α expression in the hippocampus and striatum of the gerbil brain 1h following a 10 min global ischemia. Of interest, TNF α has been considered as a specific link in apoptotic pathways. Thus, apoptosis has been shown triggered by occupation of TNF receptors, which in turn stimulates the Fas-associated and TNF receptor1-associated death domain proteins FADD and TRADD. These proteins recruit the caspase-8 molecule, which through its own actions and activation of caspase-3 potentiates a number of apoptotic processes including DNA fragmentation.

Minami et al. [345] also reported a transient increase in IL-1 β mRNA expression shortly following ischemia that appeared inhibited following a 24 h reperfusion period.

Similarly, other researchers demonstrated enhanced IL-1 expression at the hippocampus, striatum and cerebral cortex 3 h following global ischemia [465,525]. Both TNF α and IL-1 are thought to play a key role in the inflammatory response induced by an ischemic brain injury. This inflammatory reaction includes the infiltration of leukocytes, such as neutrophils [159], which could have a number of potentially damaging effects including obstruction of capillaries, damage to the blood–brain barrier and vasoconstriction [188]. Actions of IL-1 have also been shown to include enhancement of intracellular calcium levels in cultured neurons [70], stimulation of free radicals production [367], NO synthase and free fatty acids such as arachidonic acid [31,189,211]. Furthermore, IL-1 is a potent pyrogen able to promote hyperthermia, a state known to exacerbate ischemic damage [161].

Several studies have reported that ICV injections of IL-1 or TNF α in animals exacerbate brain damage in focal models of ischemia [304,519,582]. Conversely, injections of IL-1 antagonists confer significant neuroprotection (over 50%) when injected centrally [304,444,518,519,582] or systemically [158,295,443]. Similarly, IL-1 blockade, by central injection of IL-1 antibodies produces a significant reduction of ischemic injury [582].

Despite this evidence, there is still caution in attributing a direct neurotoxic role to IL-1 and TNF α , since thus far neither of these pro-inflammatory cytokines has been shown to produce neurotoxicity *in vivo* when administered centrally, in the absence of any other challenge. Furthermore, *in vitro* studies using cultured neurons failed to reproduce the neurotoxic or neuroprotective effects of IL-1 or IL-1 antagonists, respectively [517]. It should be reiterated that to date, the effects involving TNF α and IL-1 modulation have

been exclusively demonstrated in focal models of ischemia. Similar demonstrations have not yet been observed in global ischemic models and it is unclear whether the same processes would be involved.

3.3.5 Other mediators of the ischemic cascade

In addition to established biochemical reactions in the physiological cascade leading to neuronal degeneration, a number of other putative signals have been proposed to play a role in ischemia-induced cellular damage. Among the most commonly studied are heat shock proteins and genes of the BCL family. Heat shock proteins (HSP) were first identified and studied in the context of cellular tolerance to heat shock. They are among a plethora of intracellular proteins that are synthesized following physiological stressors and thought to increase the cells' resistance to future stressors. In addition to thermal stressors, HSP increases in response to a wide range of challenges or toxins including heavy metals, viral infections, oxidants and cerebral ischemia [326,398].

Although often thought to play a role in rescuing endangered cells following ischemia, their exact participation remains a matter of debate. Thus, among the members of the HSP family, HSP60 has been shown to promote the maturation of procaspase-9 and its conversion into the apoptotic-mediating caspases [574]. HSP60 also specifically interacts with caspase-3 to promote DNA fragmentation [254]. However, the extent of its role in ischemia-induced cell death remains undetermined, as the potential neuroprotective effects of HSP60 blockade has yet to be tested. At present, HSP60 appears mainly involved in apoptotic cell death, and its participation in necrotic death is not established.

Other HSP molecular fragments are also induced following a focal or global ischemic insult but they appear to inhibit rather than promote apoptotic processes. For instance, HSP27 binds to cytochrome *c* upon its release from the mitochondria and thus prevents its interaction with apoptotic-activating factor (Apaf-1) [62,63]. Pandey et al. [396,397] have also recently reported that HSP27 can directly bind and inhibit caspase-3 processing. Moreover, HSP70 appears to inhibit apoptosis through direct binding with Apaf-1, preventing the recruitment of procaspase-9 [34,467] and by inhibition of the release of cytochrome *c* [290]. In addition, HSP70 has been shown to interact with anti-apoptotic molecules such as BCL-2 (described below) and to prevent caspase-3 interactions with nuclear membranes [222]. Increased expression of HSP70 and HSP27 has been observed in association with a variety of insults, including cerebral ischemia, and these proteins have been proposed to play a role in rescuing cell population, promoting recovery following injury [440,484,573].

In addition to HSP, anti- and pro-apoptotic members of the BCL family, *BCL-x* and *BCL-2*, have been implicated in ischemia. Activation of these genes is initiated by the transcription factor NF-kappaB which itself increases in response to both global and focal ischemia [329]. The *BCL-2* gene is expressed in different brain loci and has been shown to promote cell survival by inhibiting apoptosis under a variety of conditions. Thus, studies using *BCL-2* gene knockout mice showed that the absence of *BCL-2* increases the generation of reactive oxygen species in the brain [201]. BCL-2 antagonism was also shown to reduce oxidative stress [53]. As well, virus-mediated transfer of the *BCL-2* gene into the brain inhibits neuronal cell death induced by ischemia [276,296,486]. The

delivery of the *BCL-2* and *BCL-x* genes into hippocampal slices of 5-week old rats via a particle gun was also shown to prolong neuronal survival [14].

The *BCL-x* gene itself is alternatively spliced into a long and short form. The long form (*BCL-xL*) appears to retard cell death whereas the short form (*BCL-xS*) acts directly as a *BCL-2* and *BCL-xL* antagonist, promoting apoptosis [51,125,144]. Interestingly, the short variant was specifically shown to increase in the CA1 hippocampal region following a 30 min global ischemic insult [125]. Other members of the *BCL-2* gene family, including *bad*, *bak* and *bax*, also appear to promote apoptosis. *Bad* and *bax* selectively antagonize *BCL-xL*, and to a lesser extent *BCL-2*, and thus inhibit their anti-apoptotic effects [181]. *Bak* also promotes apoptosis by direct interaction with survival proteins or by activating pro-apoptotic pathways [596].

Research into the potential role of these and other putative mediators of neurodegeneration produced by global ischemia is ongoing. Future research will likely attempt the manipulation of these compounds through various gene therapies to further establish the significance of their role in the mediation of cell death.

3.4 Global ischemia and behaviour

In addition to effects on neuronal integrity, global ischemic insults provoke a number of behavioural changes. Although behavioural consequences of ischemia have not received as extensive a characterization as that of cellular and molecular changes, they nonetheless represent a critical component of the evaluation of putative neuroprotective strategies. Indeed, successful neuroprotective compounds would necessarily require the reversal of ischemia-induced functional impairments as well as neuronal damage. Thus

far, the vast majority of pharmacological studies involving global ischemia have assessed the effects of neuroprotectants using two main types of behavioural measures, namely hyperactivity and spatial memory tasks. Other tasks have also been used to provide further understanding of discrete functional impairments.

3.4.1 Hyperactivity

Locomotor activity is a relatively easy model to implement, which has generally been assessed by placing animals into a plexiglas chamber in which the floor is divided into squared units or the chamber is equipped with infrared beams. Counting the number of squared units being crossed or the number of beams having been interrupted in a given period of time provides a measure of the locomotor activity. Using such measures, it has been a common and long-standing observation that global ischemic insults induce transient locomotor hyperactivity [79,160,270,424]. The increased activity in ischemic animals is prominent within the first days following reperfusion, but eventually diminishes until it is equivalent to that of sham-operated controls [47,234].

In most studies, such ischemia-induced hyperactivity has been attributed to hippocampal neuronal damage [72,160,270] and some studies have even reported a correlation between hippocampal damage and locomotor activity [160,241]. Increasing the duration of ischemia has also resulted in a simultaneous increase in hippocampal damage and locomotor activity [72]. Consequently, inhibition of hippocampal damage through pharmacological treatments has led to attenuation of activity in ischemic animals [18,89,411].

Despite these findings, some concerns have been raised with the attribution of increased activity levels selectively to hippocampal damage. A major issue is the existing temporal dissociation between the two phenomena. Thus, while neuronal cell death progressively develops over 72-96 h following global ischemia, locomotor hyperactivity is observed within 24h following reperfusion. It thus appears that rather than neuronal death per se, intermediate physiological mediators potentially involved in subsequent neuronal damage may play a role in initiation of this transient increase in locomotor activity.

In this context, some researchers have argued that neuronal hyperactivity of hippocampal pyramidal cells preceding neuronal death may account for the locomotor hyperactivity [160]. Thus far however, the degree to which such neuronal hyperactivity actually occurs and its potential effects on behavior remains uncertain. Brain structures such as the striatum could also be implicated, this brain region being sensitive to ischemic insult and known to directly mediate locomotor behaviour. However, no correlation between striatal damage and hyperactivity has yet been found and research in this area is limited [343]. Thus, although locomotor hyperactivity represents an acknowledged consequence of ischemia, its physiological basis remains unidentified. In the present thesis, one of the pursued objectives involves further elucidation of the source of such behaviour, attempting to document how it may be linked to ischemia-induced neuronal degeneration and/or other behavioural alterations.

3.4.2 Learning/conditioning paradigms

Numerous behavioural paradigms have been designed to assess the ability of an animal to learn associations. Among these is the passive avoidance model, a learning task that employs an apparatus with two compartments, usually separated by a guillotine-type door. The floor of one compartment delivers a footshock, while the other compartment is considered 'safe', possessing no aversive stimuli. In the initial acquisition stage, rats are placed in the safe compartment and allowed to explore. Eventually, the animals enter the second compartment and receive the footshock. At a remote period (approximately 24 h) rats are again placed in the safe compartment and the latency to enter the footshock compartment measured. Normal or sham-operated rats show a natural avoidance response on the second trial as indicated by a longer latency to enter the footshock chamber. However, ischemic animals have, in many studies, shown a significantly shorter latency to enter the footshock compartment [12,234,239,584]. This impairment in avoidance learning has been shown attenuated by cerebral metabolic enhancers [584] as well as neuroprotective agents including GABAergic agonists [234] and glutamate antagonists [319].

Ischemia-induced learning deficits have also been assessed using a number of other tasks. Recently, Mado et al. [349] tested ischemic rats in a conditioned discrimination paradigm. In this model, animals learned to travel down one arm of a Y-maze in response to one set of intramaze visual cues or choose the other arm using a different set of cues. In this task, a 25 min global ischemia, associated with significant cell loss within the hippocampal CA1 and CA3 pyramidal layers and the striatal region, induced significant impairment in the rats' ability to use these cues to select the proper arm.

Similarly, a number of studies have demonstrated deficits in delayed alternation using a T-shaped maze. In this task, subjects must learn the rules (alternation) to obtain a food reward available at the end of one arm of a T-maze. The food reward is initially placed at the end of one arm, and after a time delay, the food becomes available in the other arm. In this task, rats having undergone a 15 min global ischemia, or gerbils with a 4 min ischemia made significantly more choice errors than control animals [7,180].

Although these findings suggest interesting possibilities regarding functional deficits following global ischemia, further characterization is at this stage warranted. Indeed, aside from passive avoidance tasks, learning and conditioning deficits following global ischemia have not been widely replicated. Nor has there been widespread validation of their relationship to ischemic damage using classical neuroprotective strategies such as hypothermia or glutamate antagonists.

3.4.3 Object Recognition tasks

Ischemia-induced brain damage has also been associated with impairments involving visual or tactile recognition. A particularly prominent non-spatial memory task involves the delayed non-matching to sample (DNMS) task. This paradigm is considered to mimic deficits analogous to those observed in amnesic human patients with ischemic injuries [572]. As the name suggests, subjects performing this task must distinguish a novel from a familiar stimulus after a specific time delay. Thus, during the acquisition stage, a sample object is presented and placed over a reward-baited well or tray. The subject must displace the object to obtain the reward. The experimenter then hides the sample and following a few seconds, presents the initial sample object plus a novel one.

The subject must then displace the novel object to obtain the reward. The position of this object is determined randomly at each trial. After a large number of trials, the subject eventually reaches a learning criterion (approximately 90% correct). Subjects are then re-tested at different intervals, ranging from a few seconds to up to 10 min. The number or percentage of correct responses in the second trial (the recognition task) becomes the dependent variable.

In this task, a 20 min forebrain ischemia inducing pronounced CA1 neuronal loss accompanied by damage to other hippocampal areas, such as the dentate gyrus, produced significant impairments in both the acquisition and recognition portions of the DNMS task at retention intervals up to 5 min [572]. Even extensive pre-ischemic training did not reduce post-ischemic deficits. Such learning and memory deficits have also been documented in non-human primates, as Zola-Morgan et al. [595] showed significantly impaired recognition in the DNMS task in monkeys following a 15 min global ischemia.

Of interest, recent evidence has suggested that DNMS deficits may largely be mediated by damage outside of the hippocampus. Indeed, partial or complete ablations of the hippocampus produce milder DNMS deficits than cerebral ischemia and in some instances, no recognition deficits at all [132,364]. Thus, some researchers have speculated that hippocampal damage has little, if any impact on object recognition abilities (e.g. Mumby et al., [363]). By contrast, selective damage to extrahippocampal regions, such as the perirhinal cortex, significantly impairs object recognition in rats [365]. These findings suggest that extra-hippocampal regions, which to date have received little attention in research, may play an important role in ischemia-induced recognition deficits. Testing in non-human primates further supports this contention. For

instance, ablations of the perirhinal cortex alone, or of the combined entorhinal and perirhinal cortices, produce significant visual recognition deficits in rhesus monkeys, whereas the hippocampus itself appears to play a limited role [66,179,339,340]. Several researchers have recognized the contribution of brain regions outside the hippocampus, which might also include corticostriatal regions such as the caudate putamen, in mediating habit or stimulus-response learning (potentially including object recognition tasks) whereas hippocampal regions are more likely to contribute to spatial learning tasks [122,346,410].

3.4.4. Spatial memory

Among the most documented post-ischemic impairments are deficits in spatial memory. Spatial memory tasks assess the ability of subjects to learn to navigate to distinct positions in an environment defined by their position relative to distal extramaze cues [355]. Global ischemic rats demonstrate a significantly longer learning acquisition profile in spatial memory tasks and reliably make more errors than sham-operated animals in working and reference memory when tested on the radial arm maze, a well-validated test of spatial memory [111,171,258]. Correlation analysis has revealed a strong negative correlation between the number of pyramidal cells in the CA1 section of the hippocampus and number of errors in the radial arm maze [258]. Furthermore, neuroprotective strategies significantly reducing hippocampal cell death are accompanied by concomitant reductions in the number of errors generated in the radial arm maze [171,216,292,570].

Similar findings have been obtained using another well-validated spatial memory task, the Morris Water Maze (MWM). In this test, rats can escape the water by using extramaze cues to find a platform hidden just beneath the water surface in a large pool. A number of parameters are assessed including escape latency, swim speed and the distance traveled to reach the hidden platform. In a number of studies, ischemic rats required significantly more time to reach the platform than sham-operated rats [48,373,382,383]. A deficit in the ability to use spatial cues is supported by the observation that increased escape latency is not a function of swim speed, but rather is attributable to the length of the path taken to reach the platform (i.e. the distance traveled).

Demonstrations of a deficit in the MWM have not been completely uniform, however, as other studies using escape latency as the dependent variable have failed to find impairments among ischemic rats [16,258]. Indeed, unlike findings with the radial arm maze, some studies have reported poor or no correlation between CA1 hippocampal cell death and performance deficits in the MWM [373,377,383]. Furthermore, the deficit in the ability of ischemic rats to use spatial cues in the MWM does not appear long lasting and, learning of the platform position is often accomplished within a few trials in both sham and ischemic animals. Under some conditions, it thus appears that the radial maze can provide more reliable findings, better correlated with the extent of the CA1 pyramidal loss and allowing for the evaluation of the impact of putative neuroprotective compounds on recovery of spatial memory function.

3.5 CRH and Global ischemia

In addition to mediating numerous behavioural and regulatory systems, the 41-amino acid peptide CRH has recently been identified as a potential mediator of ischemia-induced neuronal death. Thus, CRH has been shown to enhance neuronal excitability in various brain regions that contain CRH and CRH receptors. These include the locus coeruleus [150,543], hippocampus [5] and cerebral cortex [134]. Locally administered CRH increases the spontaneous discharge rate of the locus coeruleus in rats and increases excitability of pyramidal neurons of the CA1 region of the hippocampus via norepinephrine release [104,388].

At higher doses, CRH can induce seizure activity indistinguishable from that observed following electrical kindling of the amygdala [27,88,137,203,561]. The action of CRH is characterized by epileptiform activity in both the amygdala and hippocampus. Of particular relevance to the current thesis, CRH significantly enhances excitatory activity at the hippocampus. In this context, it has been well established that exogenous CRH administration causes significant increases in the firing rate of the large pyramidal neurons in the CA1 region of the hippocampus [5,203], an effect prevented by pretreatment with CRH antagonists. This observation, coupled with the finding by Lee et al. [281] that CRH has excitatory effects on NMDA receptors at the hippocampus, has made this peptide a focus of attention with respect to ischemia-induced hippocampal damage.

There are specific mechanisms linking the excitatory effects of CRH to CA1 neurons of the hippocampus. Hippocampal CRH neurons are largely interneurons, which form axosomatic and axodendritic synapses on pyramidal and granular cells [25]. CRH in

these interneurons is colocalized with GABA and, to a lesser extent, neuropeptide Y [172,504]. In the hippocampus, actions of CRH appear to involve suppression of slow Ca^{2+} activated K^{+} currents (also called slow afterhyperpolarizations (sAHP)), the net effect being to increase the spontaneous neuronal discharge frequency at the hippocampus [5]. This appears to represent an intrinsic action of CRH as CRH antagonist administration has been shown to partially promote sAHP [439]. As well, CRH antagonists inhibit other substances involved in suppressing sAHP, such as hippocampal norepinephrine (NE) and serotonin (5-HT) [176,177,221]. If such a system is activated following ischemia, it could provide a mechanism by which CRH induces its excitatory and potentially neurotoxic effects in the CA1 region of the hippocampus.

However, it should be added that not all CRH effects at the hippocampus are excitatory. For instance, CRH and CRH agonists administered *in vitro* exert an inhibitory effect on long-term potentiation (LTP) at the hippocampal CA1 region. Higher concentrations of CRH have less depressing effects on neuronal activity, yet they still prevent the occurrence of LTP [442].

In addition to direct effects of CRH on hippocampal neurons, the role of CRH in ischemia is suggested by the peptide's interactions with a number of biochemical signals known to mediate ischemia-induced neuronal damage. Among these factors, the increased glutamate production associated with excitatory amino-acid-induced neurotoxicity in hypoxia and ischemia may be particularly relevant. Thus, Piekut & Phipps [417] found that kainate-induced neurotoxicity, which stimulates a subset of ionotropic glutamate receptors, resulted in a significant increase of CRH-immunolabeled fibres, particularly in those regions with cellular degeneration. As well, a number of

studies have shown that glutamate can directly stimulate amygdala and hypothalamic neurons to release CRH [155,226].

CRH also interacts with a range of other physiological signals within the ischemic cascade that have been implicated in neurodegeneration. For instance, a number of studies have demonstrated the ability of CRH to stimulate increases in intracellular Ca^{2+} levels [233,249,250,279] an effect specifically blocked by CRH antagonists [249]. In addition, interleukin-1 (IL-1), which has pro-inflammatory effects and a demonstrated capacity to exacerbate ischemia-induced damage, has been shown to induce a pronounced increase in CRH synthesis and release [495]. In this context, Rothwell et al. [463] hypothesized that IL-1 stimulation of CRH might represent a mechanism associated with ischemia-induced neurodegeneration. CRH also interacts with other pro-inflammatory cytokines and has been shown to induce a significant increase in $\text{TNF}\alpha$ release [556], an important trigger of the apoptotic cascade. Finally, CRH has also been shown to significantly promote the phosphorylation of phospholipase C [251], a critical enzyme involved in the breakdown of phospholipids within the cellular membrane following global ischemic insults.

To date, the involvement of CRH activation in ischemia-induced cell death has been inferred from studies demonstrating neuroprotective effects of centrally administered CRH antagonists in response to focal and global ischemia, with neuronal protection maximally reaching 80% of normal controls in some studies [311,516]. Although these studies did not identify the mechanism by which CRH antagonists confer neuroprotection, Lyons et al.[311] reported that increases in forebrain EEG activity induced following global ischemia were reduced in animals that received the CRH

antagonist, suggesting that CRH's effects on neuronal excitability may contribute to its neurotoxic effects. CRH antagonism also produced a 40% reduction in cell death produced by NMDA activation [516] and conferred neuroprotection in models of kainic-acid induced seizures [314].

Despite evidence that CRH induces a number of effects and interactions suggesting it may play a mediating role in ischemia, the participation of CRH in excitotoxic processes remains ambiguous. Thus, it has yet to be demonstrated that a global ischemic insult does stimulates CRH activity. Moreover, research has failed to show direct neurotoxic effects of *in vivo* CRH administration, and CRH does not appear to exacerbate glutamate-induced neurotoxicity [98]. In fact, a number of studies have even shown that CRH and CRH receptor activation can produce neuroprotective effects in models studying oxidative stress [402], hypoxia [152] and Alzheimer's disease [401]. Furthermore, Elliot-Hunt et al. [140] recently demonstrated that CRH administration could protect against glutamate-induced neurotoxicity by inhibiting the phosphorylation of a number of potentially destructive enzymes.

3.6 Specific research objectives

A second major objective of the current thesis aimed to further elucidate the role of CRH in global ischemia. This will be accomplished in part through assessment of endogenous fluctuations in CRH expression in distinct regions of the brain associated with a prolonged ischemic insult, using a variety of techniques including brain micropunch and *in vivo* microdialysis. Using pharmacological tools, an attempt will also be made to further elucidate interactions of CRH with other physiological signals, such as

glutamate and/or NMDA activation, which have well-characterized roles in global ischemia. In light of the suggested participation of CRH as a physiological signal in ischemia, the impact of modulation of CRH transmission (through the use of selective as well as non-selective CRH receptor antagonists administered systemically and/or centrally) on behavioural and physiological events associated with ischemic states will also be determined. To date, studies demonstrating neuroprotection through CRH modulation have primarily relied on peptide-based antagonists, which do not penetrate the blood-brain barrier and cannot be administered systemically (a limiting factor for clinical treatment). These antagonists are also non-specific, inhibiting both post-synaptic CRH receptor subtypes. The CRH2 receptor is known to share a high affinity with other endogenous peptides including urocortin [498], which has known excitatory effects in the hippocampus of rats [500]. Thus, it remains possible that the neuroprotection conferred by CRH antagonists involves attenuation of CRH and/or other endogenous compounds. In addition, although CRH antagonism has conferred neuroprotection in models of global ischemia, whether this neuronal protection translates into attenuation of behavioural deficits is not known. To address this, a battery of behavioral tests was developed, some of which subsequently served to properly evaluate the efficiency of CRH antagonists in protecting against ischemia-induced behavioural impairments. It is hoped that such behavioral assessments combined with cellular and molecular research findings can help broaden our understanding of the role of CRH in ischemia and of its potential value in the development of new therapeutic strategies.

4. Scientific contribution

Combined with the research work of the first part of this thesis, these studies shall together provide a significant contribution to the understanding of the physiological and behavioural functions of CRH in two strategic areas of research regarding this peptide: anxiety and global ischemia. More specifically, these studies should help 1) elucidate some of the physiological mechanisms by which CRH may mediate anxiety and ischemic processes, 2) identify discrete brain loci where endogenous alterations of the peptide content are occurring in response to these states, 3) further characterize behavioural and cognitive alterations associated with anxiety-provoking events and global ischemia and, 4) determine the effect of CRH antagonists in reversing the anxiety response as well as neurodegenerative and behavioural deficits observed following global ischemia.

Preface to Experiment 1

Prior to evaluating the role of CRH in mediating the behavioural response to processive stressors, experiment 1 will characterize a neophobia-based feeding paradigm with features highly characteristic of processive stressors. This “novel cage paradigm” is based on the observation that a novel environment engenders an anxiety-like state in rodents, related to the existing conflict between the needs to explore the new environment for food as well as exert vigilance against potential threats in the unfamiliar environment. In their home cage, non-food deprived rats readily approach and consume a familiar palatable snack. However, when the same snack is presented in a novel environment, the latency to approach it gets longer and the amount consumed declines. One advantage of this paradigm is that it affords the ability to continuously monitor the release of neurochemicals (using *in vivo* microdialysis) such as CRH, whereby samples could be collected in the home cage environment and then continue to be monitored during and after their transfer into the novel (and presumably stressful) environment.

Experiment 1

Characterization of novelty-induced feeding suppression.

Abstract

The intention of this chapter was to standardize the use of novelty as an anxiety model, as it adequately meets the criteria for a processive stressor. Consistent with previous findings, the current study demonstrated that rats placed in an unfamiliar environment were reluctant to consume a familiar snack. This suppression of intake exceeded 50% and lasted at least 6 days. In addition, a prototypical anxiolytic drug, the benzodiazepine diazepam, dose-dependently reversed this suppression. At anxiolytic doses, diazepam did not increase consumption of the palatable snack in the non-stressful environment of the home cage, even with extended feeding times, although it did effectively increase consumption of other diets, namely powdered sugar. Thus, snack consumption in an unfamiliar environment appears to represent a sensitive and reliable index of anxiety, which allows for the use of *in vivo* monitoring techniques warranting dynamic assessment of neurochemical changes (such as CRH release) in situations presenting different anxiety levels. Moreover, the paradigm possesses high ethological validity and easily lends itself to the evaluation of putative anxiolytic compounds, such as CRH antagonists.

Introduction

Although CRH has been closely linked to the stress response, its role in mediating processive stressors or anxiety is less clear. Specifically, it remains to be determined whether CRH antagonists would attenuate the behavioural response to certain processive-type stressors, and whether CRH activity at the amygdala is indicative of an anxious state. In order to investigate both questions, it is first necessary to define a valid anxiety model, which would also allow for pharmacological and physiological interventions necessary to examine the specific role of CRH.

Processive stress or anxiety models, by definition, involve stimuli that require a degree of processing before they are interpreted as threatening. For survival purposes, overt or explicit threats, such as toxicity or predator exposure, generate rapid responses with minimal appraisal. Situations with greater ambiguity regarding the nature of the threat require more appraisal or evaluation. Numerous paradigms lend themselves as potential anxiety models, including restraint, immobilization, noise stress, light/dark exploration, the elevated plus maze and neophobia models. These generally involve conditions or stimuli which themselves do not challenge the organism's physical integrity, but nonetheless induce a perceived sense of threat.

Of these paradigms, neophobia models contain perhaps the fewest explicit cues (or the greatest ambiguity) regarding the nature of the threat. Neophobia paradigms involve changing an animal's environment from a familiar to an unfamiliar one, such as moving them from their home cage to a novel cage. There are no harmful or painful stimuli associated with the new environment, nor does it involve overt sensory cues (as in restraint or noise stress), aversive or unpleasant stimuli (e.g. light or odours) or explicit

threats of danger (elevated planes). Nonetheless, it is able to invoke behavioural and physiological changes characteristic of a stress response, including behavioural withdrawal [597], defecation [107,146,182], inhibition of feeding [28,425,485,508] as well as activation of the autonomic nervous system [546] and the hypothalamic-pituitary-adrenal (HPA) axis [148,403,578].

The inhibition of feeding behaviour produced by novel environments has been well-replicated [54,485,508] and is reversible by a variety of anxiolytic compounds including benzodiazepines, serotonergic and noradrenergic drugs [49,50,446,515,534]. Assessing food intake in an unfamiliar environment is a relatively simple and practical behavioural paradigm with an easily quantifiable end point. It also allows for the evaluation of putative anxiolytic compounds, such as CRH antagonists, and for invasive physiological techniques (such as *in vivo* microdialysis), which would permit measurement of endogenous fluctuations in CRH release.

Despite its advantages, certain methodological issues have limited the use of neophobia models. For instance, some studies have included food deprivation or the presentation of a novel food, both of which confound the anxiogenic attributes of the unfamiliar environment. As well, the presentation of regular chow in some studies has yielded low basal feeding levels, thus making further reductions of food intake less discernible. To circumvent these concerns, we applied modifications to the neophobia paradigm, including the use of non-food deprived animals and the presentation of a highly palatable, familiar snack in both the home and novel environment. This ensured a sufficiently high level of intake and avoided additional stresses associated with physiological deprivation or unfamiliar food.

To replicate previous findings, it was also necessary to demonstrate that the inhibition of feeding is reversible by established anxiolytic drugs, which themselves do not stimulate appetite or food intake. Thus, in addition to characterizing novelty-induced feeding suppression with the conditions described above, the current chapter will evaluate the response to pre-treatment with the benzodiazepine diazepam. Diazepam has shown potent anxiolytic effects in a wide range of exploratory, conflict, social interaction and defensive models of anxiety. In fact, it has been the drug of reference for validating numerous prospective anxiety models [103,118,548] and for evaluating putative anxiolytic drugs [57,81,169,259,321,445]. Together, these studies will hopefully establish the utility of using a neophobia-based feeding paradigm as a model of processive stressors.

Materials and Methods

Animals

Adult male Sprague-Dawley rats (400-550 g) obtained from Charles River Laboratories (Rochefort, Quebec, Canada) were used in all experiments. Subjects were individually housed and maintained on a 12-h light/dark cycle (lights on at 7:00 AM), with free access to water and standard (Purina) rat chow. The room temperature was maintained at 21-23°C with 60% relative humidity. Water and food were available *ad libitum*, and the experimental subjects were non-food deprived at the time of testing.

Apparatus (Test environment: home- vs. novel-cages)

The animal's home cage consisted of a shoebox style clear Plexiglas container (24 x 30 x 18 cm) with the bottom lined with bedding material (beta chips about 1 cm deep). The roof of the home cage was made of a removable, stainless steel grill with a triangular indentation (about 10 cm deep) to hold rat chow pellets and the water bottle. The novel environment (test cage) consisted of a Plexiglas box with dimensions different from those of the home-cage (48 x 30 x 40 cm). The front of the box served as a hinged door. The floor consisted of an elevated stainless steel grid 3 cm from the Plexiglas bottom. Both the home- and the test-cages were housed in the same room where the testing took place.

Drug Preparation

Diazepam (Sabex, Boucherville, Quebec) was dissolved in a vehicle of 50% propylene glycol, 40% distilled H₂O and 10 % ethanol and injected subcutaneously (s.c.) in a volume of 0.1-ml/100 g body weight.

Behavioural measures

Animals' behaviour was monitored in the home cage in vehicle pre-treated animals and in the novel cage in vehicle and diazepam (2.0 mg/kg) pre-treated animals. The behaviour was observed and recorded at 20 sec intervals for 15 min in all environments. Monitoring was conducted by the experimenter using a portable PC computer (Toshiba 4250) and datalogging software. Animals were monitored for frequency of feeding

behaviour, locomotor activity, sniffing, grooming, rearing and stillness/resting (absence of activity).

Experimental procedures

Experiment 1: Effect of novel cage exposure on food intake

Ad libitum fed animals ($n = 6$) were given pre-weighed quantities (approximately 7-10 g) of Graham crumbs (Honey Graham Crumbs, Christie; 70.6% carbohydrate, 10.6% fat, 7.1% protein) in their home cage, once daily for a 15 min period. This snack food was presented in a round ceramic dish (10 cm in diameter). After 6 successive days, the snack intake reached a plateau such that the intake over the last 3 days (baseline) did not vary by more than ± 0.5 g. On the 7th day, rats were placed in the novel cage, and presented with the now familiar graham crumb snack. After 15 min, they were returned to their home cage and the amount of food consumed immediately measured. After 6 consecutive days of this palatable food presentation in the novel cage, the rats were again presented with that snack in their home cage (once daily for 15 min) for the final 2 days.

Experiment 2: Effect of diazepam on novelty-induced suppression of food intake

Subjects ($n = 6$) were injected with vehicle or diazepam (0.5, 1.0, 2.0 or 3.0 mg/kg; sc in a randomized order) and placed in either the home-cage or the novel-cage. The graham crumb snack was presented 20 min later. As in Experiment 1, the food was removed after 15 min and weighed. The home-cage represented a familiar or non-stressful environment. It was contended that if diazepam increased food intake due to non-anxiolytic or non-specific effects (e.g. enhanced palatability or orexigenic actions), it would also be reflected by altered food consumption in the home-cage. However, in the

home cage, subjects (controls as well as diazepam treated) consumed the snack for most of the 15 min test period, and thus it was thought that the time provided for food consumption might not have been long enough to let potential differences manifest (i.e. there might have been a ceiling effect confound). Therefore, three distinct rat groups ($n = 6-8$ / group) were given food in their home cage for 15, 25 min or 40 min following an injection of vehicle or diazepam (2.0 mg/kg; the dose deemed most effective in the novel cage portion of the study).

As well, given that under certain conditions (see [41] for review) benzodiazepine drugs have been reported to stimulate feeding, it was of interest to determine whether the absence of such effects in the current study was attributable to the specific diet selected in the present study. To test this possibility, three independent groups of rats ($n = 7-10$ /group) were served different diets varying in their carbohydrate content. The groups received either powdered Rat Chow (50% carbohydrate), Graham crumbs (70% carbohydrate) or granular sugar (100% carbohydrate). Subjects were first habituated to the diet and then given vehicle or diazepam (2.0 mg/kg) 20 min prior to food presentation in the home-cage.

Statistical analysis

The amount of food consumed across sequential test days (under home and novel cage conditions) was analyzed using either one- or two-factor analyses of variance (ANOVA). In cases where animals were repeatedly tested within the home-cage or the novel cage condition, or tested under vehicle and drug condition, these variables (i.e. cage and drug condition) were considered as within-group factors. When these variables were

assessed using distinct groups of animals they were treated as between-group factors. Post-hoc analyses of significant main effects or simple effects of significant interactions were conducted using Newman-Keuls multiple comparisons. In figures, error bars represent the standard error of the mean (SEM).

Results

Experiment 1: Effect of novel-cage exposure on food consumption.

The ANOVA revealed significant variations of food intake across testing days ($F_{(13,60)} = 27.685, p < 0.001$). Post hoc analyses revealed that this was attributable to a significant and long lasting drop in food intake upon presentation of the familiar palatable snack in the novel-cage (day 7-12). This novel cage-induced suppression was evident for each of the 6 consecutive test days. When the rats were re-tested under the home-cage condition once more (days 13 and 14), the intake increased sharply to levels comparable to those of the original baseline (see Fig. 1).

Experiment 2: Effect of diazepam on snack consumption in home-cage or novel-cage conditions.

Repeated measures ANOVA revealed a significant effect of diazepam on food intake in the novel cage ($F_{(4, 24)} = 8.51, p < .001$). Post-hoc analysis revealed that the effects to enhance food consumption reached significance at the 2.0 and 3.0 mg/kg dose (see Fig. 2; top). In the home-cage condition, only a slight increase in food consumption was noted, which in contrast to the novel-cage condition occurred at the lower (0.5 mg/kg; $p < .05$) but not at the higher (2 and 3 mg/kg) diazepam doses (see Fig. 2; bottom). It is of interest

to note that at these higher doses of diazepam, the amount of food consumed in the novel and home-cage conditions were almost identical.

A subsequent experiment assessed whether the lack of diazepam (2 mg/kg) effect in the home-cage was attributable to insufficient time to ingest differential amounts of the snack (i.e. due to the potential ceiling effect). Hence, three groups of rats were provided with the palatable food in their home cage for 15, 25 or 40 min. The data were analysed using a two-way ANOVA. The dependent variable was the amount of Graham crumbs consumed. The between-group factor was the time of snack access including the 3 levels (15, 25 and 40 min), while the within-group factor was the treatment with 2 levels (vehicle or diazepam: 2 mg/kg). The analysis revealed that there was a significant effect of Time ($F_{(2,16)} = 45.05, p < .0001$) but no Treatment effect ($F_{(1,16)} = 1.36, p = 0.26$), nor a significant Time X Treatment interaction ($F_{(2,16)} = .646, p = 0.537$). Thus, food intake appeared to increase progressively with access time, although diazepam failed to enhance food intake in the home cage at any duration (see Fig. 4; top).

Lastly, we assessed whether the effects of diazepam were dependent upon the carbohydrate content of the snack. Different snacks (individually) that varied in their carbohydrate content (Rat Chow (50%), Graham crumbs (70%) or granulated sugar (100%)) were used. The results were analysed using a 2-way ANOVA and revealed a significant Snack type X Treatment interaction ($F_{(2,22)} = 3.7, p < .05$). Post hoc analysis (Newman-Keuls) revealed that this effect was attributable to differential consumption of the various snack types. Specifically, food consumption appeared greatest for Graham crumbs followed by granulated sugar while very little amount of rat chow was consumed. A significant interaction was attributable to the fact that whereas diazepam failed to affect

the intake of Chow or Graham crumbs, it significantly increased the consumption of granulated sugar (see Fig. 4; bottom).

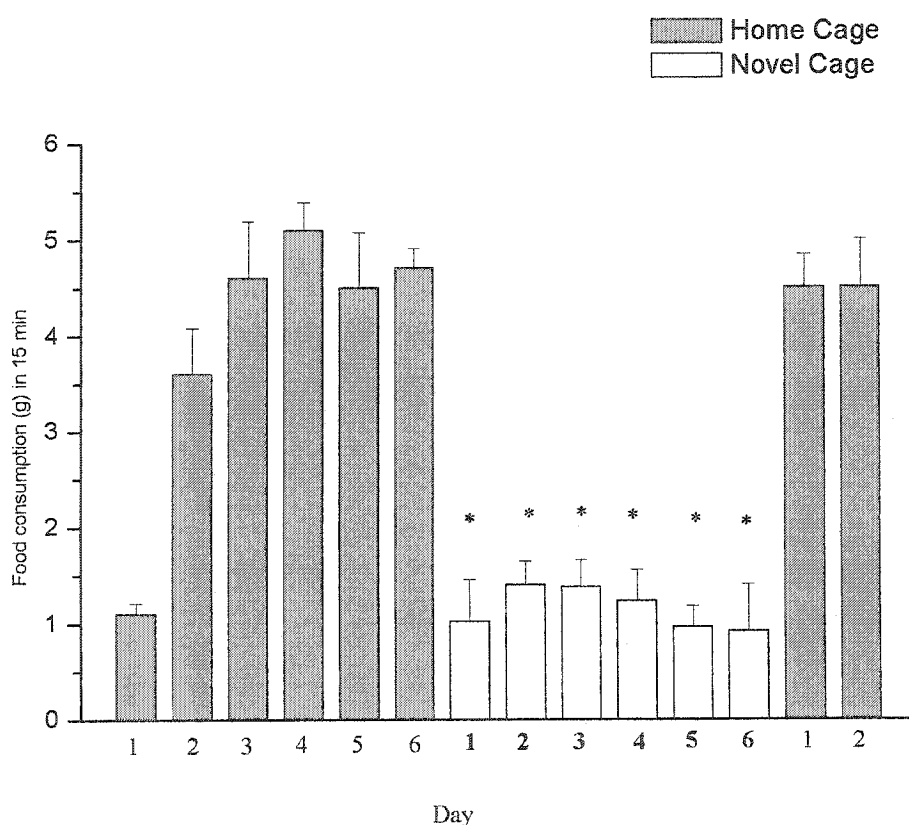


Figure 1: The effect of an unfamiliar environment on snack intake. Consumption of an initially novel snack (Graham crumbs) in the home cage is depicted in gray columns, whereas open columns represent that in the novel cage. The ordinate reflects the amount of snack consumed (g) over the 15 min interval. The abscissa represents consecutive test days; thus, on successive days, rats were tested in the home cage (6 days) then the novel cage (6 days) and then returned to the home cage (2 days).

* Significantly different from the final home-cage baseline values (Day 6) at $p < 0.01$.

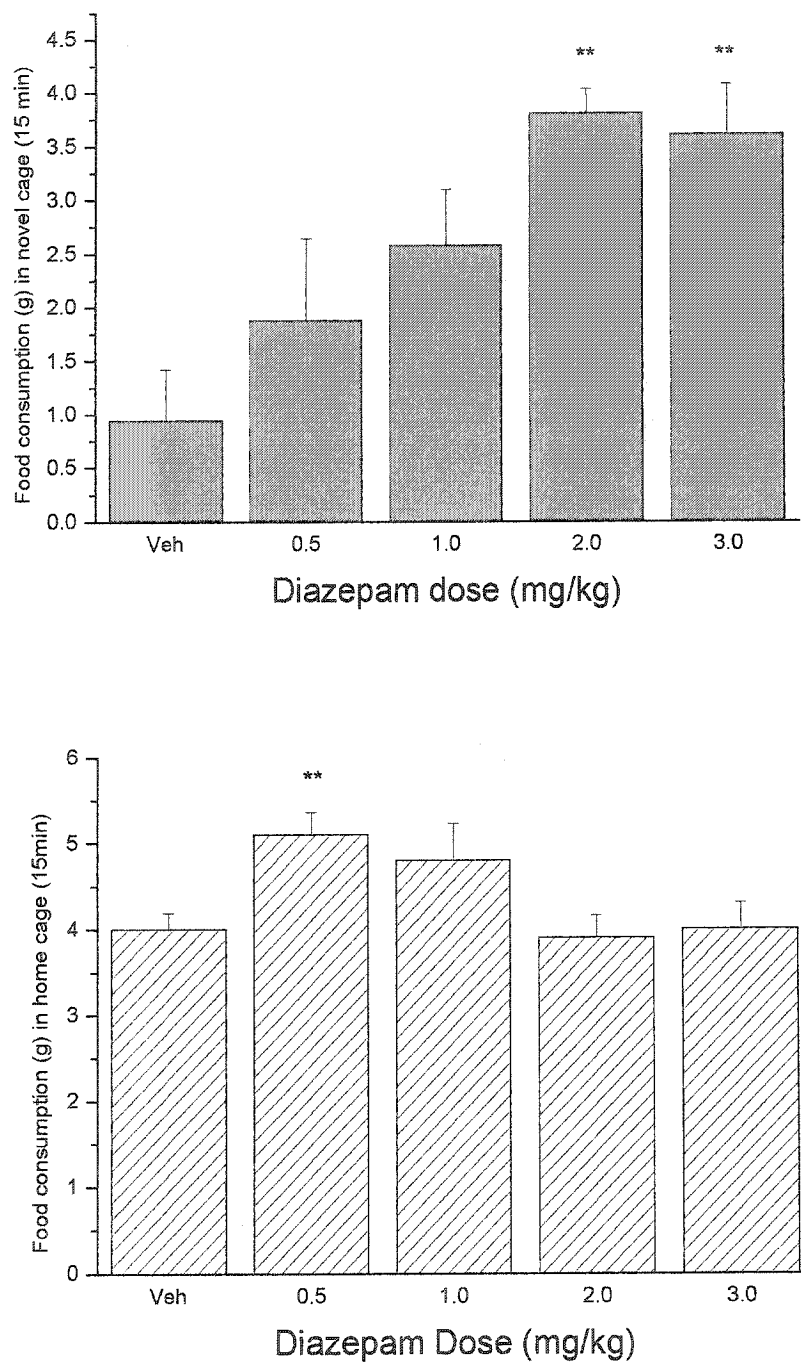


Figure 2: Effect of diazepam on snack consumption in the home cage and novel cage conditions. Top figure depicts snack consumption in the novel cage, whereas the bottom figure shows consumption in the home cage. The ordinate depicts the amount of snack consumed (g) over 15 min. The abscissa depicts the treatments: control (vehicle) or diazepam (0.5 to 3.0 mg/kg).

****** Significant within group differences (from respective home cage or novel cage baseline controls), $p < 0.01$.

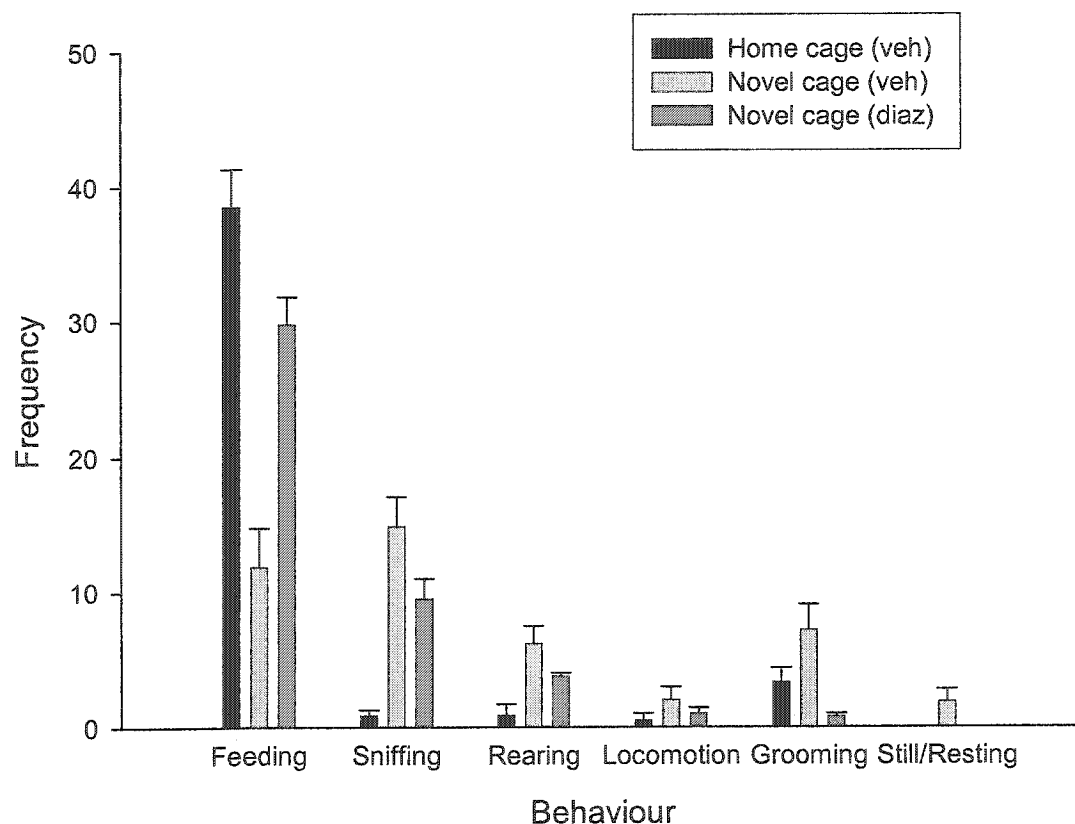


Figure 3: Behavioural profile of animals in the home or novel cage over 15 min. As compared to the home cage (black bar), vehicle-treated animals in the novel cage (light-gray) showed reduced incidence of feeding behaviours, but higher exploratory type behaviours such as sniffing and rearing. When pre-treated with diazepam (2.0 mg/kg; dark-gray), feeding behaviour in the novel cage increased dramatically while exploratory type behaviours decreased slightly. In contrast to what might be expected from a sedative or muscle-relaxing effect, diazepam-treated animals did not show an increase in stillness/resting behaviours.

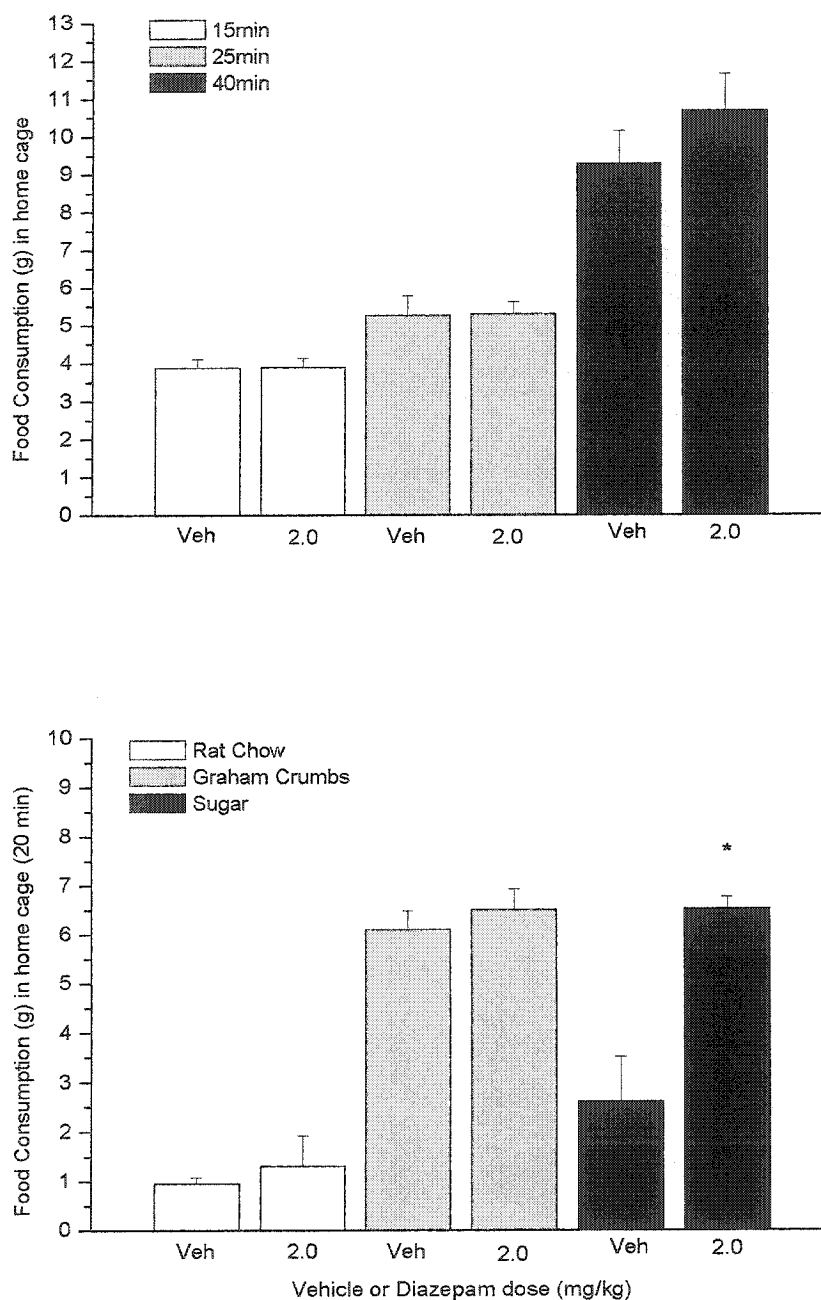


Figure 4: Effects of diazepam on home-cage snack consumption as a function of duration of snack access and test diet. In the home cage, groups of rats (6-8 per group) were given access to snack over various durations: 15, 25 or 40 min (top figure). Amount of snack consumed is represented on ordinate. In each case, rats were pre-treated with the vehicle or diazepam (2.0 mg/kg). Bottom figure represents the impact of carbohydrate content on the amount of snack consumed in the home cage over 20 min, in vehicle or diazepam pre-treated rats. Carbohydrate content for each diet is: Rat chow: 50%; Graham crumbs: 70%; Powdered sugar: 100 %.

* Significantly different from the matched vehicle condition, at $p < 0.001$.

Discussion

The intention of this study was to standardize the use of novelty as an anxiety model, as it closely meets the criteria of a processive stressor. It also offers numerous practical advantages over other anxiety models which often require extensive training (fear-potentiated startle), sophisticated equipment (acoustic startle), or simply have physical structures (such as an open field or elevated plus maze) which make it difficult to perform dynamic monitoring of physiological changes using procedures such as *in vivo* microdialysis.

Consistent with earlier research [50,446,485] the present study showed that when placed in an unfamiliar environment, rats were reluctant to consume a familiar snack. Thus, the introduction of novelty (by placing rodents in a novel cage) produced a robust suppression in the consumption of palatable food in non-deprived rodents. This suppression was greater than 50% and lasted for 6 days in the conditions tested (15 min exposure/day). This suppression was also dose-dependently reversed by the benzodiazepine diazepam. As an increase of consumption necessarily involved the initiation of an active response, this outcome could not be attributed to motor side effects or lethargy elicited by the anxiolytic.

Interestingly, with regards to the animals' behavioural profile, animals showed a decrease in the incidence of feeding behaviours when moved to the novel cage as compared to the home-cage condition, but an increase in exploratory-type behaviours such as sniffing and rearing. Contrary to what might be produced by a sedative effect, when pre-treated with diazepam, animals in the novel cage showed an increase in feeding behaviours but no increase in stillness or resting-type behaviours.

At anxiolytic doses, diazepam did not increase consumption of this particular palatable snack in the non-stressful environment of the home cage, even with extended feeding times, although it did effectively increase consumption of other diets, namely powdered sugar. This strongly suggests that although diazepam may have orexigenic effects, its effectiveness at reversing feeding suppression in the current conditions were likely due to its anxiolytic properties. Thus, it appears that snack consumption in an unfamiliar environment is a sensitive and reliable index of anxiety, and the simplicity of this paradigm permits the concurrent and dynamic assessment of neurotransmitter changes under conditions of high ethological validity, as well as the assessment of other potential anxiolytic compounds, such as CRH antagonists. Indeed, partially based on the results from this study, the current model was recently validated for use in studying neurobiological mediators of anxiety in mice [337].

Preface to Experiment 2

To assess the mediating role of CRH in a processive stress model, Experiment 2 determined the impact of CRH inhibition on the behavioural response produced by novel cage exposure. In addition, the effect of the novelty-stressor on the release of CRH, glutamate and GABA at the central nucleus of the amygdala (CeA) is assessed. It was hypothesized that if the novel environment was indeed acting as a stressor, it should increase the release of CRH at the CeA. Likewise, if the effects of the novel cage on behavior were mediated through CRH at the CeA, then diazepam, which prevented anxiogenic-type behavioral responses in the novel environment, should attenuate this increase in release. Finally, if the behavioural response in the novel cage is attributable to stressor-induced changes in central CRH concentrations, then pretreatment with CRH receptor antagonists should attenuate or block this anxiogenic response.

Chapter 2

The mediating role of CRH and amygdala CRH in novelty-induced inhibition of feeding.

Abstract

The corticotropin-releasing hormone (CRH) system within the brain is activated by stressful stimuli and may contribute to behavioral and emotional responses. The present investigation assessed anxiety-like responses and neurochemical alterations at the central nucleus of the amygdala (CeA) evoked by exposure to an unfamiliar environment. As indicated in chapter 1, placement into an unfamiliar environment (novel cage) markedly suppressed ingestion of a palatable snack, and this effect was antagonized in a dose-dependent manner with diazepam pretreatment. As such, this behavioural change was utilized as an index of anxiety in the rodent. This stressor also stimulated the release of immunoreactive-CRH (ir-CRH) and glutamate at the CeA. However, various selective and non-selective CRH antagonists (e.g. α h-CRF, CP-154, 526, antisauvagine-30, preproTRH₁₇₈₋₁₉₉) did not attenuate the behavioral effects elicited by the stressor. Despite the potent anxiolytic effect of diazepam in this paradigm, it failed to prevent the stressor-associated release of ir-CRH and glutamate at the CeA. Thus, although exposure to an unfamiliar environment may be interpreted as being “stressful” by neural circuits involving CRH and/or glutamatergic receptors at the CeA, the data do not support the view that activation of CRH receptors is necessary for the expression of anxiety-like behavioral responses. Rather than provoking anxiety, it is suggested that these systems might serve to draw attention to events or cues of biological significance, including those posing a threat to survival.

Introduction

The participation of CRH in stress responses has been studied for almost two decades. Close to twenty distinct psychological and physiological stressors activate the HPA axis as a coping or adaptive mechanism via CRH activation (see [198] for review). The principal anatomical structures mediating these effects include the amygdala and the hypothalamus. Intracerebroventricular infusions of CRH also produce a constellation of behavioural, physiological and endocrine changes similar to those produced by natural stressors [58,59,139,452,453].

With respect to processive stressors or anxiety, CRH activity at the amygdala is believed to play a particularly important mediating role. The HPA response to processive stressors is attenuated or blocked by lesions to the amygdala. It is thought that the amygdaloid complex in general, and the central nucleus of the amygdala (CeA) in particular, contribute to emotional responses such as the acquisition and expression of fear and anxiety [112,562]. They also appear to influence processes by which sensory stimuli gain motivational and emotional significance [156,229,277].

CRH mRNA, protein and receptors are also prominent at the amygdala [317,335,427,468,528]. Different stressors have been shown to increase CRH mRNA activity and peptide expression within the amygdala [206,232,305,341] and manipulations of amygdaloid CRH seem to be closely related to initiation of anxiety [130,528]. As well, various aversive conditions (such as withdrawal from alcohol, cocaine or cannabinoids) also influence the levels and/or turnover of CRH at the central nucleus of the amygdala [414,448,449,456,591].

Despite the seemingly close associations between CRH, amygdala CRH and stress, the role of this peptide in processive-type stressors or anxiety has, of late, come into question. One issue has been the inconsistent effect of CRH antagonists as anxiolytics in behavioural stress models. For instance, in the elevated plus maze, which tests the willingness of animals to walk on an elevated plane, some studies have shown an anxiolytic effect for CRH antagonists [194,510] while others have not [22,43,170]. Moreover, when CRH antagonists have shown anxiolytic activity in this model, this typically occurred when testing was preceded either by exposure to another stress or exogenous CRH infusion. Systemic administration of a CRH1 antagonist was also ineffective in influencing the anxiety state caused by other 'exploratory' stressors such as the light/dark exploration task [380]. On the other hand, under more threatening conditions, including conditioned fear [200], shock-induced freezing [528], defensive withdrawal [170] or conditioned defeat [224], CRH antagonists appeared to be effective in attenuating anxiety-related behaviours.

Thus, CRH systems may play a mediating role in the behavioural manifestations of stimulus-specific fear as produced by pain or predator exposure, or in the response to intense stressors. However, it is unclear whether this extends to ambiguous circumstances such as induced by a novel environment.

A second issue is the degree to which amygdala CRH activation is anxiety-specific. Although CRH in this region responds to anxiogenic stimuli, it may also respond to more general stimuli or conditions. Makino et al. [317] found that amygdala CRH also responds to aversive conditions which are not stressful enough to provoke HPA activation. In their study, non-stressed rats were placed in a chamber with rats that had

previously been footshocked. This condition was not stressful enough to invoke an HPA based-coping response in non-footshocked rats, as indicated by failure of this paradigm to induce changes in hypothalamic CRH or plasma ACTH and corticosterone. However, such a procedure did increase amygdala CRH mRNA expression. The authors suggested that changes in amygdala CRH might reflect arousal associated with a negative event.

However, it could be argued that arousal produced by negative events or emotions may be fundamentally similar to a stress effect. Recent evidence suggests however, that conditions associated with rewarding events also promote amygdala CRH activity. Merali et al. [338] demonstrated that consumption of palatable food provoked an increase in CRH release at the CeA equivalent to that produced by a 20 min restraint stress. Thus, as with the amygdala in general, amygdala CRH may respond to stimuli of emotional significance regardless of whether they are positive or negative.

In the preceding chapter, it was demonstrated that consumption of palatable food was suppressed when subjects were placed in a novel cage. This suppression was reversed when subjects were pretreated with the anxiolytic drug diazepam. The extent to which CRH activation mediates this effect will now be examined via administration of a number of CRH antagonists prior to placement in the novel environment. These antagonists will include a CRH release inhibitor, non-specific CRH antagonists (i.e. which simultaneously block CRH1 and CRH2 receptors), as well as antagonists selectively blocking CRH1 or CRH2 receptor subtypes. If CRH activation mediates the behavioural response to anxiety, as assessed in this model, then inhibition of this activity should have an anxiolytic effect in the form of increased food intake.

The current chapter will also examine whether amygdala CRH is responsive to changes in the anxiety state. Firstly, it will be examined if transition from a familiar to an unfamiliar environment is sufficient to provoke an increase in amygdala CRH release. If so, it would then be of interest to determine if this increase is attenuated by pre-treatment with diazepam. As diazepam attenuates the behavioural response to anxiety in this model, a concomitant reduction in amygdala CRH release might be expected.

Thus far, much of the evidence for a role of CRH in the stress response has come from indirect measures such as *in situ* analysis of mRNA or protein expression in post-mortem tissue. These techniques can be used as indicators of gross changes in CRH concentrations, but one cannot know if fluctuations in mRNA expression or peptide content in brain tissue is accompanied by changes in the actual peptide release. Furthermore, these techniques represent more static assessments that do not allow for dynamic monitoring of CRH changes, across time and conditions, within subjects. The experiment thus proposed offers a significant contribution in that it will provide a dynamic profile of amygdala CRH release associated with anxiety, using *in vivo* microdialysis combined with a solid-phase radioimmunoassay (RIA).

In addition to the role of CRH, the release of the amino acids glutamate and GABA from the amygdala, in response to stress and diazepam pre-treatment, will be measured. Glutamate and GABA respectively represent the two principal excitatory and inhibitory neurotransmitters in the amygdala. Like CRH, both systems may also influence anxiety. Benzodiazepine infusion in the amygdala produces profound anxiolytic effects [334] and anxiolytic actions of systemically administered benzodiazepines can be blocked by intra-amygdaloid injections of GABA antagonists [113]. Benzodiazepines facilitate the ability

of endogenous GABA to stimulate GABA_A receptors, via partial allosteric modulation of the receptor. In addition, when microinfused locally into the amygdala, glutamate directly stimulates CRH release at the median eminence. Thus, glutamate may contribute to stress responses by affecting CRH release at the amygdala and median eminence [155,466]. Together, the proposed studies will attempt to assess the involvement of CRH and amygdala CRH in mediating the behavioural response in a neophobia-based model of anxiety.

Materials and Methods

Animals

Adult male Sprague-Dawley rats (400-550 g) obtained from Charles River Laboratories (Rochefort, Quebec, Canada) were used in all experiments. Subjects were individually housed and maintained on a 12-h light/dark cycle (lights on at 7:00 AM) with free access to water and standard (Purina) rat chow. The room temperature was maintained at 21-23°C with 60% relative humidity. Water and food were available *ad libitum* and the experimental subjects were non-food deprived at the time of testing.

Surgical procedures

All surgeries were carried out in rats anaesthetized with pentobarbital (65 mg/kg *i.p.*), according to the procedures and guidelines approved by the Animal Care Committee. Animals were placed in a stereotaxic instrument with leveled skull. For microdialysis studies, rats were implanted with a 20-gauge guide cannula containing a removable 24-gauge obturator aimed at the CeA using the following co-ordinates: 2.1mm posterior to

bregma, 4.2 mm lateral to the midline and 7.35 mm ventral to levelled skull surface. For microinjection studies, guide cannulae (22 gauge) aimed at the 3rd ventricle were positioned using the following coordinates: 4.3 mm posterior to bregma, 0.0 mm lateral to the midline and 4.3 mm ventral to the skull surface. All coordinates were derived from the atlas of Paxinos & Watson [400]. Guide cannulae were anchored to the skull using 4 stainless steel screws and acrylic dental cement.

Apparatus

The home and novel cages are the same as those used in Chapter 1. For the microdialysis studies, the top of the home cage was modified to accommodate a microdialysis swivel, which allowed the animal to move freely within the cage during the sampling period. When subjects were transferred to the novel cage, the swivel was detached from the home cage and mounted on top of the novel cage.

Drugs and Peptides

The CRH release inhibitor, prepro-TRH₁₇₈₋₁₉₉ (Phoenix Pharmaceutical, Blue Mountain, CA) and the CRH receptor antagonist [D-Phe¹², C α MeLeu³]CRH (12-41) (kindly provided by W. Vale, Salk Institute, La Jolla, CA) were dissolved in saline, whereas α -helical CRH (9-41) (Phoenix Pharmaceutical) was dissolved in saline containing 0.05 % acetic acid. The CRH1 antagonist CP-154,526 (kindly donated by Pfizer Inc., Groton, CT) was dissolved in a vehicle of 90% saline, 5% ETOH and 5% Tween 80 injected intraperitoneal (*i.p.*) in a volume of 0.1 ml/100g. The CRH2 antagonist anti-sauvagine-30 (ASV-30; Phoenix Pharmaceutical) was dissolved in sterile

water. Except for the CRH1 antagonist (CP-154,526), all compounds were administered intracerebroventricularly (*i.c.v.*) into the 3rd ventricle.

Neurochemical analyses

The detection and quantification of CRH from the microdialysis studies was achieved using a highly sensitive solid-phase RIA, as described by Maidment & Evans [315]. Briefly, Protein A/G (Calbiochem, La Jolla, CA)-coated Immulon-4 wells (Dynatech, Chantilly, VA) were incubated with anti-CRH serum (rC70 kindly provided by W.Vale, the Salk Institute, La Jolla, CA) for 2 h at 20°C. Samples, standards (reconstituted in the KRB solution, ranging from 0.61 to 1250 fmol/well) or blanks were incubated for 24 h at 4°C. Next, 25 µl of assay buffer containing 4500-5000 CPM of [¹²⁵I-Tyr⁰] rCRH (Amersham, Oakville, Ontario, Canada) was added to each well and incubated for an additional 24 h period at 4°C. Finally, the wells were rinsed, separated, and their residual radioactivity counted using a gamma counter (Cobra II Auto-gamma). A four-parameter logistic curve fit model was used for interpolation of the standard curves. Sensitivity of the assay was typically ~0.1 fmol/well.

The interstitial levels of glutamate and GABA were also measured in the amygdala. All *ex vivo* analyses were performed on the Beckman P/ACE Capillary Electrophoresis System 5010 (Fullerton CA, USA) equipped with a laser-induced fluorescence detector (488 nm Beckman P/ACE System Laser Module). Separation of GABA and glutamate were achieved using fused silica capillary with 50 µm i.d. (Polymicro Technologies Inc., Phoenix, AZ, USA) and 47 cm in length. The run buffer consisted of a 100 mM boric acid adjusted with NaOH to the final pH of 9.5. Between runs, there were 30 s and 90 s

rinses of 1 M NaOH and run buffer, respectively. The 2 s pressure injection sample plugs were assayed at a separation voltage of 30 kV.

The amino acids GABA and glutamate were assayed in 5 μ l aliquots from each sample and reacted with 1.3 μ l of 1.0 mM fluorescein-5-isothiocyanate (Molecular Probes Inc., Eugene, Oregon, USA) in acetone. The reaction was allowed to proceed for 16 to 18 h before dilution in 100 μ l of run buffer. Each sample was analyzed in triplicate, and peak heights were averaged and quantified from the working curves of respective amino acid standards (Sigma, St. Louis, MO, USA). All samples from an individual subject were assayed during the same day. Unless otherwise stated, chemicals were purchased from VWR (Ville Mont-Royal, QC, Canada).

Histology

At the end of the microdialysis and ICV experiments, animals were briefly anesthetized with halothane and then sacrificed so that their brains could be extracted, sectioned, and stained for histological verification of probe placement. The correct placement of the 3rd ventricular guide cannulae was readily observable without staining under low magnification. Only data from correctly placed probes were used for statistical analyses.

Experimental procedures

Experiment 1: Effect of CRH antagonists on novelty-induced suppression of feeding

This set of experiments comprised 5 groups of rats, four of which were implanted with a guide cannulae aimed at the 3rd ventricle. The first group (n = 8) was injected *i.c.v.* with vehicle, or α -helical CRH (20 μ g) and the second group (n = 7) received vehicle and a higher dose of α -helical CRH (50 μ g). The third group (n = 6) was injected *i.c.v.* with vehicle, the CRH antagonist CRH (12-41) (10 μ g) and prepro-TRH (6 μ g). The fourth group (n = 10) was injected *i.c.v.* with vehicle and ASV-30 (2 and 10 μ g), while the final group (n = 10) was injected *i.p.* with vehicle or CP-154,526 (10 and 40 mg/kg). The subjects with cannulae were injected in a volume of 5 μ l delivered over 1 min interval, using a 1.0 ml Hamilton syringe controlled by an electronic syringe driver (Harvard 22; Harvard Apparatus, South Natick, MA). The Hamilton syringe was connected via PE 20 tubing to a stainless steel injector, which protruded 0.5-0.75 mm below the cannula tip and into the 3rd ventricle. Following injections, subjects were returned to their respective home cages for 20 min. They were then presented with the Graham crumb snack in the novel-cage for a 20-min period.

Experiment 2: Effects of novel cage exposure on interstitial levels of CRH, GABA and glutamate at the central nucleus of the amygdala (CeA)

Subjects (n = 8 per group) implanted with permanent guide cannula aimed at the CeA, were briefly anaesthetized with halothane and the obturator within the guide cannula was replaced with a microdialysis probe. The concentric microdialysis probes (with 2.0 mm length of active membrane, 250 μ m outer diameter) were made of

regenerated cellulose (6000 molecular weight cut-off; Spectrum Medical Industries) that protruded into the CeA. Each probe was secured with a retaining screw and connected via polyethylene tubing (Intermedic, Clay Adams, NJ) to a swivel and a 2.5 ml infusion syringe attached to an infusion pump. Microdialysis probes were perfused at 3 μ l/min with filtered Kreb's Ringer phosphate (KRB) solution consisting of (in mM): 2.7 K⁺, 145 Na⁺, 1.35 Ca²⁺, 1.0 Mg²⁺, 150 Cl⁻, 0.05 % ascorbate, pH 7.4 and BSA (0.1%). Samples (60 μ l) were collected in the subject's home cage every 20 min for 140 min. 20 min following vehicle injection, subjects were then gently transferred into the novel cage where sample collection continued every 20 min for 140 min. Samples were frozen on dry ice and stored at -80°C immediately upon collection.

Experiment 3: Effect of diazepam on amygdala CRH, GABA and glutamate following novel-cage exposure

The procedure was almost identical to that of Experiment 2. In this study, after 140 min of sample collection in the home cage, the subjects (n = 8) were injected with 2.0 mg/kg of diazepam and left in the home cage for 20 min. The subjects were then transferred to the novel cage for 140 min, during which the samples were continually collected. The dialysates accumulated over 20 min intervals were frozen on dry ice and stored at -80°C. Subsequent analysis of peptide and amino acid content was done using RIA and capillary electrophoresis procedures, respectively (as described earlier).

Statistical analysis

For the studies examining the effects of the various CRH antagonists, the amount of food consumed was analyzed by either one- or two-factor analyses of variance (ANOVA). In cases where animals were repeatedly tested within the home-cage or the novel cage condition, or were tested under the vehicle and drug condition (the various CRH antagonists), these variables (i.e. cage and drug condition) were considered to be within-group factors. When these variables were assessed in separate groups of animals, they were treated as between-group factors. The concentration of the baseline levels of ir-CRH, GABA and glutamate observed were averaged for individual rats and defined as 100%. All values were then expressed as a percent of that baseline, and analyzed by a mixed measures ANOVA with Drug treatment (vehicle vs. diazepam) as a between-groups factor and Cage (Home vs. Novel) and Sample (5 home cage and 5 novel cage) serving as the within-group factors. Post-hoc analyses performed on significant main effects or interactions were conducted using Newman-Keuls multiple comparisons or Bonferonni adjusted t tests in the case of the *in vivo* microdialysis data. In figures, error bars represent the standard error of the mean (SEM).

Results

Experiment 1: Effect of CRH antagonists on novel cage induced suppression of snack intake

As indicated in Fig. 5a, analysis of the effects of vehicle vs α -helical CRH (20 μ g; ICV) indicated that treatment failed to alter snack consumption ($F_{(1, 7)} = 0.440$, $p = 0.531$). Similarly, in a follow-up study involving a separate group of animals ($n = 7$),

treatment with a higher dose of α -helical CRH (50 μ g;ICV) also failed to influence consumption in the novel cage ($F_{(1,6)} = 0.037$, $p = 0.855$). Likewise, treatment with distinct CRH antagonists, namely CRH (12-41) (10 μ g) and prepro-TRH (6 μ g), did not influence snack consumption in the novel cage condition ($F_{(2,10)} = 1.28$, $p = 0.32$). In rats pretreated with CP-154,526 (10 or 40 mg/kg; i.p.) the novel cage reduced snack intake similar to vehicle injected rats so that a main effect of Cage remained ($F_{(4,18)} = 20.61$, $p < 0.001$), but no effect of Drug treatment ($F_{(1,4)} = 0.97$, $p = 0.43$) (see Fig. 5b). Similarly, in rats injected with ASV-30 (2 and 10 μ g; ICV) the novel cage still suppressed the consumption of a palatable snack so that a main effect of Cage remained ($F_{(4,9)} = 8.55$, $p < 0.001$) but there was no effect of drug treatment (see Fig. 5c).

Experiment 2 & 3: Effects of novel cage exposure on interstitial levels of CRH, GABA and glutamate at the CeA

The ANOVA performed on interstitial ir-CRH values (transformed as a percentage of baseline) revealed a significant effect of Cage x Sample interaction ($F_{(4,44)} = 4.45$, $p < 0.01$). Post hoc analysis revealed that ir-CRH levels remained relatively stable within the home cage but increased significantly in the novel environment in both diazepam- and vehicle-injected rats. As seen in Figure 4 and confirmed by the multiple comparisons, interstitial ir-CRH levels increased progressively over the course of the collection period in both the vehicle- and diazepam-pretreated animals (see Figure 6).

The analysis of the extracellular glutamate concentrations revealed a significant effect of Sample ($F_{(4,44)} = 4.27$, $p < 0.01$) marked by a progressive rise of glutamate that was most notable following transfer to the novel cage. The effects of diazepam on glutamate

concentrations were variable and non-significant. In fact, levels of glutamate might appear slightly higher in the diazepam than vehicle treated rats. The endogenous fluctuations in GABA levels appeared similar to those of glutamate. Thus, relative to the home cage, the release of GABA increased following placement in the novel cage to a similar extent in both vehicle- and diazepam-pretreated rats, $F_{(1, 11)} = 18.78$, $p < 0.001$ (data not shown).

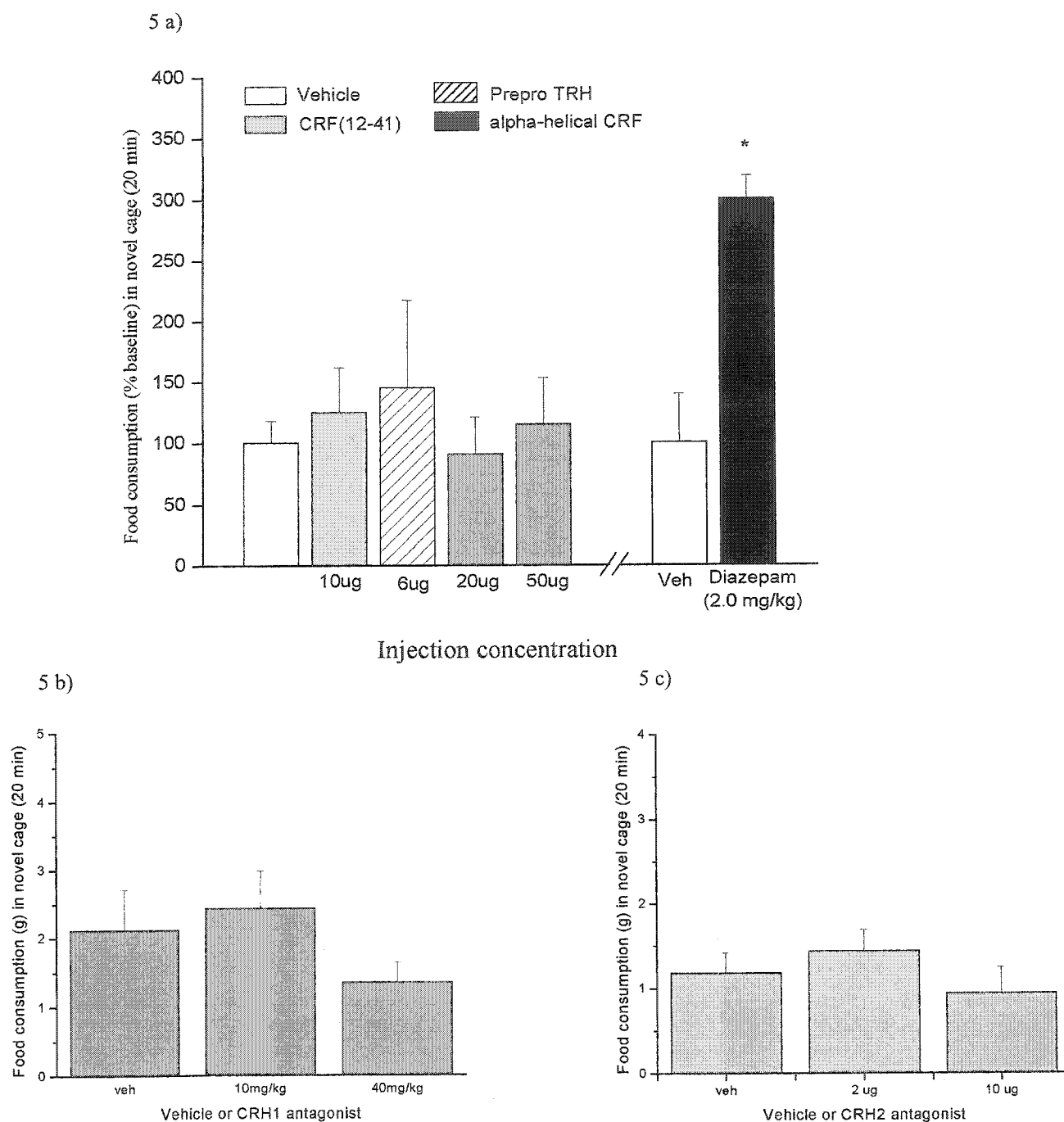


Figure 5: Effect of central and peripherally administered CRH antagonists on snack consumption in a novel cage. Figure 5a (top panel) compares ICV administered vehicle (100% baseline) to the effects of a CRH release inhibitor (prepro TRH) and two non-specific CRH antagonists (CRF 12-41 and alpha-helical CRF) on powdered graham crumb intake in the novel cage. This is also compared to the effects of vehicle and diazepam (2.0 mg/kg; IP). Ordinate reflects food consumption as percent of baseline (vehicle animals) while the abscissa shows the injection concentration.

Figure 5b (bottom left) compares IP administered vehicle and CRH1 antagonist (CP-154, 526; 10 & 40 mg/kg), while Figure 5c (bottom right) compared ICV administered vehicle and the CRH2 antagonist ASV-30 (2 and 10 μ g), on graham crumb intake in the novel cage. In the bottom figures the ordinate reflects absolute snack consumption (g) over 20 min.

* Indicates differences in food consumption between diazepam and vehicle-injected animals in the novel cage, $p < 0.01$.

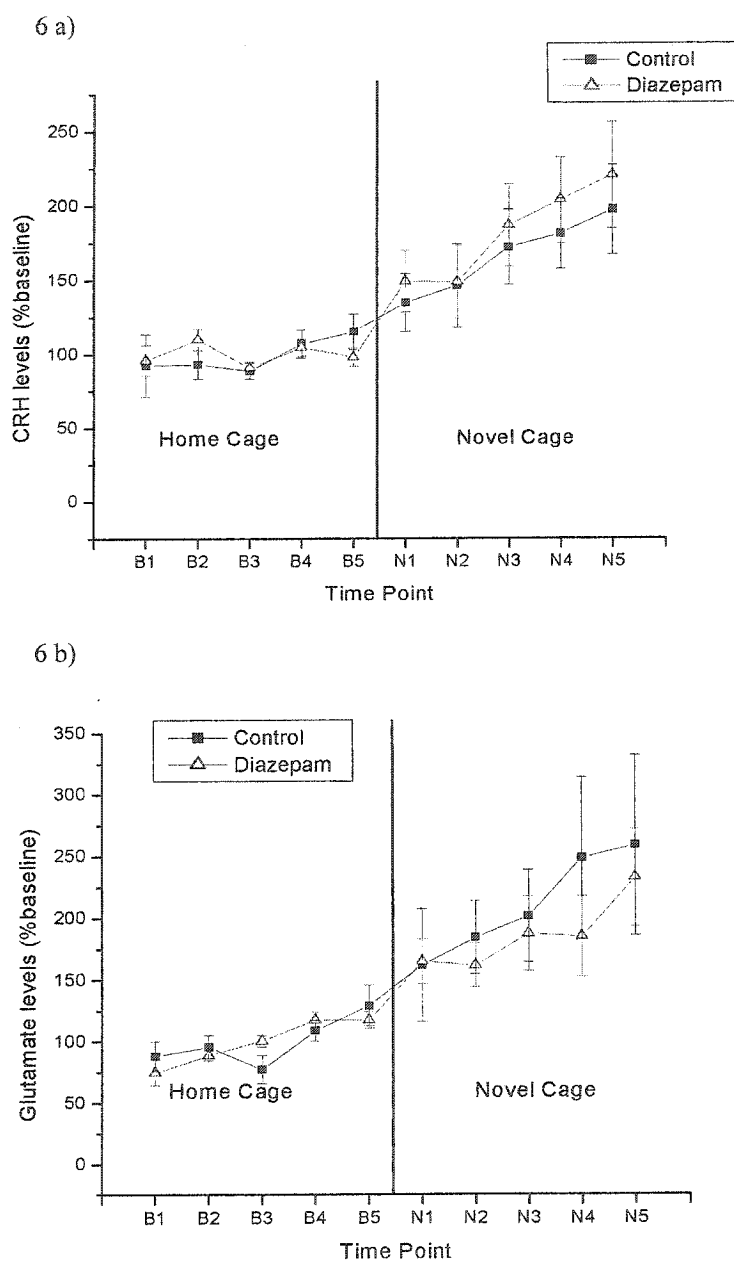


Figure 6. Effect of novel vs home cage environment on the *in vivo* release of corticotropin-releasing hormone (CRH) (6a) and glutamate (6b) at the CeA. The ordinate represents neurochemical concentrations, expressed as a percentage of baseline. Dialysate samples were collected every 20 min in the home and novel cage. Following collection of the fourth sample in the home cage, rats ($n = 8-10$ / group) were injected with either vehicle (Controls; black line) or diazepam (2.0 mg/kg; gray line). In the home cage, interstitial CRH and glutamate levels remained stable (first 5 samples). In the novel cage, levels increased progressively in both vehicle and diazepam-treated rats over the course of 5 samples. Treatment with diazepam failed to alter novel environment-induced release of CRH or glutamate.

Discussion

The amygdaloid complex, particularly the CeA, may be fundamental in the mediation and/or coordination of the stress response. Manipulation of this structure influences the emotional and HPA response to psychological stressors [9,20,130,199,232,316] and stressors enhance amygdala CRH release [93,338,449]. Likewise, in the present investigation, anxiety associated with a novel environment was accompanied by a robust *in vivo* release of CRH at the amygdala. However, although the anxiety associated with unfamiliar environments was attenuated by diazepam, the elevation of CRH release remained unaffected. Furthermore, various CRH antagonists were ineffective in attenuating the anxiety displayed in the unfamiliar environment. This was the case in response to alpha-helical CRH and CRH (12-41) (non selective CRH1 and CRH2 receptor antagonists), prepro-TRH (inhibitor of CRH release) as well as the more selective CRH1 (CP-154,526) or CRH2 (anti-sauvagine-30) receptor antagonists. This is concordant with findings of Okuyama et al. [380] and Griebel et al. [170] who reported that CRH receptor antagonists (CRA1000, CRA1001 and CP-154,526) failed to antagonize the anxiogenic-like behaviours expressed in the light/dark exploration task or the elevated plus maze. This suggests that, while stressors are associated with enhanced amygdaloid CRH release, CRH increase does not appear necessary for the expression of anxiety-like responses engendered in the unfamiliar environment.

Under more threatening conditions, including conditioned fear [200], shock-induced freezing [528], conflict models [263], conditioned defeat or defensive withdrawal [224], CRH antagonists appeared to be effective in attenuating anxiety. However, in tests involving exploration or novelty, the effects of CRH antagonists have been inconsistent

[170,194,380,510]. Moreover, in instances where CRH antagonists attenuated anxiety, this typically occurred when the test was preceded either by exposure to another stress [22,43,193] or exogenous CRH infusion [510]. Thus, CRH systems may be more relevant in the behavioural manifestation of stimulus-specific fear or in response to intense stressors, whereas under more ambiguous circumstances, such as that produced by a threatening (or novel) environment, central CRH systems may not be the primary mediator of anxiety.

If amygdaloid CRH release is increased in response to an unfamiliar or threatening environment but does not mediate anxiety, one wonders what its functional role might be. It is possible that increased CRH release may reflect arousal, rather than fear or anxiety [193] particularly as CRH at the amygdala may be released in response to the saliency or emotional significance of stimuli, regardless of whether they are positive or negative [338]. In effect, amygdala neurons mediate processes by which sensory stimuli gain emotional and motivational significance [9,277]. Thus, it is likely that emotional changes (hyper-arousal, vigilance) independent of anxiety are associated with exposure to a novel environment and CRH release contributes in this respect.

Alternatively, CRH may be fundamental in priming or preparing the organism for action. Part of the effect of increasing CRH activity, particularly at the level of the pituitary, is to increase release of adrenal glucocorticoids. A move to an unfamiliar, potentially threatening environment may engage preparatory responses. However, a glucocorticoid response does not solely occur following negative stimuli (distress) but is also elicited by positive events that threaten homeostasis, such as following a rewarding or appetitive stimuli [338,412]. Such a response is not unique to HPA functioning, as

catecholaminergic activity at the prefrontal cortex or nucleus accumbens is also affected by both stressful and rewarding stimuli [531,563].

It is unclear to what extent stressor-activated CRH systems involve other CRH-like peptides. Indeed, two new members of this neuropeptide family, namely urocortin II (also known as stresscopin-related peptide) and urocortin III (known as stresscopin) appear to be selective agonists for CRH2 receptors [108,206,289]. Both CRH1 and CRH2 receptor subtypes are present in stress-relevant regions such as the amygdala, paraventricular nucleus and ventromedial hypothalamus [135]. Inasmuch as CRH1 deficient mice exhibit diminished anxiety [503,536] and CRH antagonists reduce anxiety associated with certain stressors [117,178,242], it was suggested that CRH1 receptors contribute to stressor-related anxiety [512]. However, in more recent studies, CRH1 receptor knockout mice failed to display diminished anxiety in elevated plus maze and open field tests, questioning the contribution of CRH1 receptors to this emotional state [24]. At present, the contribution of CRH2 receptors remains more ambiguous. Stressor exposure fails to affect CRH2 mRNA expression [318,447], but mice lacking the CRH2 receptor displayed a hypersensitive response to stressors as reflected by increased anxiety-like behaviour [23] and a more rapid termination of stressor-elicited HPA response [97]. This profile could result either directly from the absence of CRH2 receptors or indirectly from developmental adaptations, such as increased CRH and/or urocortin mRNA at the CeA or in other structures in these mice [21]. Interestingly, among double mutant mice (lacking both CRH1 and CRH2 receptors), aberrant anxiety in the elevated plus maze was not evident in males, but was apparent in females. However,

the contribution of these receptor subtypes appeared to vary not only as a function of gender, but early-life experience as well [24].

The role of amygdaloid glutamate in the mediation of anxiety is also of particular interest, as activation of glutamatergic projections to the amygdala has been shown to facilitate CRH release and HPA axis stimulation [155]. Consistent with an NMDA receptor-driven event, glutamate-stimulated CRH release was blocked by NMDA receptor antagonism [99], implicating glutamatergic mechanism in regulating amygdaloid CRH release. The present investigation confirmed that along with CRH release, interstitial glutamate concentrations at the CeA were increased when rats were transferred to the unfamiliar cage. However, as with CRH, the glutamate elevation was not attenuated by diazepam. Thus, it seems that the amygdala glutamate changes do not underlie the overt anxiogenic effects of the stressor, although it may play some other role in the overall stress response.

The present results indicate that psychogenic stressors increase the *in vivo* release of CRH, but the precise role of this peptide in subserving anxiety requires reconsideration. In an attempt to reconcile the disparate findings concerning the contribution of CRH to anxiety, it is important to consider that different types of stressors (natural or unlearned vs. conditioned responses) may involve distinct neural substrates (CRH-dependent vs. CRH-independent). The current literature is consistent with the view that neurogenic stressors and/or conditioned fear cues elicit a CRH-mediated anxiety, which is reversible by CRH antagonists. Likewise, systemic stressors (e.g. staphylococcal enterotoxin B) elicit an anxiety response coupled with amygdala CRH activation, although it is not known whether anxiolytics or CRH antagonists modify the emotional response [271].

Our findings suggest that, in addition to CRH activation, unconditioned psychogenic stressors (e.g. threatening novel environments) may stimulate distinct pre-wired CRH-independent neural circuits. In this context, it has been reported that preshocked rats display long-term increased anxiety-like behavior in the defensive withdrawal test but that CRH is unlikely to be involved in its expression [64]. In the case of monoaminergic systems, it has been reported that different neural circuits may be activated by conditioned and unconditioned stressors [192,356], further supporting the notion of independent systems mediating these different stressor types. Indeed, anxiety disorders comprise distinct state and trait characteristics that involve different neural circuits modifiable by different types of treatments. Thus, simply attenuating the effects of CRH may not be sufficient to promote an anxiolytic response. Ultimately, it may be necessary to distinguish between different forms of stressor-related anxiety, each of which may necessitate different treatment strategies.

Preface to Experimental 3

The purpose of the second half of this thesis is to investigate the physiological response of CRH to global ischemic insults, and its mediating role in subsequent neurotoxicity and behavioural impairments. Experiment 4 will be devoted to characterization of region and time-dependent changes in CRH following a global ischemia, while Experiment 5 will investigate the effectiveness of selective and non-selective CRH antagonists in reversing ischemia-induced cell death and behaviour deficits.

However, prior to investigating the potential therapeutic effects of CRH antagonists, the nature of certain ischemia-induced behavioural deficits need to be better characterized. At present, the assessment of putative neuroprotective compounds rely largely on two behavioural measures, namely ischemia-induced hyperactivity and spatial memory deficits (the latter usually assessed using the Morris water maze or radial arm maze). In addition to a small number of validated tasks, the precise nature and basis of existing impairments remain poorly understood. Thus, experiment 3 aimed to expand knowledge on the range of potential deficits associated with ischemia, by assessing the performance of ischemic animals in novel behavioural tasks, while also examining the relative contribution of cognitive/emotional factors underlying existing deficits.

Experiment 3

Characterization of behavioural activity, anxiety and habituation profiles following global ischemia in rats.

Abstract

Despite considerable research detailing the physiological consequences of global ischemic insults, comparatively fewer studies have characterized the impact of ischemia on behavioural functions. The purpose of the current study was to document the behavioural profile of ischemic rats in novel tasks including the elevated plus maze (EPM), the Vogel/conflict model of anxiety and novelty-induced feeding suppression paradigm as well as to further characterize, using behavioral monitoring, the response of ischemic animals in existing paradigms such as the open field. Our findings revealed that ischemic animals spent significantly more time and made more entries in the open arm of the EPM as compared to sham animals, indicative of a decreased anxiety level. Of interest, however, this anxiolytic effect appeared restricted to exploratory models of anxiety, as no differences in punished licking rate were observed between groups in the Vogel/conflict test. In the open field, behavioral monitoring suggested that ischemia-induced hyperactivity is transient and limited to the initial 15 min of a 30 min testing period. Increased activity in ischemic animals was primarily characterized by increased exploration and sniffing behavior with no significant alterations in rearing and grooming frequencies. Finally, using feeding behaviour, our findings revealed a comparable rate of habituation to a novel environment in ischemic and sham rats. Taken together, these results suggest that ischemia-induced hyperactivity may involve a disinhibition to explore unfamiliar and/or mildly anxiogenic environments. However, the basis of such hyperactivity and the presence of an habituation deficit following ischemia require further study and/or validation.

Introduction

Four-vessel occlusion (4-VO) is a widely used experimental model for global cerebral ischemia in rodents. This model allows for investigation of endogenous processes mediating neuronal damage following major disruptions in systemic blood flow (such as through cardiac arrest). Forebrain ischemia produces selective neuronal degeneration, particularly affecting the CA1 pyramidal layer of the hippocampus. Research into the post-ischemic biochemical cascade has identified a number of potential mediators of cell death, including excessive release of excitatory amino acids, intracellular Ca^{2+} entry as well as other primary and secondary factors including activation of caspases and nitric oxide synthase [86,209,371,490].

In contrast to studies assessing cellular and molecular events, there have been comparatively fewer studies characterizing the impact of ischemia on behavioural functions, despite the fact that functional consequences of an ischemic insult may be considered of comparable importance to its effects on endogenous processes. Thus, in order to represent viable therapeutic strategies, potential neuroprotective agents would require the reversal of ischemia-induced behavioural impairments as well as cellular damage. In this context, the goal of the current study aims to characterize and expand upon behaviours used to assess hyperactivity, while also investigating novel tasks that might further the understanding of ischemia-induced behavioural deficits.

Along with spatial memory impairments, hyperactivity is perhaps the most commonly studied behavioural change following ischemia [46]. However, despite its prominence, a detailed behavioural characterization of ischemia-induced hyperactivity has yet to be conducted, and questions remain regarding its underlying physiological basis. Chandler,

DeLeo and Carney [79] first noticed that ischemic animals spontaneously displayed elevated activity levels shortly following reperfusion. This hyperactivity appeared particularly noticeable when subjects were placed in a novel environment [90,554]. Most studies have specifically linked ischemia-induced hyperactivity to memory impairments resulting from hippocampal cell death [8,72,270]. It has been argued, for instance, that memory deficits delay familiarity with new environments, in part through an inability to use spatial cues, thus requiring further exploration and increased locomotor activity. Evidence of a memory deficit also comes from observations that ischemic animals take longer to habituate to a novel environment when tested across days, which might be expected from a delayed familiarity [95,127].

However, it also remains possible that global ischemia influences general behavioural or locomotor activity through processes other than memory. By nature, unfamiliar environments tend to be threatening or anxiogenic to rodents. Indeed, exposure to novel environments produces a range of behavioural and physiological consequences characteristic of natural stressors. This includes activation of the autonomic nervous system [546] and hypothalamic-pituitary-adrenal (HPA) axis [148,403,578] as well as spontaneous defensive withdrawal [597] and inhibition of non-defensive behaviours [45], including an inhibition of feeding behaviour [28,425,485,508]. Thus, although a delay in familiarity could promote exploratory activity, it is equally plausible that it would inhibit it by prolonging the sense of novelty. In this context, it is interesting to observe that one of the consequences of ischemia-induced hyperactivity is to overcome the natural inhibition of rodents to explore unfamiliar and/or anxiogenic environments.

Of interest, open field activity is widely used in the assessment of exploratory anxiety (see [37,100,390] for reviews). Thus, increased exploratory activity and/or increased exploration of the center of the open field versus the periphery, are considered strong indices of decreased anxiety levels [430,459,493,507]. In the assessment of exploratory anxiety, it is also common to combine measures of open field behaviour with open-arm activity in the elevated plus maze (EPM), as both assess the willingness of rodents to explore anxiogenic environments (see [37]). Indeed, studies examining anxiety commonly combine measures of open field and open-arm activity in the EPM to characterize genetic, environmental and pharmacological mediators of anxiety [268,269,286,581]. As well, anxiogenic agents (such as CRH administration) induce behavioural inhibition in both the open field and the EPM [499,510]. Conversely, effective anti-anxiety agents, such as benzodiazepines, simultaneously increase activity in a novel open field and open-arm activity in the EPM [74,325].

To further elucidate the basis of ischemia-induced hyperactivity, an initial step in the current study aimed to characterize, using behavioral monitoring, the different behaviours of sham and ischemic animals in a novel open field. Given the close association between behavioural changes in the open field and other models of anxiety, a second objective aimed to examine whether ischemia-induced hyperactivity is accompanied by response changes in exploratory and non-exploratory anxiety tests. To address this, the performance of ischemic animals in the elevated plus maze and the Vogel Test (a conflict based anxiety model)[506] will be assessed. Finally, the long-term habituation profile of ischemic animals will be investigated using novelty-induced feeding suppression.

Dowden & Corbett [127] and Corbett et al., [96] observed that when ischemic rodents

were tested for 10 minutes/day across several days, their behavioural activity remained consistently higher than that of sham-operated controls. This was taken to indicate an impaired habituation, possibly resulting from memory deficits. Thus far, habituation deficits in ischemic animals have only been inferred from a hyperactive response in the open field. To clarify the nature of this deficit and its generalization under different situations, it appears of interest to evaluate this behavioural response using other paradigms. For instance, the extent to which ischemia-induced hyperactivity or habituation deficits would represent a dysfunction may also depend on whether it impairs functional or biologically necessary behaviours. Thus, a third objective of this study aimed to characterize the habituation profile of sham-operated and ischemic rats to a novel cage, as determined through assessment of the recovery over days, of their basal consumption of a familiar palatable snack in a new environment.

Materials and Methods

Animals

Male Wistar rats ($n = 38$) weighing between 250-320g at time of surgery were obtained from Charles River Laboratories (Rochefort, Quebec, Canada). They were individually housed and maintained on a 12 h light/dark cycle (lights on at 7:00 AM), with free access to water and standard (Purina) rat chow. The room temperature was maintained at 21-23°C with 60% relative humidity. One group of sham ($n = 12$) and ischemic ($n = 10$) animals were tested in the open field task, the Vogel test and in the novelty-induced feeding suppression paradigm. A different set of sham ($n = 7$) and ischemic ($n = 9$) animals were used for testing on the elevated plus maze. Upon their

arrival, animals were acclimated to the animal facility for at least one week before surgery. All procedures were carried out in accordance with the guidelines set by the Canadian Council of Animal Care and received ethical approval from the University of Ottawa Animal Care Committee.

Surgical procedure

Forebrain ischemia was performed using the four-vessel occlusion model as previously described by Pulsinelli & Brierley [431]. Briefly, rats were deeply anesthetized by inhalation of 1.5% halothane in oxygen. The core temperature was kept at $37^{\circ}\text{C} \pm 0.5^{\circ}$ throughout the surgery by means of a feedback regulated heating blanket connected to a rectal thermometer (Homeothermic Blanket Control Unit, Harvard Instruments, Natick, MA). Subjects' body temperature was supported with a heating pad in the hours following surgery and reperfusion. The vertebral arteries were irreversibly occluded by electrocoagulation, and a small-diameter silk thread looped around the carotid arteries to facilitate subsequent occlusion. Twenty-four hours later, common carotid arteries were clamped with microvascular clamps for 10 minutes in awake and spontaneously ventilating animals. Rats lost their righting reflex within 1 min of carotid clamping. Sham-operated animals underwent anesthesia, and received the same dorsal and ventral surgical incisions as the ischemic group with the exception of electrocoagulation of the vertebral arteries. Twenty-four hours later, carotid arteries were exposed, but not clamped. In figures, error bars represent the standard error of the mean (SEM).

Experimental apparatus and behavioral procedures

Novel openfield: The observation arena was made of gray Plexiglas (LWH: 75 X 75 X 30cm) with a clear Plexiglas floor. The floor was painted with a grid comprising 25 10X10 cm squares. The entire arena was kept on a table 90 cm above the floor in the same room as the rats' housing.

Between 4-7 days following reperfusion, sham and ischemic subjects were placed in the open field and behaviour was monitored for 30 min by the experimenter using a portable PC computer (Toshiba 4250) and datalogging software. Subjects were monitored for frequency of line crossing, sniffing, grooming and rearing behavior, as well as stillness/resting (absence of activity).

Elevated plus maze: The elevated plus maze consisted of two opposing open arms (50 X 10 cm with a 5 mm clear plexiglas lip), an open 10X10 cm area in the center and two opposing closed arms (50 X 10 cm with 40 cm high walls). The entire maze was elevated 60 cm from the floor.

Initially, each rat was placed in an open field (75 X 75 cm with 30 cm high walls) for a 5 min habituation period. Immediately thereafter, the rat was placed in the center of the elevated plus maze facing one of the open arms of the maze. The time spent and number of entries in the open or closed arms as well as risk assessment behaviour was recorded during a 5 min test interval. The rat was considered to be in the open or closed area of the maze if at least its head and two front paws were in that area. Risk assessment was

operationally defined as elongation of the neck with at least the snout protruding into the open arm, while the paws and body remained in the closed arm. Black curtains surrounded the maze and monitoring of the behaviour was possible using an overhead video camera and monitor.

Vogel Test (or thirsty-rat conflict test): The Vogel test chambers were comprised of stainless steel cages 38 X 38 X 30 cm, with the front and back walls made of plexiglass. The floor is a stainless steel grid and a water bottle with metal drinking tube is inserted at one end of the cage. A photobeam passes in front of the drinking tube which, when broken (through tongue protrusions) records the animals' lick rate. The cage was connected to a shocker (Precision Regulated Animal Shocker; Colbourne Instruments) linked via circuits (Colbourne Labline) to a computer. A custom-designed software (Graphic State Notation 2, v.2002) allowed the investigator to record the lick rate and to control the parameters of the test.

Subjects were initially given a two-day habituation period in the test chamber and exposed to an unpunished drinking schedule. Following 20 h of water deprivation, subjects were placed in the test chamber and permitted to drink freely from the metal drinking tube. Lick rate was automatically recorded during each daily session. On the third day, subjects were exposed to the conflict schedule. A pair of shocker leads was attached between the metal drinking tube and the stainless steel grid floor. Every 5th lick, a 0.3mA shock was delivered to the drinking tube. Daily sessions lasted for 10 min, after which subjects were returned to their home cage and permitted to drink water freely for 4 hours.

Long-term habituation to novelty (novelty-induced feeding suppression paradigm):

The novel environment consisted of a cage different in various aspects from the subjects' home-cage. Entirely made of plexiglass, the dimensions of the novel cage were 48 X 30 X 40 cm (compared to 24 X 30 X 18 cm for the home cage) with the front of the box serving as a hinged door. The floor of the novel cages consisted of a stainless steel grid fixed 3 cm above the plexiglas bottom. The animal's home cage consisted of a shoebox style clear plexiglas container with the bottom lined with bedding material (beta chips about 1.5 cm deep). Both cages were housed in the same room where testing took place.

Two groups of rats received powdered graham crumbs once per day in their home cage for a 20 min period. When intake stabilized (after approximately 5-6 days), the rats started receiving the graham crumbs once per day for 20 min, this time in the novel cage. Exposure to the novel cage continued for 11 consecutive days. Amount of graham crumbs consumed was recorded daily in the novel environment and used to determine the rate of habituation of both animal groups to the novel environment through the progression of the animal intake to reach back similar food consumption as displayed in its home cage.

Histology: Upon completion of the behavioural tests, subjects were euthanized and the brains removed, frozen on dry ice and stored at -80°C. Serial coronal sections (14 µm) of the hippocampal regions were subsequently obtained using a cryostat (Leica Instruments) and stained with cresyl violet. Neuronal degeneration was then determined using computer-assisted light microscopy.

Analysis of neuronal density on cresyl violet stained sections: The neuronal density of the hippocampal CA1 subfield was determined by the method of Kirino et al. [257]. Analysis of neuronal density was performed on coronal sections between 3.14 and 4.16 mm posterior to bregma. The total linear length of the CA1 pyramidal cell layer was measured by means of a digitizer. The number of living neurons in the stratum pyramidale within CA1 subfields was counted using a LEICA DAS microscope attached to SONY digital camera and computer-assisted cell counting performed using Norton Eclipse software (v 6.0). Neurons that had shrunken cell bodies with surrounding empty spaces were excluded. The neuronal density of the CA1 sector, i.e. the number of intact pyramidal cells per 1 mm linear length of the CA1 stratum pyramidale observed in each 14 μ m section, was quantified. Then, a mean value for each hippocampal CA1 substructure was obtained from 6 bilateral measurements per animal in each of the experimental groups. The neuronal density for a given animal represents the average of both the right and left hippocampal neuronal cell densities. Neuronal density values are expressed as mean $\pm$ standard error of the mean.

Statistical Analysis: The frequency of each behaviour (line-crossing, sniffing, rearing, grooming, stillness) in the open field was calculated for the two groups (sham and ischemic animals), every 5 min over the entire 30 min monitoring period. Primary analysis involved repeated measures analysis of variance investigating the effect of Group, Time and the Group X Time interaction on total activity level. Subsequent ANOVA analysis was conducted to assess the main effect of Group on the frequency of

individual behaviours. Tukey post hoc analyses (adjusting for multiple comparisons) were used to compare activity levels between sham and ischemic animals at each 5 min interval. For the EPM, one-way ANOVAs were used to analyze the number of open and closed-arm entries, total time spent on the open and closed arms and risk assessment behaviour. Similarly, for the Vogel test, a one-way ANOVA assessed the impact of Group on punished and unpunished lick rate. For analysis of novelty-induced feeding suppression, food intake in the novel cage was first converted to percent of baseline (home-cage) values. Repeated measures ANOVA was then performed on food intake in the novel cages as measured across the 11 test days, and the effects of Group, Time and the Group X Time interaction were analyzed. Analysis of neuronal density (number of pyramidal cells /1mm CA1 tissue) was performed using a one-way ANOVA comparing sham and ischemic animals. In figures, error bars represent the standard error of the mean (SEM).

Results

Novel open field: Fig. 7 shows the global activity of sham and ischemic animals in the open field every 5 min over the 30 min monitoring period. The general activity profile is obtained from pooling the frequencies of four of the individually monitored behaviours (line-crossing, sniffing, grooming and rearing behavior). Repeated measures ANOVA revealed significant main effects of Group ($F_{(1,20)} = 6.148$, $p = .022$), Time ($F_{(5,20)} = 46.415$, $p < .001$) and a significant Group X Time interaction ($F_{(5,80)} = 2.55$, $p = .034$). Findings indicated a significantly higher activity level in the novel open field in ischemic as compared to sham-operated animals. Post-hoc comparisons revealed that this

increase was attributable to enhanced activity during the initial 15 min ($p < .05$).

Breakdown of the overall activity into the specific behaviours monitored demonstrated that the difference in activity level was due to significant group differences in frequency of line-crossing ($F_{(1,20)} = 4.458$, $p = .001$) and sniffing activity ($F_{(1,20)} = 4.245$, $p = .002$) among the ischemic rats.

Elevated plus maze (EPM): Fig. 8 highlights the performance of sham and ischemic animals in the EPM. Monitoring included recording of the number of open-arm entries and risk assessment behaviour, and the amount of time spent on the open and close arms of the maze. Statistical analysis revealed significant differences between groups in open arm entries ($F_{(1,14)} = 21.67$, $p < .001$) and time spent in the open arm ($F_{(1,14)} = 22.51$, $p < .001$). These differences were attributable to increased numbers of entries and time spent in the open arms in ischemic as compared to sham animals, a finding indicative of decreased anxiety levels. Conversely, ischemic rats made significantly fewer closed arm entries ($F_{(1,14)} = 11.236$, $p = .005$) and spent less time in the closed arms ($F_{(1,14)} = 21.993$, $p < .001$) than sham animals during the 5 min testing period (data not shown). No significant differences were observed in the number of risk assessment behaviours between the two groups ($F_{(1,14)} = 1.648$, $p = .22$).

Vogel Conflict Test: Fig. 9 shows comparable licking rate in sham and ischemic animals under unpunished and punished drinking schedule. In this paradigm, a higher lick rate during the conflict schedule is indicative of reduced anxiety. Our findings failed to demonstrate significant differences between the sham and ischemic groups in punished

drinking ($F_{(1,13)} = 1.116$, $p = 0.3$), suggesting equivalent anxiety level in the two animal groups.

Novelty-induced feeding suppression paradigm: Fig. 10 shows the recovery profile of palatable food intake across the 11 testing sessions in the novel environment. Repeated measures ANOVA failed to indicate a main effect of group in food consumption in the novel cage across days ($F_{(1,15)} = 1.948$, $p = .183$). However, a significant effect of Time ($F_{(1,10)} = 7.284$, $p = .022$), but no Group X Time interaction ($F_{(1,15)} = .805$, $p = .62$) was observed. These findings suggest comparable rate of habituation to the novel cage in ischemic and sham animals as assessed with palatable food intake. The first 10 days of novel cage exposure was subdivided into two 5 day intervals (early and late), since food consumption in the novel cage appeared to recover a bit faster for ischemic as compared to sham animals in the initial exposure period. Statistical analysis of food intake however failed to demonstrate a significant Group difference ($F_{(1,15)} = 1.967$, $p = .18$) or a Group X Time interaction ($F_{(4,15)} = 1.244$, $p = .335$) during the initial 5-day period.

Histopathological changes at the hippocampus : Our findings showed that a 10 min global ischemia induced significant neuronal damage in the CA1 hippocampal sector ($F_{(1,18)} = 164.47$, $p < .001$). As compared to sham-operated rats, between 70-75 % of CA1 pyramidal cell populations showed signs of neuronal damage in ischemic animals 28 days following ischemia. Averaged cellular density was 175.5 ± 3.0 and 53.22 ± 9.1 cells per 1 mm linear length of hippocampal CA1 tissue in sham and ischemic animals,

respectively. Comparable histological findings were observed in the second cohort of animals test, where a significant difference in cellular density was observed ($F_{(1,14)} = 87.04$, $p < .001$), with 175.2 ± 8.6 and 55.833 ± 9.05 cells per 1 mm linear length of hippocampal CA1 tissue in sham and ischemic animals, respectively.

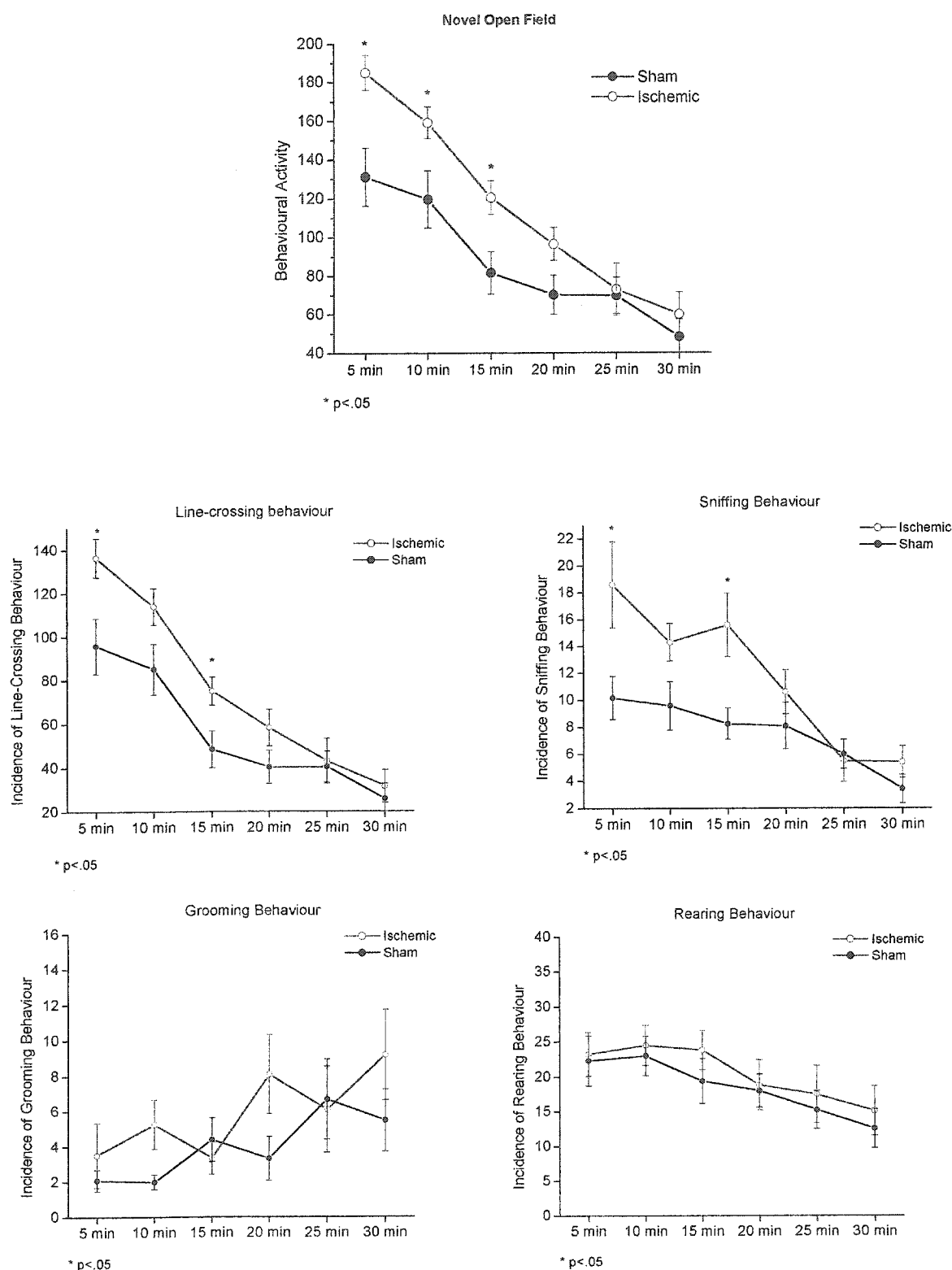


Figure 7. Behavioural profile of sham and ischemic rats in a novel open field.

The ordinate represents the incidence of the respective behaviours, while the abscissa indicates the time. Significantly higher activity levels were found in the novel open field in ischemic as compared to sham animals. Post-hoc analysis revealed that the difference was significant in the first 15 min testing period. Further breakdown of the behaviours demonstrated that this difference in global activity is mainly attributed to higher levels of line-crossing (ie. locomotor) and sniffing behaviour among ischemic animals. * Significantly different from sham rats, $p < 0.05$.

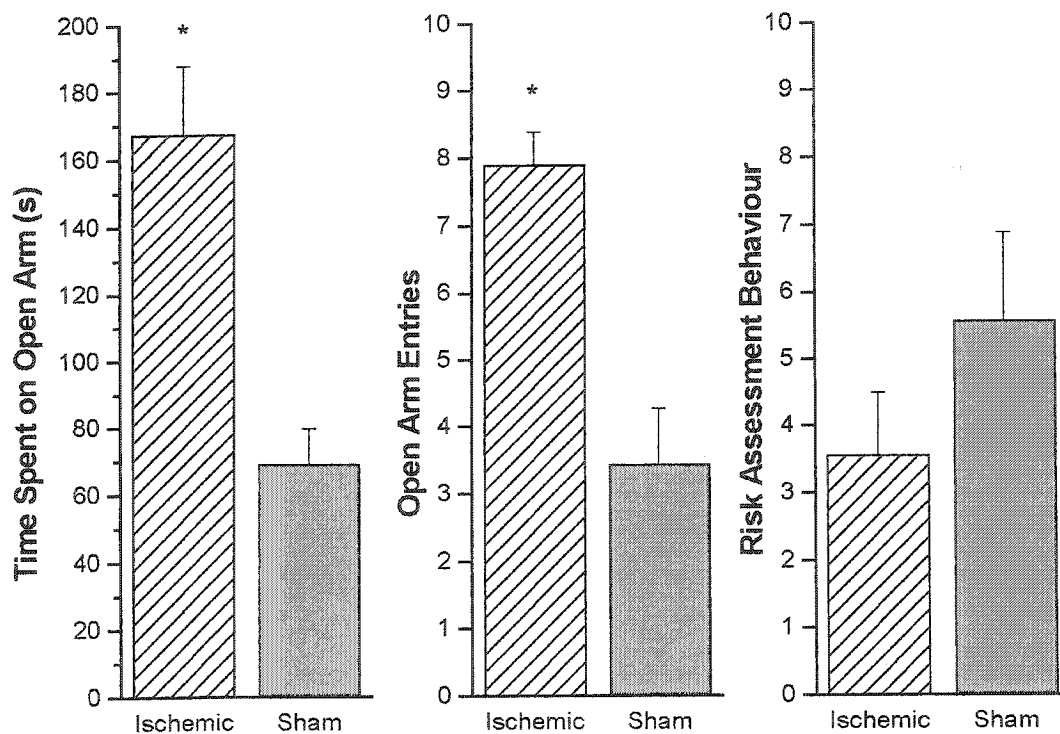


Figure 8: Behaviour of sham and ischemic rats in the Elevated Plus Maze (EPM). In comparison to sham-operated animals, ischemic animals made significantly more open arm entries and spent significantly more time on the open arms of the EPM. There were no significant differences in risk assessment behaviour.

* Significantly different from sham-operated rats ($p < .05$).

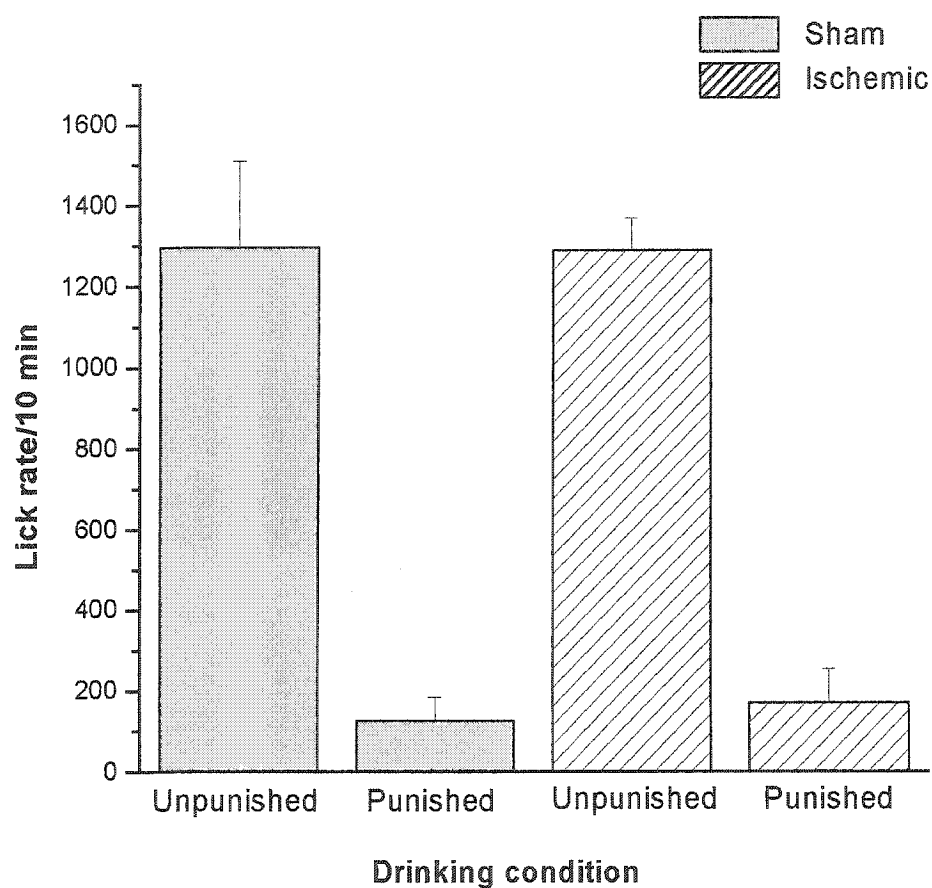


Figure 9: Performance of sham and ischemic animals in the Vogel (thirsty-rat conflict) test. Lick rate/ 10 min (the ordinate) is measured with (punished) and without (unpunished) pairing with a 0.3 mA shock. Sham and ischemic animals show a near identical suppression in lick rate when comparing punished with unpunished responses.

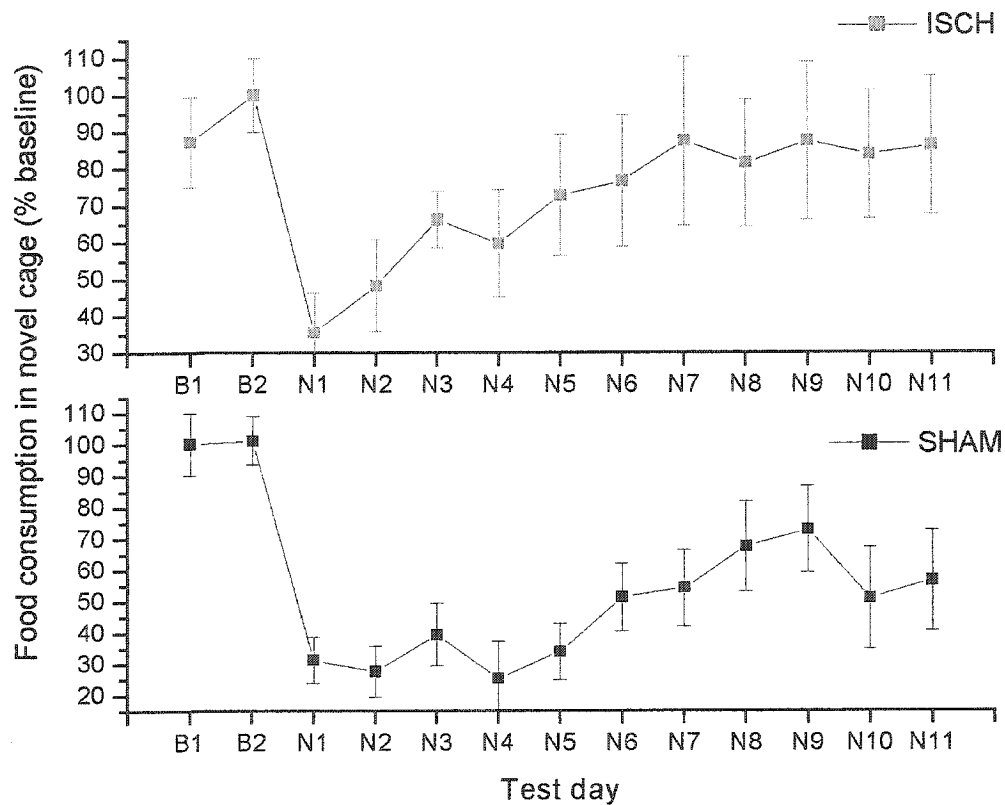


Figure 10: Habituation to novelty in sham and ischemic animals assessed using palatable snack intake. Ischemic (top figure) and sham (bottom figure) animals were presented a familiar palatable snack (powdered graham crumbs) in the novel cage (20 min/day) for 11 days (N1-N11). In this model, sham and ischemic animals showed comparable rates of habituation to the novel environment, as assessed with snack intake. Thus, intake in the novel cage reached 75% of home cage baseline levels (B2) by day 7 and day 8 for ischemic and sham animals respectively.

Discussion

To our knowledge, the current study represents the first attempt to assess the behavioural response of ischemic animals in multiple models of anxiety as well as characterize the rate of habituation to a novel environment using novelty-induced feeding suppression. Our findings suggest differences in the responses of sham and ischemic animals in the novel open field and the elevated plus maze (EPM), but not in the Vogel/conflict test. Furthermore, the present study demonstrates that sham-operated and ischemic animals do not differ in their rate of habituation to a novel environment, as assessed using novelty-induced feeding suppression. Comparable performance between sham-operated and ischemic animals in this latter task suggests that habituation deficits previously observed in the open field, and assessed using general activity levels, may not generalize to other situations or other behavioural indices, such as those involving appetitive stimuli in a novel cage.

Global ischemic insults of various durations reliably produce pronounced neurodegeneration, particularly affecting the CA1 pyramidal layer of the hippocampus. A number of functional impairments have been associated with this damage in animals and humans, including anterograde amnesia, working and spatial memory deficits [19,90,91,216,594] as well as significant increases in locomotor activity usually observed within 24 h of the ischemic insult [18,79,160]. This hyperactivity is particularly noticeable when subjects are placed in a novel environment [90,554]. In the present study, behavioural monitoring in the open field revealed that ischemia-induced hyperactivity is primarily characterized by increases in exploratory-type behaviours such

as locomotion and sniffing with no significant alterations in grooming and rearing behaviours.

In a previous study, Colbourne et al. [90] compared locomotor activity between sham and ischemic rodents in an open field over three 10 min testing periods, each separated by one hour. Throughout the first two sessions and the initial 5 min of the third session, ischemic animals displayed higher overall activity levels. The within-session differences in activity levels have been taken to represent a habituation rate impairment among ischemic animals [91,95], presumed to result from memory impairments which delayed familiarization to the novel environment in animals with hippocampal damage. However, the extent of this deficit might not have been fully captured by the short time intervals used for observation in these studies. In the current study, we thus extended testing time to a 30 min session in the open field in order to determine whether habituation deficits could be overcome by prolonged exposure to the novel environment.

Concordant with Colbourne et al's [90] findings, activity levels in the present study were initially higher in ischemic animals during the first 15 min of exposure to a novel environment. However, following that period, activity levels of ischemic animals rapidly stabilized to reach levels comparable to that of sham rats in the last 15 min testing period. Interestingly, Wang and Corbett [554] demonstrated that rodents with significant pre-ischemic exposure to the test environment did not demonstrate post-ischemic hyperactivity. These findings are consistent with the suggestion that increased exposure to the test environment can assist in overcoming this particular impairment. Thus, it appears possible that in prior studies, shorter periods of testing (10 min) might not provide sufficient time for the ischemic animals to overcome the novelty of the

environment, leading to a difficulty of habituation despite repeated exposure to the same environment.

In addition to examining the nature of within-session habituation impairments in the open field, the present study aimed to further characterize long-term habituation deficits. When tested at 3, 7, 10 and 30 days post-ischemia for 10 min sessions, Dowden & Corbett [127] and Corbett et al. [96] observed persistently higher activity levels among ischemic rats in an open field. The increased activity across several days provided further support for a deficit in habituation to novel environments in ischemic animals. Thus, one goal of this study was to determine the extent of this deficit and whether it could interfere with other functions, for instance biologically reinforcing or functionally necessary behaviours such as feeding. Rodents who consume a palatable snack in the familiar (and presumably non-stressful) environment of the home cage tend to consume less of that same food when it is presented in a novel cage [247]. However, with repeated exposure, as the novel environment becomes more familiar, food intake begins to approach home cage levels. Using this novelty-suppressed feeding paradigm, ischemic animals did not require more time to familiarize with the novel environment in the current study, and even appeared to return to home-cage (baseline) feeding levels somewhat more quickly than normal controls. These findings suggest that ischemic animals do not present habituation deficits in a model of novelty-induced feeding suppression. As measured by intake of palatable food, ischemic animals required comparable time to habituate to a novel cage than sham-operated controls. These findings suggest that if a memory deficit is influencing habituation to a novel environment, it is not of sufficient magnitude to interfere with highly motivated behaviors such as palatable food intake. Furthermore,

under the present conditions, ischemia-induced hyperactivity did not appear robust enough to interfere with feeding behavior, although this could likely be attributable to reduced size of the novel environment in this paradigm.

As indicated, the underlying cognitive/emotional basis for increased activity levels in ischemic rats in novel environments has most commonly been attributed to a deficit in spatial memory and/or spatial navigation (e.g.[8,127]). Much of the basis for this theory has relied on the close link between damage to the CA1 area of the hippocampus and errors in traditional spatial memory tasks such as the radial arm maze [111,171,258]. As for spatial memory deficits, ischemia-induced hyperactivity has been shown to correlate with the amount of neuronal loss in the hippocampal CA1 region [160,241,270]. Miles and Schwartz [343] further suggested that post-ischemic changes in locomotor activity can serve as a reliable predictor of CA1 damage.

However, recent findings have suggested a more complex relationship between CA1 neuronal death and hyperactivity. For instance, Piera et al.[418], in testing the neuroprotective effects of various serotonergic compounds, found no correlation between the amount of neuroprotection conferred to hippocampal CA1 pyramidal cells and the level of hyperactivity. Similarly, Corbett & Crooks [95] showed that a cerebral ischemic preconditioning strategy that protected over 80% of CA1 pyramidal cells in preconditioned animals failed to reduce ischemia-induced hyperactivity. Poignet et al. [424] obtained comparable results following administration of the calcium channel blocker flunarizine. Moreover, environmental manipulations such as post-ischemia enriched housing environments [434] can eliminate ischemia-induced hyperactivity, while having little impact on hippocampal cell death. Thus, the degree to which

ischemia-induced hyperactivity is mediated by CA1-dependent memory processes remains ambiguous.

Of interest, in addition to assessing changes in locomotor activity, the open field is also a widely used task in the assessment of exploratory anxiety (see [38,100,390] for reviews). Thus, a variety of pharmacological compounds, physiological states or environmental manipulations have been assessed for their ability to increase activity levels in the open field as an index of decreased anxiety levels [430,459,493,507]. In some cases, a mere increase in total activity is considered indicative of decreased anxiety, while others analyze the willingness of an animal to explore the center of the open field versus the periphery. It is also common to combine measures of open field behaviour with open-arm activity in the elevated plus maze (EPM) to assess the effectiveness of putative anxiolytic treatments in affecting exploratory anxiety (see [38]).

Interestingly, findings from the current study showed that ischemic animals made significantly more open-arm entries, and spent more time on the open arms of the elevated plus maze as compared to sham animals. These findings appear consistent with the general disinhibition to explore unfamiliar and/or anxiogenic environments observed in the open field. Along with certain other tasks, such as light/dark exploration, the open field and EPM fall into a category of anxiety models that assess an animal's spontaneous or natural reactions to stressful stimuli (e.g. flight, avoidance, freezing). These stressors do not invoke overt or explicit forms of pain or discomfort and are further characterized by the use of unlearned or unconditioned aversive stimuli (e.g. exposure to a novel open chamber or an elevated platform). The anxiolytic response profiles of ischemic animals in these two tasks believed to measure similar anxiety processes further suggest that a

basis of ischemia-induced hyperactivity in novel environments may be reduced anxiety when facing unconditioned/naturalistic stressors.

The anxiolytic response profile of ischemic animals appeared limited to exploratory models and did not extend to conflict-based models. Thus, in the current study, the performance of the two groups did not differ in the Vogel paradigm, a test involving anxiety-inducing stimuli (i.e. mild electrical shocks) and deprived drinking. This differential response in exploratory and conflict models of anxiety is not uncommon. A number of studies have suggested that different processes may mediate exploratory and stimulus-specific anxiety. For instance, selective CRH1 antagonists significantly increase punished responses in the Vogel test, but failed to increase open arm activity in the EPM [344]. Numerous other compounds such as buspirone, glucagon-like peptide, glutamate antagonists as well as certain 5-HT₃ antagonists also selectively influence anxiety in the EPM but not the Vogel test or vice versa [147,170,235,352].

It has been suggested that the level or intensity of the stress may be different in a conflict versus exploratory model, and thus may be mediated by distinct physiological systems. For instance, Moller et al. [353] found that amygdala lesions affect behaviour in relatively low-stress conditions, such as the elevated plus maze, but had a very limited effect in models involving fear-induced behavioural inhibition or stressful conflict paradigms. Similarly, others have suggested that the EPM and the Vogel test assess different components of anxiety [184]. These authors have suggested that continuous fear stimuli, which often represent complex environmental cues that signal unknown or undefined threats (such as a novel open field) may be mediated by one set of processes. On the other hand, easily identifiable or controllable events such as the shocks used in

most conflict procedures are likely mediated by separate processes, which might explain why different compounds or neuroanatomical lesions have differing effects in the two types of models. Merali et al. [336] also suggested that the ambiguity of stressors may influence which physiological systems are recruited. Thus, the behavioural response to stimulus-specific fear or to intense stressors are modulated by some types of anxiolytics [200,224,263,528], and may be mediated by more rapid or hard-wired neural mechanisms. In contrast, these same compounds appear ineffective in influencing the response to more ambiguous circumstances such as that produced by a threatening or novel environment [170,344,380], which may be mediated by limbic circuitry involved in cognitive-emotional processing, such as the amygdala [198]. Our findings suggest that although ischemia may influence the response to ambiguous and relatively mild anxiogenic environments, such as an open field or an elevated plane, it may not produce physiological changes relevant to more intense and/or stimulus-specific fears. Research assessing the effectiveness of various anxiolytic compounds or neuroanatomical interventions in influencing these behavioural changes might help determine the specific pathways mediating these effects.

Together, the findings from the current study suggest that, although robust, ischemia-induced hyperactivity may represent a transient phenomenon that stabilizes within a relatively short period of time. The observation that it is accompanied by anxiolytic behaviours in the elevated plus maze further suggest that such increase in exploratory activity in a novel open field may partly be due to altered anxiety levels following ischemia. Our findings also suggest that long-term deficits in habituation in ischemic animals appeared context-dependent and were absent in ischemic animals exposed to

highly motivating stimuli such as palatable food. The neuroanatomical basis of ischemia-induced increased exploratory activity and anxiolysis requires further study to determine how these behavioral responses specifically relate to neurodegeneration.

Preface to Experiment 4

Though CRH has been implicated in neurotoxicity associated with cerebral ischemia, thus far a detailed characterization of the CRH response to global ischemic insults has yet to be conducted. Using post-mortem analysis of CRH concentrations, the following chapter will provide an analysis of region and time-dependent changes in CRH concentrations in 10 distinct brain nuclei, at three separate post-ischemic intervals. In addition, the mediating role of NMDA receptor activation in some of these changes will be assessed. Finally, using in vivo microdialysis, a dynamic monitoring of CRH release at the CA1 region of the hippocampus and the central nucleus of the amygdala (CeA) before, during and after a global ischemic insult will be conducted.

Experiment 4

Time-dependent changes in CRH concentrations and release in discrete brain regions
following global ischemia: Assessment of the role of NMDA receptors.

Abstract

CRH's excitatory actions in the brain and the neuroprotective effects of CRH antagonists in ischemia suggest a role for this peptide in the cascade of events leading to cellular damage. The present study aimed to characterize endogenous activation of CRH in discrete brain regions following global ischemia. Time-dependent changes in CRH concentrations were assessed in 10 brain regions including hippocampal, parahippocampal and hypothalamic regions, the amygdala and frontal cortex at 3 post-ischemic intervals: 4, 24 and 72 h. To assess the potential contribution of glutamate, the impact of pretreatment with the NMDA antagonist MK-801 on ischemia-induced CRH concentrations was determined. In vivo microdialysis was also used to assess dynamic fluctuations in CRH release at the dorsal hippocampus (CA1 layer) and central nucleus of the amygdala (CeA). Our findings revealed a rapid elevation of CRH concentrations at the piriform cortex and hypothalamic nuclei following global ischemia. This was followed by decreased CRH concentrations at the amygdala, frontal cortex, CA3 and hypothalamus 24 h post ischemia. These fluctuations were reversed by MK-801 pretreatment in hypothalamic nuclei. 72 h post-ischemia, CRH levels returned to control values in all regions, except for the dentate gyrus. In vivo, a significant increase in CRH release in response to global ischemia was found at the CeA with no alterations at the CA1. These findings support region-specific ischemia-induced CRH alterations in the brain, and further suggest that CRH actions to mediate neuronal damage at the hippocampal CA1 layer may be indirect.

Introduction

In adult rodents, four-vessel occlusion is a widely used model for global ischemia, developed to mimic the neuronal damage observed following a heart attack [46]. Forebrain ischemic insults of various durations have been shown to induce selective and delayed cellular degeneration, particularly affecting CA1 pyramidal neurons of the hippocampus. The proposed cascade of biochemical reactions following ischemia includes sustained depolarization, release of endogenous excitatory amino acids (such as glutamate), and extensive intracellular Ca^{2+} entry. Among other effects, these serve to potentiate free radical formation, activation of caspases and nitric oxide synthesis, ultimately leading to excitotoxic neuronal death by necrosis and/or apoptosis [86,209,371,490]. Neuroprotective strategies aimed at blocking this cascade have been effective in some rodent models of ischemia, but have failed to provide reliable treatments in human populations [124]. Thus, the characterisation of molecular and cellular processes underlying ischemia-induced neuronal degeneration continues aiming at the identification of new targets for intervention.

In this context, a role for the 41-amino acid polypeptide, corticotropin releasing hormone (CRH) in ischemia has been proposed [85,516,571]. CRH, among other properties, plays a well-established role in regulating the behavioural and neuroendocrine responses to stress [263], and is implicated in learning [281], arousal [568] and motor activity [133]. In addition, central administration of CRH exerts excitatory effects in the brain and induces limbic seizures in rats [561]. CRH has also been shown to depolarize hippocampal neurons *in vitro*, and increase the spontaneous discharge frequency of CA1 and CA3 pyramidal neurons [4,5].

More recently, evidence has suggested that CRH activation following ischemia may be implicated in subsequent neuronal damage. For example, administration of CRH antagonists in the brain, prior to, or just following an ischemic insult, appears to confer significant neuroprotection [311,516]. Despite these early results, the role of CRH in excitotoxic processes remains ambiguous. Indeed, research has failed to show any neurotoxic effects of CRH administered *in vivo*, and CRH does not appear to exacerbate glutamate-induced neurotoxicity [98]. In fact, support even exists for a neuroprotective role for CRH in hypoxia [152] and Alzheimer's disease [401].

To further elucidate the participation of CRH in ischemia-induced processes, the present study aimed to characterize endogenous activation of CRH systems in discrete brain regions following a global ischemia. There is substantial basis for suspecting ischemia-induced CRH activation in a number of brain areas. In the hippocampus, actions of CRH involves suppression of slow Ca^{2+} activated K^{+} currents (also called slow afterhyperpolarizations (sAHP))[502], which has the net effect of increasing the spontaneous neuronal discharge frequency at the hippocampus [5]. This appears to be an endogenous action of CRH, as administration of CRH antagonists alone have been shown to partially promote sAHP's [439]. As well, CRH antagonists inhibit other substances involved in suppressing sAHP, such as hippocampal norepinephrine (NE) and serotonin (5-HT) [176,177,221].

Para-hippocampal regions, including the piriform cortex and amygdala, are also of interest as bilateral excitatory connections exist between these structures and the hippocampus. The piriform cortex has been closely linked to hippocampal-mediated limbic seizures [157], and kainate-induced neurotoxicity increases CRH mRNA

expression in this region at a magnitude similar to that produced at the hippocampus [417]. Increases in CRH mRNA expression at the amygdala have also been reported following both ischemia and traumatic brain injury [457,571]. Wong et al. [571] have demonstrated significant increases in CRH mRNA expression at the amygdala 60 min following an ischemic insult. The central nucleus of the amygdala has received particular attention in regard to some of the neurotoxic effects of CRH. Thus, exogenous administration of CRH in this region has been shown to promote seizure activity [137], and glutamate induces specific excitation of amygdalar CRH release [155]. In addition to these excitatory actions, CRH may also exert other physiological functions in response to ischemia beyond a potential role in neurotoxicity. Thus, CRH plays important homeostatic functions in the nervous system, particularly in hypothalamic nuclei, affecting metabolism, energy balance and thermogenesis, and the regulation of neuroendocrine processes [393,461].

Of interest, both glutamate and NMDA administration have been shown to potentiate hypothalamic and amygdalar CRH release in vitro [99,226]. Gabr et al. [155] also demonstrated rapid activation of the hypothalamic-pituitary-adrenal (HPA) axis and enhanced CRH release from the median eminence following amygdalar glutamate administration in stressed rats. Glutamate receptors have also been localized on subpopulations of hypothalamic CRH neurons [261]. Thus, it is possible that increased glutamate release following a global ischemia may mediate CRH release in some neuronal populations.

The present study therefore aims to characterize time-dependent alterations in the response of CRH across a number of discrete brain regions following a global ischemic

insult, and verify the existence of a link between NMDA receptor and CRH activation. In addition to post-mortem analysis of peptide concentrations, we performed a more direct *in vivo* microdialysis experiment to determine the dynamic fluctuations of CRH release from hippocampal CA1 neurons, as well as the central nucleus of the amygdala, before, during and after a global ischemic insult.

Materials and Methods

Animals

Male Wistar rats ($n = 96$), weighing between 250-320g at time of surgery were obtained from Charles River Laboratories (Rochefort, Quebec, Canada). They were individually housed and maintained on a 12 h light/dark cycle (lights on at 7:00 AM), with free access to water and standard (Purina) rat chow. Room temperature was maintained between 21-23°C with 60% relative humidity. Animals were acclimated to the animal facility for at least one week before surgery. Experiments were conducted between 09:00 and 12:00 (micropunch) and initiated at 09h00 (*in vivo* microdialysis) to minimize the influence of endocrine and neurotransmitter variations attributable to diurnal rhythms. All animal experiments were carried out in accordance with the guidelines set by the Canadian Council of Animal Care and received ethical approval from the University of Ottawa Animal Care Committee.

Surgical procedure

Forebrain ischemia was performed using the four-vessel occlusion model as previously described by Pusinelli & Brierley [431]. Briefly, rats were anesthetized using 1.5 % halothane in oxygen. Core temperature was kept at $37 \pm 0.5^\circ\text{C}$ throughout the

surgery by means of a feedback regulated heating blanket connected to a rectal thermometer (Homeothermic Blanket Control Unit, Harvard Instruments, Natick, MA). Subjects' body temperature was supported with a heating pad in the hours following surgery and reperfusion. The vertebral arteries were irreversibly occluded by electrocoagulation and a small-diameter silk thread looped around the carotid arteries to facilitate subsequent occlusion. Twenty-four hours later, common carotid arteries were clamped with microvascular clamps for 10 minutes in awake and spontaneously ventilating animals. Rats lost their righting reflex within 1 min of carotid clamping. Sham-operated animals underwent anesthesia, and received the same dorsal and ventral surgical incisions as the ischemic group with the exception of electrocoagulation of the vertebral arteries. Twenty-four hours later, carotid arteries were exposed, but not clamped.

Drugs

The NMDA antagonist MK-801 (Sigma-Alrich, Oakville, ON) was freshly dissolved in 0.9% saline solution and kept on ice until the time of injection. Injections were performed i.p. at a volume of 0.1 ml/100g.

Experiment 1: Time and region-specific changes in ir-CRH concentrations following global ischemia.

Four, twenty-four and seventy-two hours following a 10 min occlusion of the carotid arteries or a sham occlusion, subjects ($n = 8$ /group/time interval) were decapitated and

the rats' brain removed and placed in an acrylic rat brain matrix (McIntyre, Carleton University, ON) on crushed ice. Serial coronal brain sections (1.0 mm thick) were then obtained and immediately placed on microscope slides, frozen on dry ice and stored at -80°C until processed as previously described [423]. Briefly, distinct brain regions were later bilaterally micropunched using a 1.0 mm diameter punch (Micro Punch MP-600, ASI Instruments, USA), subsequently boiled (80°C) in microcentrifuge tubes containing 220 µl of 0.2 M acetic acid solution, and homogenized (Kontes Micro-ultrasonic Cell disruptor, setting 5; Kontes, Vineland, NJ). A 40 µl aliquot of the homogenate was removed for protein assay using a Protein Analysis Kit (Pierce Scientific, Brockville, ON, Canada) and a spectrophotometer (Brinkmann, PC 800 Colorimeter, USA). The remainder of the homogenate was centrifuged (13000 RPM) at room temperature for 3 min and the supernatant removed, freeze-dried and stored at -80°C for subsequent peptide determination using radioimmunoassay (RIA). A total of 10 brain regions were micropunched using a modification of the method of Palkovits and Brownstein [392]. These included three hippocampal [CA1 & CA3 fields of the Ammon's horn (CA1, CA3), dentate gyrus (DG)] regions, the piriform cortex (Pir), the central nucleus of the amygdala (CeA), four hypothalamic [the paraventricular (PVN), ventromedial (VMH), dorsomedial (DMH) and lateral hypothalamic (LH)] nuclei and the frontal cortex (FC).

Experiment 2: Effects of NMDA antagonism on ischemia-induced ir-CRH concentrations.

The experimental procedure was similar to that described in Experiment 1, with the exception that a subset of sham and ischemic animals ($n = 8$ / group) received 3.0 mg/kg

(i.p.) of MK-801, 30 min prior to sham occlusion or ischemia. Existing literature indicates that this dose provides significant neuroprotection [42,384]. The negative and positive controls (sham and ischemic animals) were injected with saline. Twenty-four hours following reperfusion (or mock occlusion for the sham animals), subjects were decapitated and their brain rapidly removed. Tissue dissection, processing and radioimmunoassay for CRH were conducted as described elsewhere in the text. The 24 h reperfusion interval was selected as changes in CRH were present in various brain regions at that post-ischemic interval and CRH induction also found maximal in other neurotoxic models, such as kainate-induced epilepsy [416].

Experiment 3: Dynamic fluctuations of CRH release at the CA1 of the hippocampus and central nucleus of the amygdala following global ischemia.

Subjects ($n = 8/\text{group}$) were anesthetized throughout the experiment. Wound clips were initially removed from the throat region, and the carotid arteries exposed, following which animals were placed on an elevated stereotaxic apparatus with a level skull. An incision was made exposing the skull and a concentric microdialysis probe with 2.0 mm active membrane (250 μm outer diameter) of regenerated cellulose (6000 MW cut-off; Spectrum Medical Industries, CA), was lowered to the level of the CA1 region of the hippocampus (3.5 mm posterior to bregma, 1.9 mm lateral to the midline and 5.7 mm ventral to the skull surface) or the amygdalar CeA (2.2 mm posterior to bregma, 4.3 mm lateral to the midline and 9.0 mm ventral to leveled skull surface) according to coordinates from Paxinos & Watson [42].

Each probe was connected via polyethylene tubing (PE20, Intramedic, Clay Adams, NJ) to a dual channel swivel (Instech Laboratories, PA) and a 5.0 ml gas-tight infusion syringe attached to a delivery pump (model 22, Harvard Apparatus, MA). Microdialysis probes were perfused at 3 μ l/min with filtered Kreb's Ringer phosphate (KRB) solution (pH 7.4) [Na^+ 145 mM, K^+ 2.7 mM, Mg^{++} 1.0mM, Ca^{++} 1.2mM, Cl^- 150.0 mM; Moghaddam and Bunney [350]] containing 0.1 % BSA. Following one hour of recovery, baseline samples (90 μ l) were collected every 30 min for an additional 90-120 min prior to carotid occlusion. A 15 min carotid occlusion using microvascular clamps was then performed and samples continued to be collected every 30 min for an additional 3 h at the CA1 or CeA. Upon collection, samples were immediately frozen on dry ice and stored at -80°C for subsequent RIA analysis. The efficiency of the microdialysis probes were assessed in vitro (details described in [338]) with the average peptide recovery for CRH being $3.3 \pm 0.6\%$.

Histology

At the end of each microdialysis experiment, subjects were immediately euthanized and their brain extracted, frozen on dry ice and stored at -80°C . Coronal brain sections (30 μ m) were later obtained using a cryostat (Leica Instruments), and stained with cresyl violet for histological verification of microdialysis probe placements. Only those probes confirmed to be in the CA1 region of the hippocampus and CeA for both sham and ischemic animals were included in the data analysis.

CRH Radioimmunoassay (RIA)

The detection and quantification of CRH for micropunch and microdialysis was achieved using a highly sensitive solid-phase RIA, as described by Maidment & Evans [315]. Briefly, Protein A/G (0.1 µg/ 10 µl/ well; Calbochem, La Jolla, CA)-coated Immulon-4 wells (Dynatech, Chantilly, VA) were incubated with anti-CRH serum (rC70; final dilution 1:300,000; kindly provided by W.Vale, the Salk Institute, La Jolla, CA) for 2 h at 20°C. Lyophilised samples obtained from micropunch areas were reconstituted in phosphate buffered saline (ph 7.4). Samples and standards (ranging from 0.61 to 1250 fmol/well) were pipetted into designated wells and incubated for 24 h at 4°C. Reference and blank samples were included to determine total and non-specific binding of the radiolabelled ligand. Next, 25 µl of assay buffer containing 4500-5000 CPM of [¹²⁵I-Tyr⁰]-rCRH (Amersham, Oakville, ON) was added to each well and incubated for an additional 24 h period at 4°C. Finally, the wells were rinsed with wash buffer, blotted dry on paper and the wells broken apart into 12 x 75 mm culture tubes for gamma counting (Packard Cobra II Autogamma, model D5002). A four-parameter logistic curve fit model was used for interpolation of the standard curves. Sensitivity of the assay was typically ~0.1 fmol/well.

Statistical Analysis

In experiments 1 and 2, CRH concentrations in individual brain regions for the 4, 24 and 72 h time intervals were analyzed using one-way ANOVAs. For the 24 h time interval, data were analyzed by two factor (brain region and treatment) ANOVA followed by Tukey's post-hoc comparisons. Ir-CRH values in these post-mortem studies were expressed as pg/mg protein for the purpose of analysis. At times, the degrees of freedom

slightly varied because of missing values. For the analysis of in vivo microdialysis results (Experiment 3), the 3 baseline CRH values from each rat were averaged and defined as 100%. All values were then expressed as a percentage of that baseline value. The changes in ir-CRH levels were analyzed using repeated measures ANOVA with Group as the between factor (Sham vs Ischemic) and Time (3 baseline samples and 6 post-ischemic samples) as the within-subjects factor. Simple main effects assessed the differences between groups at relevant post-ischemic time intervals. Bonferroni corrected t-tests were used to compare post-ischemic CRH levels to baseline values at selected time intervals. In figures, error bars represent the standard error of the mean (SEM).

Results

Experiment 1: Time and region-specific changes in ir-CRH concentrations following global ischemia

Fig. 11 shows alterations in ir-CRH concentrations in the 10 sampled brain regions 4, 24 and 72 h following a 10 min global ischemia.

Four hour interval: CRH concentrations were significantly elevated in ischemic as compared to sham-operated rats at the piriform cortex ($F_{(1,11)} = 5.106$, $p = .045$) and the hypothalamic lateral ($F_{(1,10)} = 13.14$, $p = .005$), ventromedial ($F_{(1,11)} = 6.251$, $p = .029$) and paraventricular ($F_{(1,10)} = 6.001$, $p = 0.034$) nuclei. The data also revealed a significant decrease in CRH levels at the central amygdaloid nucleus ($F_{(1,11)} = 7.789$, $p = .034$). No differences between groups were present in any other assessed brain regions,

including the dorsomedial hypothalamus, prefrontal cortex, hippocampal CA1 and CA3 pyramidal cell layers and dentate gyrus.

24 hour interval: Statistical analysis showed significant differences in seven brain regions: FC ($F_{(3,25)} = 4.25$, $p = .01$), CA3 ($F_{(3,24)} = 3.564$, $p = .029$), CeA ($F_{(3,25)} = 3.29$, $p = .037$), PVN ($F_{(3,25)} = 3.356$, $p = .035$), VMH ($F_{(3,25)} = 8.3$, $p = .000$), LH ($F_{(3,25)} = 5.463$, $p = .005$) and DMH ($F_{(3,24)} = 6.145$, $p = .003$). Post hoc comparisons revealed significantly lower CRH concentrations in vehicle-injected ischemic as compared to sham-operated rats in these brain regions.

72 hour interval: The dentate gyrus was the only brain region where significant differences in CRH concentrations between sham-operated and ischemic animals were observed at that time interval. Ir-CRH concentrations in ischemic animals appeared higher in this region as compared to sham-operated animals ($F_{(1,14)} = 9.453$, $p = .008$).

Experiment 2: Effects of NMDA antagonism on ischemia-induced ir-CRH concentrations

Fig. 12 highlights the impact of MK-801 pretreatment on ir-CRH concentrations in sham and ischemic animals. Post-hoc comparisons in brain regions where CRH alterations were observed 24 h post-ischemia revealed that MK-801 reversed ischemia-induced inhibition in CRH levels in four hypothalamic nuclei (PVN, VMH, LH and DMH; $p < .05$). The NMDA antagonist exerted no intrinsic effects on CRH concentrations in sham-operated animals.

Experiment 3: Dynamic fluctuations of CRH release at the CA1 of the hippocampus and central nucleus of the amygdala following global ischemia

Fig. 13 demonstrates *in vivo* alterations of ir-CRH within the CA1 (upper panel) and CeA (lower panel). With regards to the CA1, planned comparisons using linear contrasts failed to demonstrate differences between pre- and post-baseline CRH levels in both ischemic ($p = .85$) and sham ($p = .37$) animals. Repeated measures ANOVA revealed that neither Time ($p = .332$), Group ($p = .226$) nor Time X Group interactions ($p = .945$) reached significance.

For the CeA, planned comparisons revealed significant differences between pre- and post-baseline CRH levels for the ischemic ($p = .0001$) but not for sham ($p = .14$) animals. Repeated measures ANOVA demonstrated a significant effect of Group ($F_{(1,11)} = 10.39$, $p < .01$), Time ($F_{(8,88)} = 9.17$, $p < .0001$) and a Time X Group interaction ($F_{(8, 88)} = 2.45$, $p < .01$). Simple main effects revealed that differences between groups were attributable to higher extracellular CRH release in ischemic as compared to sham-operated animals 90, 120, 150 and 180 min following ischemia (see Figure 3). With regards to time, significant alterations in ir-CRH concentrations were limited to the ischemic group ($F_{(8,40)} = 9.26$, $p < .0001$). Comparisons of post-ischemic samples against averaged baseline values revealed significant ischemia-induced CRH elevations beginning 60 min following reperfusion.

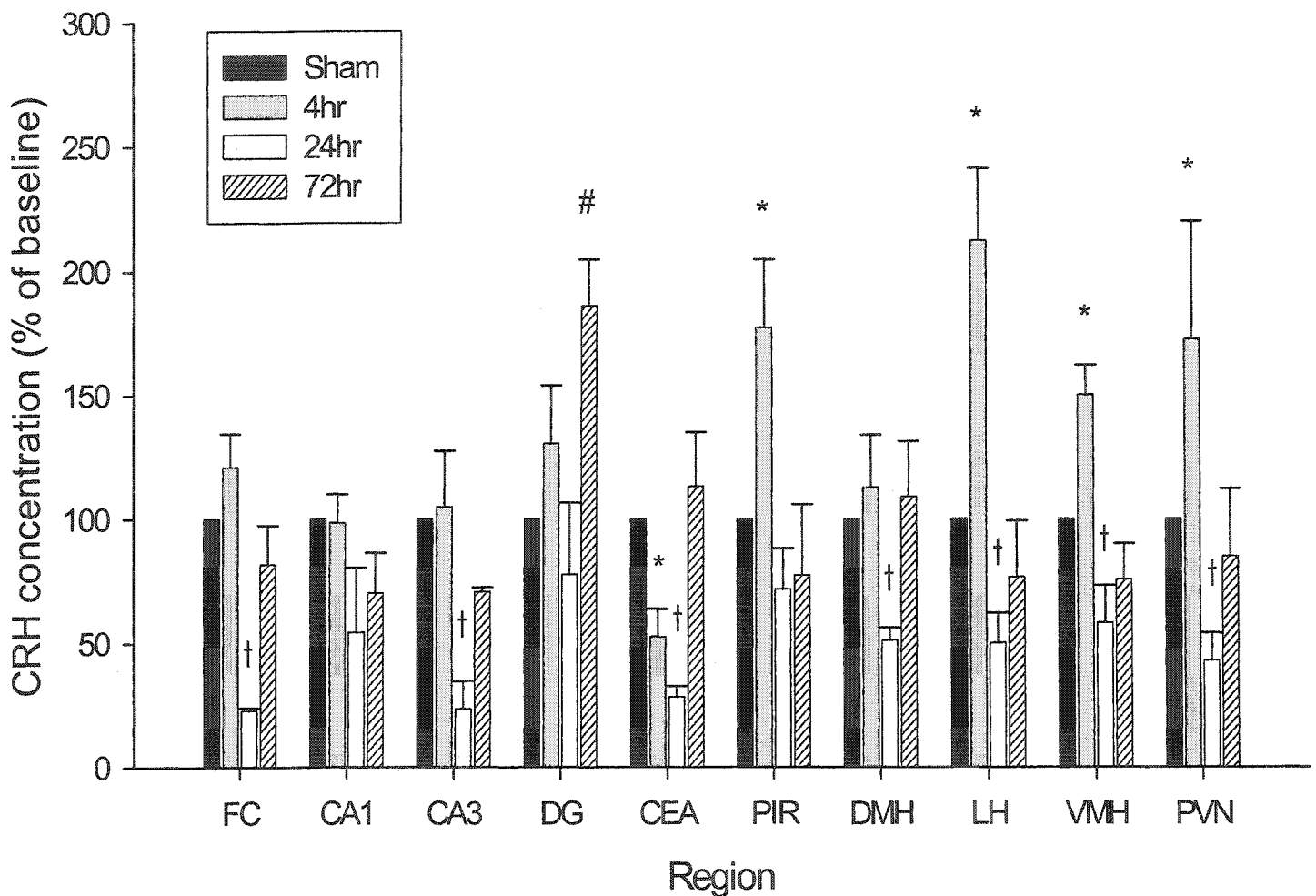


Figure 11. Alterations of ir-CRH concentrations following global ischemia in 10 discrete brain regions at 3 post-ischemic intervals. For the purpose of display, CRH concentrations were converted to % baseline (i.e. % sham controls). Brain regions include frontal cortex (FC), CA1, CA3 and dentate gyrus (DG) regions of hippocampus, central nucleus of the amygdala (CeA), piriform cortex (Pir), and dorsomedial (DMH), lateral (LH), ventromedial (VMH) and paraventricular (PVN) nuclei of the hypothalamus. Comparisons are obtained from original analysis using the raw data for each time intervals.

*, †, # significantly different from sham animals 4, 24 and 72 h post ischemia, respectively ($p < .05$).

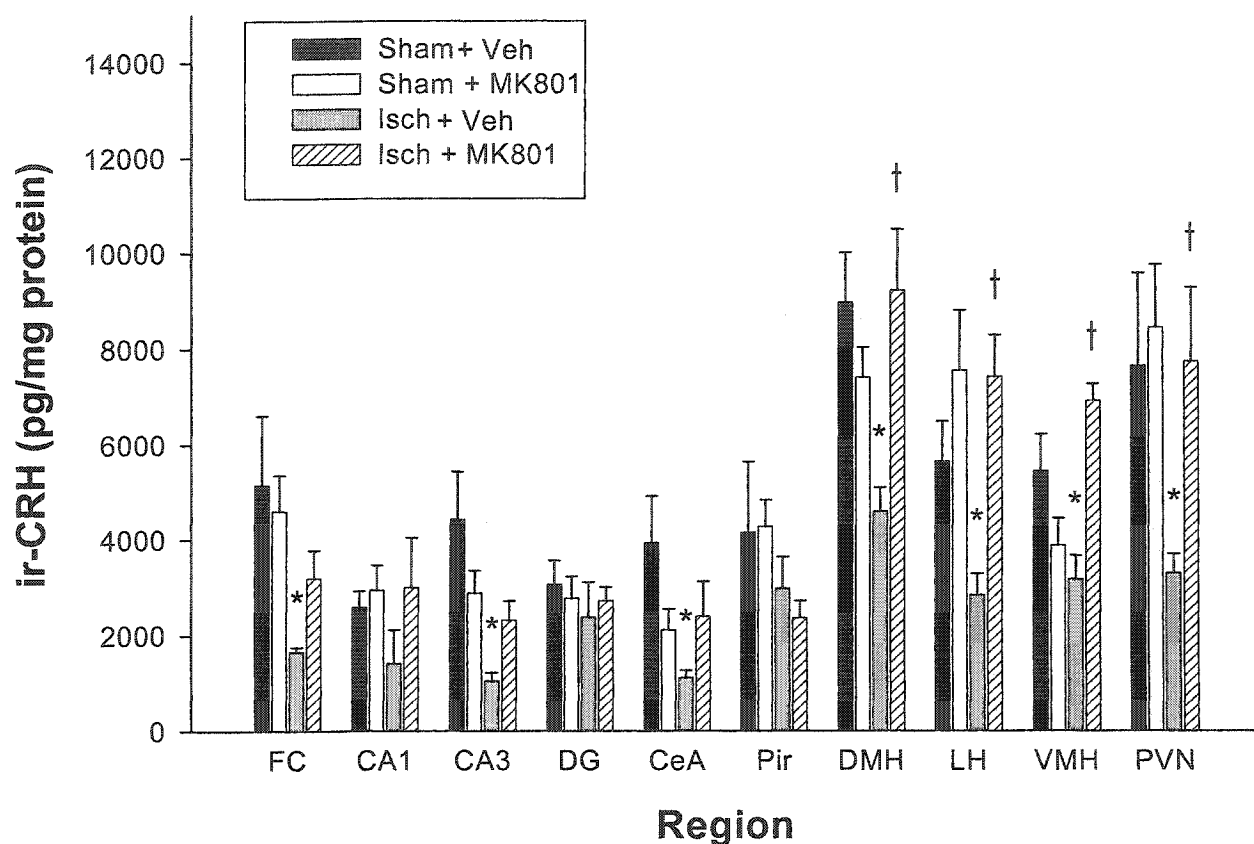


Figure 12. ir-CRH tissue concentrations assessed 24 h following global ischemia and pre-treatment with Mk-801 in the 10 brain regions.

*, † Significantly different from vehicle injected sham and ischemic animals, respectively ($p < .05$).

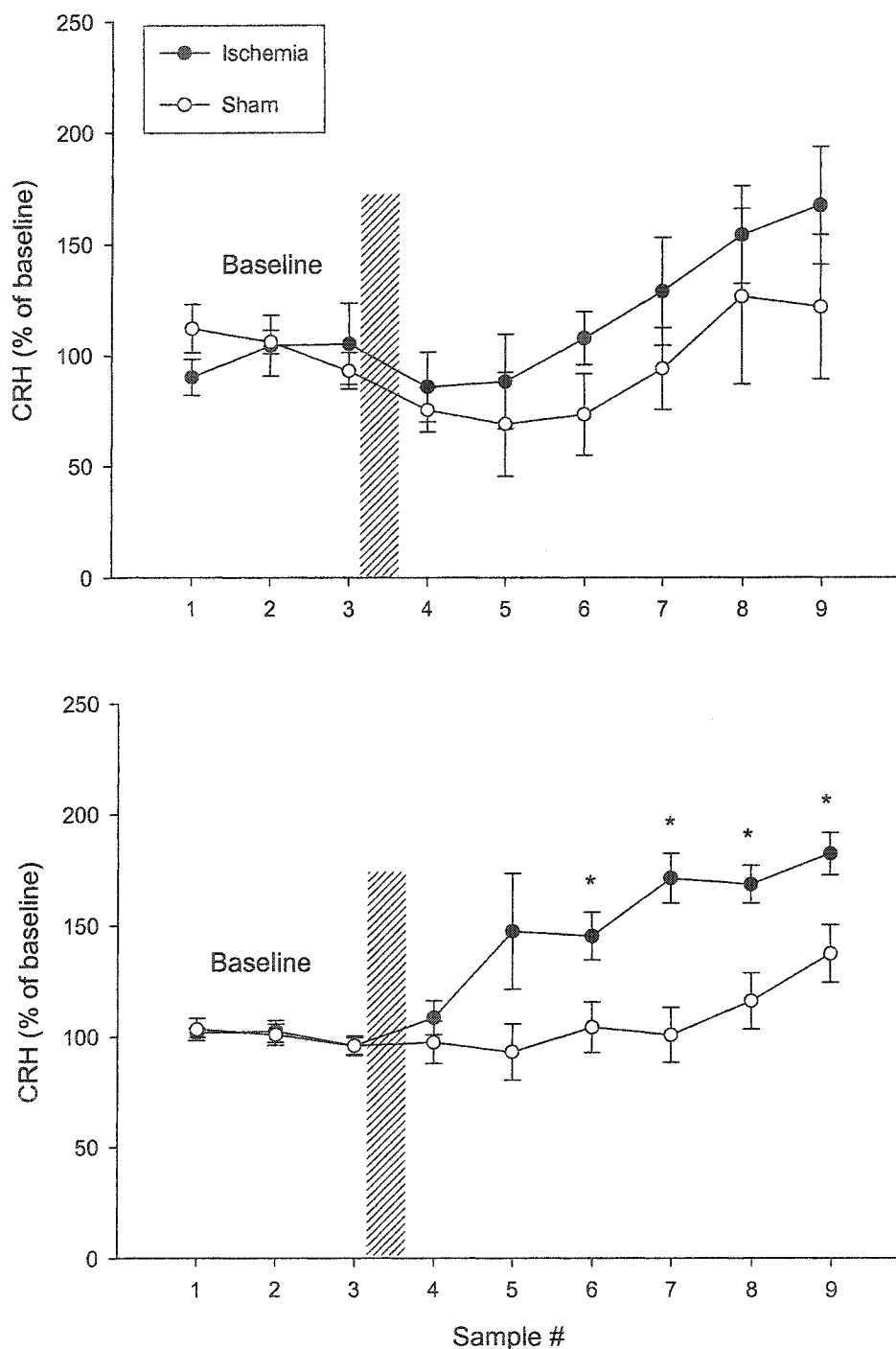


Figure 13. Pre and post-ischemic extracellular CRH levels in sham and ischemic rats at the dorsal hippocampus (CA1) and central nucleus of the amygdala (CeA). Levels of ir-CRH at the dorsal hippocampus (CA1 layer - top panel) and the CeA (bottom panel) as measured by *in vivo* microdialysis in sham and ischemic animals, before, during and following a 15 min global ischemia or sham occlusion. The baseline samples were averaged from each subject and defined as 100%. All values were then expressed as a percent of that baseline. The shaded vertical bar represents the onset and duration (15min) of the global ischemia.

* Significantly different from sham animals ($p < 0.05$).

Discussion

A number of indicators have suggested that CRH may be part of the cascade of biochemical reactions initiated by ischemia, which ultimately leads to neuronal damage. The main goal of this experiment was to further characterize the physiological response of CRH neurons to global ischemia. To do so, we assessed post-mortem fluctuations of corticotropin-releasing hormone in ten discrete brain regions 4, 24 and 72 h following a global ischemic insult as well as monitored *in vivo* ischemia-induced CRH release from the hippocampal CA1 neurons and central nucleus of the amygdala (CeA). We also investigated the participation of glutamate release and NMDA receptor activation to ischemia-induced CRH alterations. To our knowledge, this is the first study to report dynamic fluctuations in CRH release associated with an ischemic insult and to document time-dependent changes in tissue concentrations in numerous brain nuclei following a global ischemia. Previous research has characterized CRH or CRH mRNA expression in a single nucleus or in whole brain structures and has not provided dynamic monitoring of *in vivo* CRH release in ischemic models as yet.

Our findings revealed brain region-specific alterations in CRH levels following global ischemia. These changes were observed in the Pir, CeA, hippocampal CA3 and DG, FC and hypothalamic LH, VMH, PVN, DMH nuclei at different time intervals following ischemia. The piriform cortex represents one brain region where increased ir-CRH concentration was observed 4 h post-ischemia. This brain structure comprised of large pyramidal cells is among the most important brain loci involved in the development and propagation of generalized seizures to the dorsal hippocampus [244]. Neural networks between the piriform and amygdala appear particularly critical in mediating kindling

activity and epileptiform behaviour [332,333]. Thus, numerous bilateral connections have been established between this cortical area and other limbic structures, such as the hippocampus and amygdala. The piriform cortex also appears involved in the spread of excitatory waves to and from these areas in response to limbic seizures [307], which can be produced by central CRH administration [561]. In this context, central CRH administration (at seizure-inducing concentrations) has been shown to rapidly activate c-fos mRNA expression in the piriform cortex but not in the hippocampus. It is only at longer intervals following seizure induction that increased hippocampal mRNA was observed [88]. Studies have also demonstrated that the piriform cortex kindles at a faster rate than the hippocampus and requires significantly less stimulations (5-10 versus 30-40) to induce convulsions [307,331,351]. Although the exact role of CRH activation in piriform neurons following global ischemia remains unknown, the reciprocal pathways between the piriform cortex and hippocampus, and the observation of increased survival of pyramidal CA1 neurons following central blockade of CRH, suggest that increased concentration of the peptide at that site may contribute to hippocampal neuronal damage and/or disruption of cell functioning. Such findings emphasize the need to further investigate the contribution of extrahippocampal sites to ischemia-induced neuronal damage.

In addition to changes at the piriform cortex, significant increases in CRH concentrations were present at the lateral, ventromedial and paraventricular nuclei of the hypothalamus at this early reperfusion interval. These brain structures are involved in regulation of homeostatic processes, which are greatly challenged during the initial phase of the ischemic recovery process. Such post mortem fluctuations, in particular the

increased peptide level at the PVN, although not revealing the underlying mechanism of action, are consistent with the observation that other physiological stressors, such as hypoxia or ether, are associated with stimulation of the hypothalamic-pituitary-adrenal (HPA) axis via CRH-dependent mechanisms [198]. Subsequently, at the 24 h reperfusion interval, a significant decrease in CRH concentrations was observed in these hypothalamic regions, suggesting that enhanced peptide content observed at the earlier time interval had translated into increased release of the peptide leading to temporary depletion of intracellular stores. This depletion appeared to correct itself by 72 h, as hypothalamic CRH concentrations were similar in both sham and ischemic animals at that post-ischemic interval.

The current findings also identified glutamate, via stimulation of NMDA receptors, as an important mediator of ischemia-induced CRH activation in the hypothalamus. This is highlighted by a complete reversal of ischemia-induced CRH alterations in animals pre-treated with the NMDA antagonist MK-801, which showed comparable CRH levels as vehicle-injected sham-operated rats. This finding is consistent with a number of *in vivo* and *in vitro* studies that have established excitatory effects of glutamate on hypothalamic CRH release [155,226], and suggest that similar physiological mechanisms may be activated in response to a global ischemic insult. This interaction appeared selective as NMDA receptor blockade failed to alter CRH immunoreactivity in any of the other tested brain regions.

In addition to hypothalamic CRH alterations, global ischemia was associated with decreased ir-CRH at the frontal cortex 24 h following reperfusion. Although this brain structure has received little attention in excitotoxic processes, it has recently been

proposed that amygdala kindling, in addition to recruiting parahippocampal structures such as the piriform cortex, also engages the frontal cortex [244]. Given the rapid increase in CRH release at the CeA and increased ir-CRH level at the piriform cortex following ischemia, it is conceivable that such cascade of activation translated into an increase in frontal cortex CRH activity occurring between the 4 and 24 h post-ischemic time intervals. This hypothesis remains to be validated in the context of an ischemic insult.

Significant alterations were also observed at the hippocampal CA3 and DG, 24 and 72 h post-ischemia, respectively. The physiological mechanisms underlying decreased CRH concentrations at the CA3 24 h post-ischemia remain unclear. The ability of CRH to produce excitatory effects in pyramidal neurons of the CA1 is equally strong in neurons at the CA3 [5]. Hollrigel et al. [203] further observed that CA3 pyramidal neurons are more sensitive to the seizure inducing effects of centrally administered CRH than CA1 neurons. Interestingly, ischemia and epilepsy are pathological states known to share numerous physiological correlates including enhanced neuronal depolarization and glutamate release and cellular degeneration in similar brain regions [421,432,509]. A diminution in CRH concentrations at the CA3 24 h following reperfusion may thus suggest enhanced CRH activation in the hours post-ischemia, leading to a subsequent depletion of the intracellular stock of the peptide at that site, which may or may not contribute to subsequent hippocampal degeneration.

The increase in CRH at the DG 72 h post ischemia is also of interest. As in the CA1, CRH appears to produce excitatory effects in the DG through inhibition of sAHP's [504]. Wang et al. [555] further found that CRH injected into the DG produces a sustained and

dose-dependent enhancement in synaptic efficacy. Perhaps related to these effects, administration of CRH in the DG has been shown to enhance memory processes and improve retention in the passive avoidance task [210,281]. Considering these actions, one can speculate that potential CRH-mediated protective mechanisms might be activated prior to the onset of neuronal degeneration in an attempt to preserve or protect certain cellular and/or functional properties under direct threat from the ischemic insult. The DG is also among the principal brain structures involved in neurogenesis following epileptic seizures and global ischemia, and increase in the proliferation rate of neuronal precursor cells has been observed as early as 3 days following seizures [225,243]. Ischemia-induced neurogenesis has been proposed to contribute to recovery of functions, specifically as it pertains to improvement of anterograde and retrograde recent memory function that is most affected following global ischemia in animals and man [483]. A potential contribution of CRH to these effects remains to be determined.

A direct neurotoxic effect for CRH in response to cerebral ischemia has been inferred in part from the effectiveness of centrally-administered CRH antagonists in conferring neuroprotection when administered before or shortly after the ischemic insult [311,516]. Given the excitatory effects of CRH on CA1 pyramidal neurons, it has been speculated that ischemia-initiated increases in CRH release in this region might produce excitotoxic effects. In the present study, analysis of post-mortem tissue concentrations did not reveal significant alterations in ir-CRH at the CA1 at any of the tested post-ischemic intervals. This contrasts with findings highlighting enhanced CRH immunoreactivity in the CA1 and CA3 pyramidal cell layers in other neuropathological states involving hippocampal excitotoxic damage such as generalized clonic seizures induced by kainic acid [52].

However, our findings are consistent with Choi et al's [85] recent study that failed to demonstrate alterations in CRH immunoreactivity in CA1 pyramidal cells 3 h following ischemia. Increased CRH expression was only present 96 h post-ischemia in their study, suggesting that if CRH plays a direct role at the CA1, it may act as a late modulator of ischemia-induced neurodegenerative processes.

At the central nucleus of the amygdala, ischemia-related fluctuations in the content of CRH are characterized by a significant diminution of CRH peptide content 4 and 24 h following ischemia. While these findings support the physiological participation of CRH in ischemia, they do not reveal the mechanism of action of the peptide. One possibility for decreased peptide level reflects a depletion of intracellular CRH stores following a period of increased utilization of CRH at that site. Conversely, decreased peptide level can also indicate reduced peptide synthesis with no change in release.

It is difficult using post-mortem analysis of neurochemical alterations at discrete time intervals to reflect the dynamic changes that may be occurring in response to global ischemia, as those changes may be linked to a series of complex physiological processes. Moreover, because synthesis of CRH is rapid, time dependent post-mortem changes in concentrations may not always reflect dynamic activity of the peptide. Also important is the fact that the CRH antagonists that have thus far conferred neuroprotection against ischemic damage, have a relatively short time course of action (approximately 60 min [56]). Therefore, if the neuroprotective mechanism of these drugs involves direct suppression of hippocampal CRH release, this elevation should be observed rapidly following ischemia, at earlier time intervals than thus far selected in post-mortem tissue analysis.

Thus, to obtain a more dynamic profile of CRH activation in response to global ischemia, we have used *in vivo* microdialysis to measure endogenous CRH changes at the dorsal hippocampus and central nucleus of the amygdala. Our findings revealed no changes in CRH release at the CA1 in the initial 3 h sampling period following global ischemia (as compared to pre-ischemic baseline values), indicating that no short-term increases in CRH release from hippocampal CA1 neurons appear to occur following a 15 min global ischemia. Endogenous CRH release appeared comparable in sham and ischemic animals over the entire sampling period.

In contrast, global ischemia was associated with a significant increase in *in vivo* CRH release at the CeA, beginning 60 min following ischemia. This ischemia-induced increase in CRH release at the CeA combined with significant decreases in intracellular CRH concentrations observed at more remote time intervals (4 and 24 h) suggest that such inhibition of CRH concentrations may reflect a depletion of intracellular CRH levels at the CeA, following increased and/or prolonged release during the initial reperfusion period. Amygdalar increase in CRH release is concordant with rapid increase in CRH mRNA expression at the CeA reported following exposure to other acute physiological stressors, such as hypoxia and ether exposure [414]. Increased CRH mRNA at the amygdala has also been reported 60 min following focal ischemia [571], suggesting increased CRH synthesis at this site. Furthermore, the rapid activation and sensitivity of amygdala CRH neurons is interesting as CRH-induced limbic seizures appear mediated by the amygdala [26,137] and seizure activity is not uncommon following an ischemic insult.

The exact physiological mechanism involved in such activation remains to be determined. Recent findings have demonstrated that injection of glutamate into the CeA can stimulate CRH release in a dose-dependent manner [99], and glutamate release is known to occur within 1 or 2 minutes of an ischemic insult [39]. In the present study, although not reaching significance, a trend was observed towards a reversal of the CRH depletion in the CeA in ischemic animals pre-treated with the NMDA antagonist MK-801. This may suggest a partial role for glutamate in amygdala CRH activation following ischemia.

Other factors may also exert influence on CRH peptide content at the amygdala. Palkovits et al. [393] have reported the existence of an inhibitory CRH pathway from the PVN to the amygdala, that passes through the lateral hypothalamus. This pathway appears activated in conditions such as adrenalectomy, where CRH activity at the PVN is directly increased. This is of interest in the current context as short-term CRH increases were observed at both the PVN and lateral hypothalamus. It thus remains possible that enhanced activation at the PVN may also have contributed to inhibition of CRH levels at the amygdala.

Alterations of CRH in the present study suggest that if this peptide plays a role in ischemia-induced neuronal damage, it may exert an indirect action on hippocampal CA1 neurons via activation in brain structures shown to send ascendant fibers to the hippocampus. This may include the amygdala and piriform cortex, where rapid alterations of CRH release and post-mortem concentrations have been observed. Moreover, a number of studies have shown that CRH activation of locus coeruleus neurons stimulates hippocampal norepinephrine release [84,388,389], a neurotransmitter

known to inhibit slow afterhyperpolarizations (sAHP). This could potentially increase the firing rate of CA1 neurons [273]. Other studies have demonstrated that CRH receptors mediate hippocampal serotonin release [221,297,404] which also inhibit sAHP's.

In conclusion, our findings demonstrate that a global ischemic insult, capable of inducing significant damage at the CA1 region of the hippocampus, is not associated with significant alterations of CRH concentrations and/or release in this region in the initial or later phases of the ischemic process, suggesting that the neuroprotection observed thus far by CRH antagonists may be modulated through CRH alterations in regions outside the hippocampus. Early activation of CRH neurons is present in the parahippocampal region of the piriform cortex, which sends excitatory projections to the hippocampus, as well as in the hypothalamus. In vivo, a rapid increase in CRH release is observed at the CeA following ischemia, leading to decreased intracellular CRH concentrations shortly after. These endogenous CRH changes may act as excitatory signals that ultimately influence physiological processes at the hippocampus. Further study into the role of discrete extra-hippocampal regions demonstrating CRH changes and investigation into possible homeostatic mechanisms involving CRH are warranted to gain a more thorough understanding of the particular role of this peptide in ischemia-related processes.

Preface to Experiment 5

The previous chapter demonstrated widespread changes in CRH release and concentrations throughout the brain, and at various time intervals in response to global ischemia. Interestingly, there was a conspicuous absence of CRH increases in the region most vulnerable to cell death, the CA1 zone of the hippocampus. Thus, CRH antagonists that have previously conferred neuroprotection might indirectly affect excitotoxic processes at the hippocampus through inhibition of secondary molecules or systems (e.g. glutamate, norepinephrine, serotonin) or blockade of CRH in structures projecting axons to hippocampal CRH interneurons. Alternatively, CRH antagonists may inhibit the activation of receptors by other endogenous ligands (e.g. urocortin, urotensin, sauvagine). Prior studies demonstrating neuroprotection using CRH antagonists have left a number of issues unresolved, including the relative contribution of CRH receptor subtypes in neuroprotection, and the effectiveness of these compounds in treating ischemia-induced behavioural impairments. The experiments conducted in chapter 5 were designed to investigate these issues.

N.B. - For the Elevated Plus Maze (EPM) test, the sham and ischemic animals used in chapter 5 are the same as those used in chapter 3.

Experiment 5

CRH antagonism and ischemia-induced neurodegeneration and behavioural impairments.

Abstract

The current study evaluated the effectiveness of selective and non-selective corticotropin-releasing hormone (CRH) antagonists in reversing global ischemia-induced neurodegeneration and behavioural deficits. CRH has been implicated in ischemia-induced neurotoxicity, due in part to excitatory effects at the hippocampus, and the demonstrated neuroprotective effects of centrally administered, non-specific CRH antagonists. However, a number of issues remain to be clarified from these studies, including the relative contribution of CRH receptor subtypes, and the efficacy of these compounds in treating ischemia-induced behavioural impairments. In the current study, a highly selective, systemically administered CRH1 antagonist (CP-154,526) failed to reverse ischemia induced cell death at the CA1 or spatial memory impairments as assessed in the radial arm maze. Similarly, central administration of alpha-helical CRH also failed to confer protection against ischemic damage. Interestingly, CRH1 antagonism reversed ischemia-induced hyperactivity in a novel open field and increased open-arm activity in the elevated plus maze, suggesting that these behavioural impairments are largely independent of CA1 neuronal damage. Failure of the current study to demonstrate neuroprotective effects of either of the CRH antagonists tested following global ischemia challenge the proposed neurotoxic role of CRH in ischemia. These findings are discussed in relationship to recent findings reconsidering the participation of CRH in excitotoxic-mediated cellular damage.

Introduction

Global ischemia results in a loss of energy supply to the brain associated with complex physiological and genetic alterations which compromise cellular functioning, leading to cellular death by necrosis and/or apoptosis [86,209,371,490]. The CA1 pyramidal cell layer of the hippocampus is particularly sensitive to cerebral ischemic damage with brief periods of global ischemia leading to delayed loss of CA1 neurons in rodents and humans [255,407,432]. Moderate to extensive loss of hippocampal CA1 pyramidal sector has been shown to induce severe anterograde amnesia in humans [594], and a variety of functional deficits, including locomotor hyperactivity as well as working and spatial memory impairments in rodents [19,90,91,216]. A variety of putative neuroprotective strategies have endeavoured to reverse these cellular and behavioural impairments. However, these compounds have not yet translated into therapeutic successes in human populations [124] and the search for novel neuroprotective drugs continues.

Among the molecular targets suggested to play a role in excitotoxic cellular damage and global ischemia is the 41-amino acid polypeptide corticotrophin-releasing hormone (CRH) [311,516,571]. CRH-like immunoreactive cells, fibres and receptors have been identified in different hypothalamic, brain stem and limbic structures including the amygdala and hippocampus [76,354,468,527,547]. Administration of kainic acid has been shown to enhance CRH-immunoreactivity in regions that undergo neurodegeneration including the hippocampus [416], and central blockade of CRH receptors using α -helical CRH has been shown to confer significant neuroprotection when administered before or shortly after global or focal ischemic insults [311,516] or

following kainic-acid induced seizures [314]. CRH also produces a number of excitatory effects at the hippocampus including depolarization of hippocampal neurons *in vitro* and an increase in the excitability and spontaneous discharge frequency of CA1 and CA3 pyramidal neurons [4,5,203].

Despite these findings, a number of issues concerning the exact contribution of CRH to neuronal damage remain unresolved. For instance, the studies thus far demonstrating neuroprotection following CRH receptor blockade have used the peptide-based antagonist alpha-helical CRH. Being a non-specific antagonist, it blocks both known types of CRH receptors (CRH1 and CRH2). This is a particular concern since CRH2 is known to share a high affinity with other endogenous peptides such as urocortin. In fact, urocortin has a 10-fold higher affinity for the CRH2 receptor than CRH itself [498] and also produces excitatory effects in the dorsal hippocampus of rats, as assessed using EEG recordings [500]. Therefore, it is unclear whether the biologically significant effect of alpha-helical CRH in ischemia models involves inhibition of CRH or urocortin. Indeed, recent evidence has failed to show neurotoxic effects for CRH administered *in vivo* and CRH does not exacerbate glutamate-induced neurotoxicity [98]. Under certain conditions, such as hypoxia [152] and Alzheimer's [401], a neuroprotective role for CRH has even been suggested. Interestingly, recent evidence has also suggested that alpha-helical CRH exerts agonistic properties at high doses [457], which could further confound findings and conclusions about the neurotoxicity of CRH.

To address these issues, the current study will investigate the neuroprotective effects of CP-154,526, a recently developed non-peptide CRH antagonist with high specificity for the CRH1 receptor [478]. One additional advantage of this non-peptide CRH1

antagonist is its possibility to be administered systemically, which if successful, could help it qualify as a potential therapeutic agent in human populations. To directly compare the neuroprotective properties of CRH antagonists, a distinct set of ischemic animals will be pretreated with central administration of alpha-helical CRH.

Although neuroprotective effects for CRH antagonists have been demonstrated, whether such morphological and cellular preservation translates into significant recovery of behavioral and physiological functions remains unknown. In the present study, the assessment of different behavioural functions in animals injected with the CRH1 antagonist will help determine the impact of such treatment on the functional recovery of ischemic animals, and further characterize the degree of association between hippocampal neuronal damage and behavioural impairments.

Among the most commonly observed behavioural impairments in ischemic subjects are hyperactivity and spatial memory deficits. Chandler, DeLeo and Carney [79] first noticed that ischemic subjects spontaneously displayed elevated activity levels shortly following reperfusion. This hyperactivity appears particularly noticeable when subjects are placed in a novel environment [90,554]. Some authors have reported a positive correlation between hippocampal damage and locomotor activity [8,160,241] while other studies have attributed ischemia-induced hyperactivity to deficits in spatial memory [18,554]. Interestingly, increased exploratory activity in an open field has also been associated with decreased anxiety [38,100,390]. In this context, numerous studies have combined measures of open field activity with open-arm activity in the elevated plus maze (EPM) to assess environmental, pharmacological and genetic mediators of exploratory anxiety [268,269,286,581]. Effective anti-anxiety agents tend to increase

both exploratory activity in an open field as well as open-arm activity in the EPM [581], while anxiogenic compounds suppress both types of activity [499,510]. Recent findings from our laboratory have demonstrated that ischemia-induced hyperactivity in an open field is accompanied by a significant increase in the number of entries and time spent in the open arm of the EPM, suggesting decreased anxiety levels in ischemic animals [422]. The current study will thus assess the impact blockade of CRH1 receptors with CP-154,526 on ischemia-induced hyperactivity in the open field and anxiolytic response in the EPM.

The effectiveness of CRH1 antagonism in reversing ischemia-induced spatial memory deficits will also be examined. The impact of global ischemia on spatial memory is well acknowledged. Ischemic animals reliably demonstrate significant memory impairments, in part manifest through increased working memory errors, in testing paradigms such as the radial arm maze [111] where performance has been positively correlated with the number of intact pyramidal cells in the CA1 section of the hippocampus [258].

Together, we hope that this study can provide a more comprehensive evaluation of the effectiveness of CRH antagonists in reversing ischemia-induced cell death and behavioural impairments, as well as help determine the relative contribution of particular receptor subtypes to these effects.

Materials and methods

Animals

Male Wistar rats ($n = 43$) weighing between 250-320g at time of surgery obtained from Charles River Laboratories (Rochefort, Quebec, Canada). They were individually housed and maintained on a 12 h light/dark cycle (lights on at 7:00 AM), with free access to water and standard (Purina) rat chow. The room temperature was maintained at 21-23°C with 60% relative humidity. Upon their arrival, animals were acclimated to the animal facility for at least one week prior to surgery. All procedures were carried out in accordance with the guidelines set by the Canadian Council of Animal Care and received ethical approval from the University of Ottawa Animal Care Committee.

Global Ischemia

Forebrain ischemia was performed using the four-vessel occlusion model as previously described by Pusinelli & Brierley [431]. Briefly, rats were deeply anesthetized by inhalation of 1.5% halothane in oxygen. The core temperature was kept at $37^{\circ}\text{C} \pm 0.5^{\circ}$ throughout the surgery by means of a feedback regulated heating blanket connected to a rectal thermometer (Homeothermic Blanket Control Unit, Harvard Instruments, Natick, MA) and supported with a heating pad in the hours following surgery and reperfusion. The vertebral arteries were irreversibly occluded by electrocoagulation, and a small-diameter silk thread looped around each carotid artery to facilitate subsequent occlusion. Twenty-four hours later, common carotid arteries were clamped with microaneurysm clamps for 10 minutes in awake and spontaneously ventilating animals. Rats lost their righting reflex within 1 min of carotid clamping.

Sham-operated animals underwent anesthesia, and received the same dorsal and ventral surgical incisions as the ischemic group with the exception of electrocoagulation of the vertebral arteries. Twenty-four hours later, carotid arteries were exposed but not clamped.

Drug Preparation

The CRH1 antagonist CP-154,526 (generously donated by Pfizer Inc., Groton, CT) was dissolved in a vehicle of 90% dH₂O, 5% DMSO, and 5% Emulphor (ethoxylated castor oil, kindly donated by M.Owens, Emory University, Atlanta, GA), and injected *i.p.* in a volume of 1ml/100g. Alpha-helical CRH (9-41) (Phoenix Pharmaceutical; Mountain View, CA) was dissolved in saline containing 0.05 % acetic acid, and administered *i.c.v.* into the 3rd ventricle in a volume of 5 μ l.

Experiment 1: Effect of the CRH₁ antagonist CP-154,526 on ischemia-induced neuronal degeneration and behavioural deficits.

Drug Treatment

Two doses of CP-154,526 were tested: 32 and 64 mg/kg. The doses were selected based on prior demonstrations of significant CRH receptor blockade [478] and inhibition of norepinephrine (NE) and serotonin (5-HT) release at the CA1 [221]. NE and 5-HT have excitatory effects in the CA1 of the hippocampus via a similar mechanism as CRH. The 32 mg/kg dose was administered either 30 min before ($n = 6$) or 15 min after a 10 min global ischemia ($n = 5$). The 64 mg/kg dose was administered 30 min before ischemia ($n = 8$). Control groups were composed of vehicle-treated ischemic ($n = 10$)

and sham ($n = 11$) animals as well as sham-operated rats pre-treated with the high dose of CP154,526 (64 mg/kg; $n = 8$).

Apparatus and procedures for behavioural testing

Novel openfield: The observation arena was made of gray Plexiglas (LWH: 75 X 75 X 30 cm), with a clear Plexiglas floor. The floor was painted with a grid comprising 25 10X10 cm squares. The entire arena was kept on a table 90cm above the floor and in the same room as the rats' housing. Between 4-7 days following reperfusion, vehicle or CRH₁ antagonist-injected sham and ischemic subjects were placed in the open field and behaviour was monitored for 30 min by the experimenter using a portable PC computer (Toshiba 4250) and datalogging software. Subjects were monitored for frequency of line crossing, sniffing, grooming and rearing behavior as well as stillness/resting (absence of activity).

Elevated plus maze: The elevated plus maze consisted of two opposing open arms (50 X 10 cm with a 5 mm clear Plexiglas lip), an open 10 X 10cm center zone and two opposing closed arms (50 X 10 cm with 40 cm high walls). The entire maze was elevated 60cm from the floor. Ischemic subjects pre-treated with the high dose CRH₁ antagonist (64 mg/kg) were placed in an open field (75 X 75 cm with 30 cm high walls) for a 5 min habituation period. Immediately thereafter, the animals were placed in the center of the elevated plus maze facing one of the open arms. The number of entries and the time spent in each arm of the maze as well as risk assessment behaviour was recorded during a 5 min testing period. The rat was considered to be in the open or closed area of the maze

if at least its head and two front paws were in that area. Risk assessment was operationally defined as elongation of the neck with at least the snout protruding into the open zone, while the paws and body remained in the closed arm. Black curtains surrounded the maze, and monitoring of the behaviour was possible using an overhead video camera and monitor.

Radial arm maze: The maze consisted of eight arms (60 X 12 cm, with a 5 cm lip around each arm) extending radially from a central octagonal area (32 cm in diameter with a 30 cm high clear Plexiglas wall). Plexiglas sliding doors allowed for entry into each arm. The floor of the arms and central area were covered with black rubber lining. The apparatus was placed on a stand 50 cm above the floor and surrounded by extra-maze cues such as posters or calendars along the side walls. The experimenter was sitting behind a desk with a front panel where he could observe and record behaviours unobtrusively as well as manipulate the strings to open and close the maze doors. At the end of each of the maze's arm was a food cup (1 cm deep into the floor) that held a piece of fruit loop (the bait). The animals were kept on a restricted diet (4-5 rat chow pellets/day) and body weight maintained at approximately 85% of their free-feeding rate during the testing period to ensure adequate motivation.

During training, subjects were habituated to the radial arm maze for 4 daily tests each lasting 10 min and performed on successive days. The bait was initially available throughout the maze to encourage exploration, but gradually restricted to the food cup. Following this shaping period, each animal was placed individually in the center of the maze with the doors to all arms closed. Upon opening all doors, the rat was permitted to

enter any of the eight arms. When the rat had consumed the fruit loop at the end of one arm, it returned to the center of the maze and all doors were closed again, confining the rat to the center of the maze for a 10 sec delay. The doors were then reopened, and the procedure repeated. The trial continued until all baits were consumed or until 15 min had elapsed. An arm entry was counted when all four limbs of the rat were within an arm. Measures were made of the number of working memory errors (entering an arm that was previously entered) and the time taken to consume all baits. Animals were tested every day for 15 days.

Experiment 2: Effect of alpha-helical CRH on ischemia-induced neuronal degeneration.

Surgical procedure

Surgeries were performed in rats anaesthetized with pentobarbital (65 mg/kg; i.p.), according to the procedures and guidelines approved by the Animal Care Committee. Animals were placed in a stereotaxic instrument with level skull. Guide cannulae (22 gauge) aimed at the 3rd ventricle were positioned using the following coordinates: 4.3 mm posterior to bregma, 0.0 mm lateral to the midline and 4.3 mm ventral to the skull surface. All coordinates were derived from the atlas of Paxinos & Watson [400]. Guide cannulae were anchored to the skull using 4 stainless steel screws and acrylic dental cement.

After 7-10 days of recovery from cannulation surgery, subjects with 3rd ventricle cannulae then underwent surgery for forebrain ischemia as described earlier. Thirty minutes prior to ischemia or sham occlusion, subjects were injected with α -helical CRH

(100 µg) in a volume of 5 µl delivered over 1 min interval, using a 1.0 ml Hamilton syringe controlled by an electronic syringe driver (Harvard 22; Harvard Apparatus, South Natick, MA). The Hamilton syringe was connected via PE 20 tubing to a stainless steel injector, which protruded 0.5 mm below the cannula tip, and into the 3rd ventricle. Thirty minutes following infusion, common carotid arteries were clamped with microaneurysm clamps for 10 minutes.

Histology

Upon completion of the behavioural tests, subjects were euthanized and the brains removed, frozen on dry ice and stored at -80°C. Serial coronal sections (14 µm) of the hippocampal regions were subsequently obtained using a cryostat and stained with cresyl violet. Neuronal degeneration was then determined using computer-assisted light microscopy.

Analysis of neuronal density on cresyl violet stained sections

The neuronal density of the hippocampal CA1 subfield was determined by the method of Kirino et al. [257]. Analysis of neuronal density was performed on coronal sections located between 3.14 and 4.16 mm posterior to bregma. The total linear length of the CA1 was measured by means of a digitizer. The number of living neurons in the stratum pyramidale within CA1 subfields was counted using LEICA DAS microscope attached to SONY digital camera and computer-assisted cell counting performed using Norton Eclipse software (v 6.0). Neurons that had shrunken cell bodies with surrounding empty spaces were excluded. The neuronal density of the CA1 sector, i.e. the number of

intact pyramidal cells per 1 mm linear length of the CA1 stratum pyramidale observed in each 14 μ m section, was quantified. Thus, a mean value for each hippocampal CA1 substructure was obtained from 6 bilateral measurements on 6 sections per animal in each of the experimental groups. The neuronal density for a given animal represents the average of both the right and left hippocampal neuronal cell densities. Neuronal density values are expressed as mean $\pm$ standard error of the mean.

Statistical analysis

Behavioural activity in the open field (calculated from frequencies of line crossing, sniffing, grooming and rearing) was assessed in blocks of 5 min intervals for a 30 min testing period in vehicle and CRH1 antagonist-treated sham and ischemic subjects. Six animal groups were included for analysis. Ischemic subjects received either the vehicle or one dose of the CRH1 antagonist (32 or 64 mg/kg) before or 32 mg/kg following vessel occlusion. Sham rats received either the vehicle or the CRH1 antagonist (64 mg/kg) prior to sham occlusion. A repeated measures two-factor ANOVA was used to assess differences in activity levels between groups as well as time-dependent changes in activity level over the 30 min period. Tukey post hoc analyses (adjusting for multiple comparisons) was used to determine significant within and between group differences. For the elevated plus maze, one-way ANOVA was used to analyze the effect of Group on the number of open arm entries, total time spent on the open and closed arms and risk assessment behaviour. Four rat groups (vehicle- and drug-treated (64 mg/kg) sham and ischemic rats) were included in the analysis. Tukey post-hoc analysis was conducted to determine individual group differences.

In the radial arm maze, a repeated measures ANOVA followed by Tukey post-hoc analysis was used to determine differences in working memory errors between groups. The 15 day testing period was grouped into 3 day intervals (i.e. days 1-3, 4-6, 7-9, 10-12, 13-15) for the analysis. A repeated measures ANOVA investigated the effects of Group and Time on working memory errors. Thus, the effect of ischemia on memory errors is seen in the main effect of Group and the Group X Time interaction.

Analysis of neuronal density (number of pyramidal cells /1mm CA1 tissue) was performed using a one-way ANOVA followed by Tukey post-hoc comparisons. Seven groups were included for comparison, vehicle and drug-treated (64 mg/kg) shams, vehicle treated ischemics and four drug-treated ischemic groups (alpha-helical CRH (100 µg) and CRH1 antagonist: 32 mg/kg pre and post-occlusion, 64 mg/kg pre-occlusion). In figures, error bars represent the standard error of the mean (SEM).

Results

Experiment 1: Effect of the CRH₁ antagonist CP-154,526 on ischemia-induced behavioural deficits

Novel Open Field: Fig.14 shows the impact of treatment with the CRH1 antagonist on the general activity profile of sham and ischemic animals in the open field. Statistical analysis revealed a significant effects of Group ($F_{(5,50)} = 7.192$, $p < .0001$) and Time ($F_{(5,50)} = 39.081$, $p < .0001$) on overall activity level but no Group X Time interaction ($F_{(5,250)} = .800$, $p = .741$). This indicates differences in activity levels between groups over the entire 30 min interval, but no differences in the rate of habituation between groups. Post hoc comparisons revealed no significant difference in activity levels

between vehicle and drug-treated-sham animals. Vehicle treated ischemics did show significantly higher behavioural activity than vehicle-treated shams in the first 15 min of open field exposure. In addition, vehicle-treated ischemic rats also displayed higher activity levels over the initial 20 min testing period as compared to all three drug-treated ischemic groups ($p < .05$).

Elevated Plus Maze (EPM): Fig. 15 highlights the exploration profile of vehicle and CP-154, 526 treated sham and ischemic rats in the EPM. Results from vehicle-treated sham and ischemics were compared to drug-treated shams and ischemics for open and closed arm entries, time spent on the open and closed arms, and risk assessment behaviour. A one-way ANOVA revealed a main effect of Group on both the number of open ($F_{(3,26)} = 16.121, p < .001$) and closed ($F_{(3,25)} = 6.08, p = .003$) arm entries as well as total time spent on the open ($F_{(3,27)} = 7.592, p < .001$) and closed ($F_{(3,25)} = 13.108, p < .001$) arms of the maze. No significant difference was found in risk assessment behaviour ($F_{(3,25)} = 1.519, p = .234$). Post-hoc analysis revealed that vehicle-treated ischemic animals made significantly more open arm entries and spent more time on the open arm as compared to vehicle-treated sham rats. In contrast, drug-treated ischemic rats made fewer open arm entries and spent significantly less time on the open arm as compared to vehicle-treated ischemic rats ($p < .05$). No difference was observed on either measure between vehicle and drug-treated sham animals. These findings suggest that the CRH₁ antagonist inhibits ischemia-induced increases in open arm activity.

Radial Arm Maze: Three groups were tested on the radial arm maze; sham, vehicle-treated and CRH1-antagonist-treated ischemics (64 mg/kg, pre-occlusion). The 15 day period of testing was grouped into 3 day intervals (i.e. days 1-3, 4-6, 7-9, 10-12, 13-15) and analyzed using a repeated-measures ANOVA. Analysis showed a significant effect of Group ($F_{(2,25)} = 27.87$, $p < .001$), Time ($F_{(4,100)} = 22.90$, $p < .001$) and a significant Group X Time interaction ($F_{(8,100)} = 6.352$, $p < .001$). Tukey post-hoc analysis, adjusting for multiple pair-wise comparisons, revealed that the number of working memory errors for drug and vehicle-treated ischemics, were significantly greater than for sham-operated animals over the first 9 days ($p < .05$) (see Fig. 16).

Analysis of neuronal density

Table 2 shows the impact of a 10 min global ischemia and of the different CRH antagonists treatments on CA1 neuronal density. Results are expressed as number of cells/1 mm CA1 tissue.. One-way ANOVA indicated a significant effect of Group on cell density ($F_{(6,40)} = 23.575$, $p < .001$). Post-hoc comparisons revealed that this effect was due to lower cell densities in the vehicle and drug-treated ischemic groups as compared to the vehicle and drug-treated sham animals ($p < .05$). Cell densities were comparable for all ischemic groups regardless of whether they received vehicle or the CRH antagonists. Similarly, vehicle and CRH₁ antagonist-treated shams showed no differences in cellular density. These findings suggest that treatment with CRH antagonists failed to confer neuroprotection against ischemia-induced hippocampal damage.

Table 2: Hippocampal cellular density (cells/1mm, CA1 tissue; means $\pm$ SEM)

Sham (vehicle; n = 11)	182.55 $\pm$ 4.977
Sham (CP 154,526, 64mg/kg; n=6)	182.10 $\pm$ 8.29
Ischemia (n = 10)	48.33 $\pm$ 9.78 *
Alpha-helical CRH (100 μ g; n = 6)	30.8 $\pm$ 2.26 *
CP 154,526 (64mg/kg; n = 8)	41.73 $\pm$ 20.49 *
CP 154,526 (32mg/kg before ischemia; n = 6)	55.2 $\pm$ 9.6 *
CP 154, 526 (32mg/kg after ischemia; n = 5)	65.1 $\pm$ 16.5 *

Table 2. Density of hippocampal CA1 neurons in vehicle and CRH-antagonist treated sham and ischemic animals. * Indicates significantly different from vehicle-treated sham rats ($p < .01$).

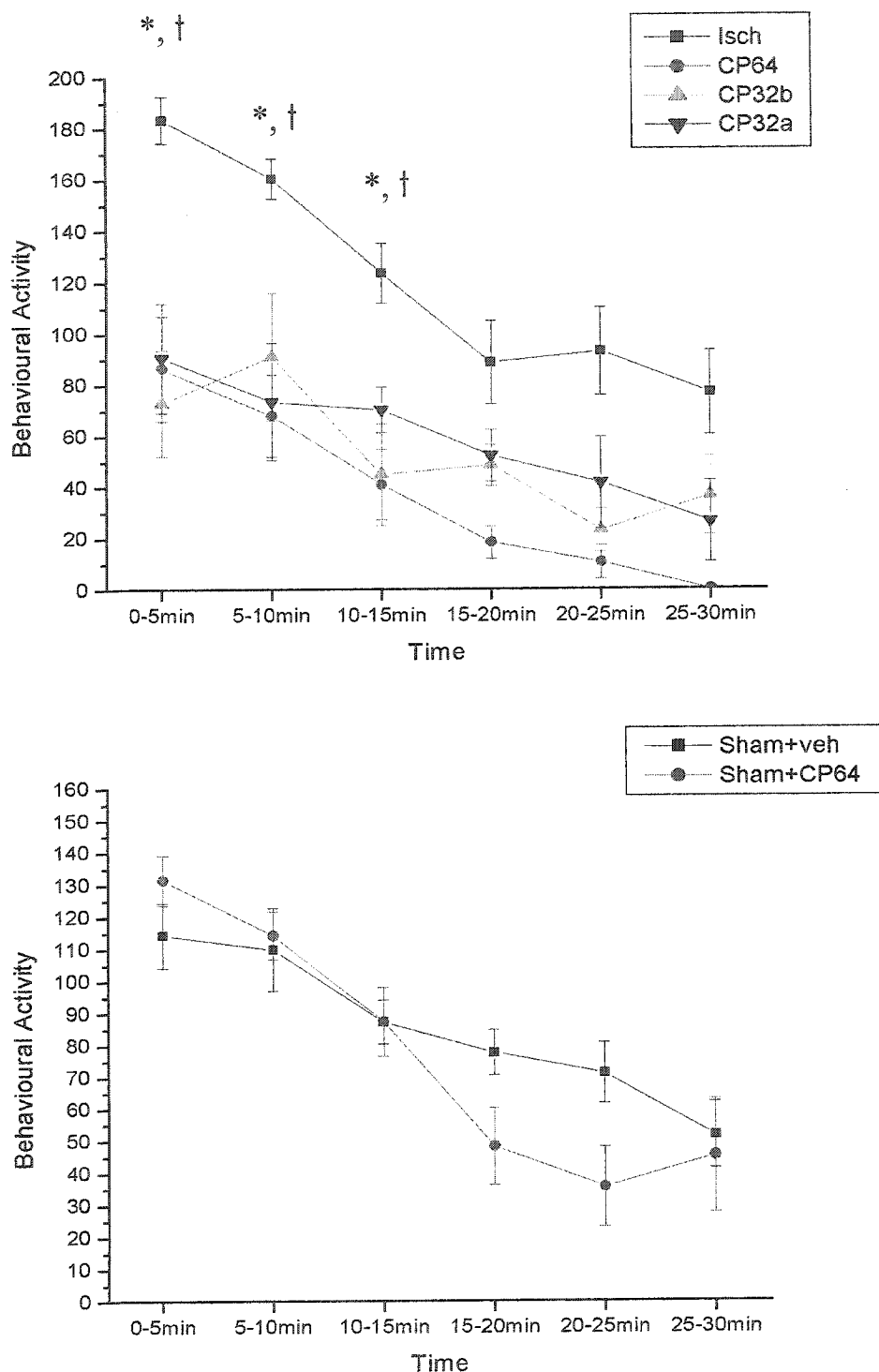
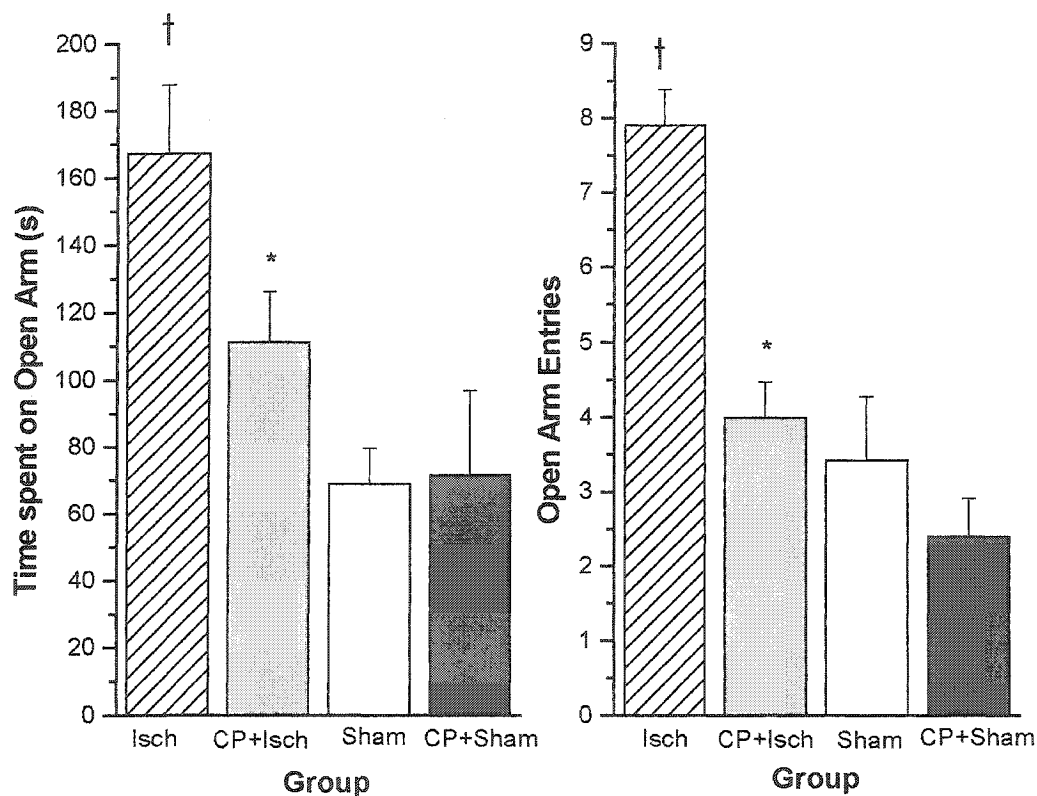


Figure 14. Behavioural activity profile of vehicle and CRH1 antagonist-treated sham and ischemic animals in the novel open field. Ischemic animals (top figure) received either vehicle or one dose of the CRH1 antagonist CP-154,526: (32 (CP32b) or 64mg/kg (CP64) before, or 32 mg/kg (CP32a) after the ischemia. Sham-operated animals (bottom figure) received either vehicle or 64 mg/kg (sham + CP64) dose of the CRH1 antagonist before sham occlusion.

* Indicates significantly higher activity levels in vehicle-treated ischemic compared to vehicle-treated shams ($p < .05$); † indicates significantly higher activity among vehicle-treated versus drug-treated ischemics ($p < .05$).



*Figure 15. Effect of CRH1 antagonism on performance of sham and ischemic rats in the Elevated Plus Maze (EPM). Vehicle-treated sham (Sham) and ischemic (Isch) rats are compared to CRH1-antagonist (64 mg/kg)-treated sham (CP+sham) and ischemic (CP+Isch) rats with regards to number of entries and time spent on the open arm of the EPM. The vehicle or drug treatments were administered 30 min prior to global ischemia or sham occlusion. * Significantly different from vehicle-treated ischemic rats ($p < .05$); † Significantly different from vehicle-treated sham rats ($p < .05$).*

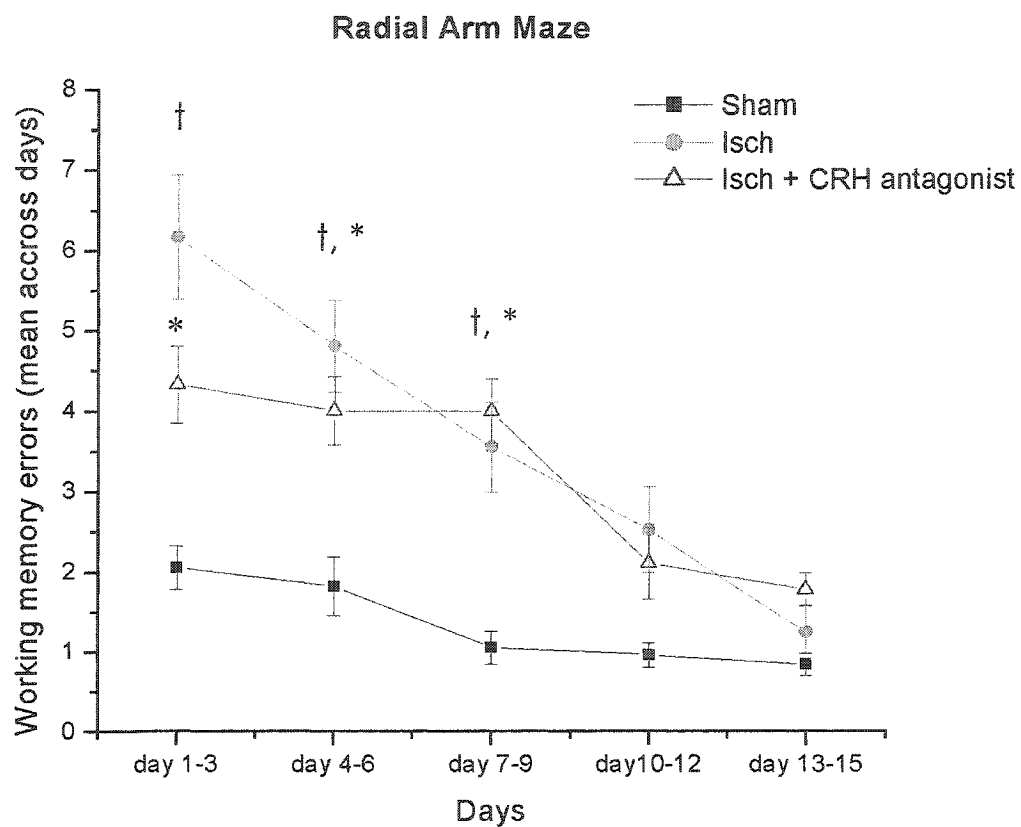


Figure 16. Effect of CRH1 antagonist pre-treatment on performance of ischemic animals in the 8-arm radial maze. Values are expressed as mean $\pm$ SEM. Drug-treated animals received 64 mg/kg of CP154,526 prior to global ischemia.

†, * Significantly more working memory errors than vehicle-treated sham (Sham), for vehicle- (Isch) and drug-treated (Isch+CRH antagonist) ischemic rats respectively, $p < .05$.

Discussion

Different studies have suggested the participation of CRH in the hippocampal CA1 neurodegeneration following focal and global ischemia [311,516,571]. A common feature of these studies is their use of non-selective CRH antagonists, leaving unresolved the elucidation of contribution of the different receptor subtypes to the observed protection. CRH1 is the most prominent CRH receptor subtype at the hippocampus, which appears to mediate activation of excitatory neurotransmitters in this region, including serotonin and norepinephrine [221,404]. CRH2 receptors have been shown to demonstrate significantly higher affinity for other endogenous ligands, such as urocortin than CRH [126]. In addition to the participation of distinct CRH receptor subtypes, the degree to which the neuroprotection observed by prior studies translated into recovery of functional impairments remained unexamined. Thus, the main objectives of the present study were to assess the neuroprotective effects of selective and non-selective CRH antagonists, and determine the ability of CRH1 antagonism to reverse ischemia-induced behavioural deficits.

In contrast to previous observations, our findings failed to support neuroprotective actions of CRH receptor blockade against damage induced in hippocampal CA1 pyramidal neurons following global ischemia. The CRH1 antagonist CP-154,526 appeared ineffective at both doses tested and regardless of whether the drug was administered prior to or following the ischemic insult. This particular CRH1 antagonist has been shown to rapidly cross the blood brain barrier, bind to CRH1 receptors with high affinity, leading to inhibition of the release of excitatory neurotransmitters at the hippocampus [221,478]. The present results thus suggest a limited contribution of CRH1

inhibition to the neuroprotection induced by CRH receptor blockade. Interestingly however, the non-specific antagonist α -helical CRH (100 μ g) in the present study also proved ineffective in preventing ischemia-induced cell death.

The absence of neuroprotective effects of α -helical CRH contrasts with the neuroprotection observed in previous studies [311,516] despite an identical duration of the global ischemic insult and same dosage of the CRH antagonist. Although these findings appear difficult to reconcile, the relationship between CRH and ischemia-induced neurotoxicity remains controversial. CRH expression in hippocampal CA1 neurons appeared significantly delayed in response to ischemia and was demonstrated only 96 h following global ischemia in rats [85], raising doubts about direct hippocampal CRH activation following ischemic insults. Data from our laboratory also failed to show significant alterations in *in vivo* CRH release in the CA1 region of the hippocampus within the initial 4 h following global ischemia, while post-mortem CRH concentrations in these neurons remained unchanged 4, 24 and 72 h post-ischemia [248]. Moreover, Craighead et al. [98] showed that high CRH doses administered directly into the brain did not induce neurotoxic effects nor did it exacerbate glutamate-induced cell death. However, these observations do not exclude possible indirect actions that CRH blockade might have to alter ischemia-induced hippocampal damage.

Interestingly, evidence is beginning to accumulate suggesting that CRH may exert neuroprotective actions. For instance, Pedersen et al. [401] found that CRH stabilizes the effect of amyloid beta on hippocampal neurons, considered a key factor in neurodegeneration produced in Alzheimer's disease, by preventing the increase in intracellular calcium. Lezoualc'h et al [288] also demonstrated that hippocampal neurons

over-expressing the CRH1 receptor received enhanced neuroprotection via CRH in an *in vitro* model of oxidative stress. Models of hypoxia-induced cell death [152] have also shown neuroprotective effects for CRH, and Hendryk et al. [196] recently demonstrated that CRH protects against increases in blood brain barrier permeability produced by cerebral ischemia. Perhaps, even more striking are findings by Elliot-Hunt et al. [140] showing that central administration of a low, physiological 2 pmol dose of CRH protected against glutamate induced neurotoxicity in hippocampal cells *in vitro*.

With regards to the relationship between glutamate and CRH, Givalois et al [162] reported that the NMDA antagonist MK-801 potentiated short-term increases in CRH mRNA expression produced by restraint stress, at the hippocampus CA1, CA3 and dentate gyrus areas. The authors suggested that at the hippocampus, glutamate (at least through NMDA activation) may actually inhibit CRH activity. This is particularly intriguing, since MK-801 is among the more reliable neuroprotective agents in rodent models of ischemia [124]. Thus, observations from the current study appear consistent with suggestions that CRH activation following ischemia may not necessarily exert neurotoxic actions.

Concomitant with the assessment of the effects of CRH blockade on ischemia-induced neuronal damage, the current study was interested in the participation of CRH in ischemia-induced behavioural impairments. Thus, behavioral recovery was assessed in the radial arm maze, elevated plus maze and open field, measuring spatial memory, anxiety and hyperactivity, respectively. Consistent with failure of the CRH1 antagonist to alter ischemia-induced CA1 neuronal degeneration, these animals showed comparable spatial memory impairments in the radial maze to vehicle-treated ischemic rats, making

significantly more working memory errors than sham-operated rats. The relationship between spatial memory impairments and CA1 hippocampal damage has been well established (see Hodges et al., [202] for review), and a strong negative correlation exists between the number of errors in the radial arm maze and the number of pyramidal cells in the CA1 section of the hippocampus [258].

Interestingly, CRH1 antagonism reversed ischemia-induced hyperactivity in the novel open field and the anxiolytic response of ischemic animals in the EPM. Thus, CP-154,526-treated ischemic rats showed reduced activity in the open field and spent significantly less time in the open-arm of the EPM as compared to vehicle-treated ischemic rats. This effect did not appear to result from a non-specific inhibition of locomotor activity, since in the radial-arm maze the activity level of CRH1 antagonist-treated ischemic rats was comparable to that of sham-operated controls and vehicle-treated ischemic rats. The disinhibition to explore unfamiliar or anxiogenic environments among ischemic rats might thus be influenced by CRH-mediated systems activated at the time of ischemia, but appeared independent of subsequent neuronal degeneration.

Ischemia-induced hyperactivity is one of the most documented behavioural impairments associated with global ischemic insults [79,160,270,424]. This hyperactivity has generally been attributed to a deficit in memory and the level of hyperactivity in ischemic animals has been shown in some studies to positively correlate with cell loss in the hippocampal CA1 pyramidal region [8,160]. Nonetheless, other studies have found that CA1 neuroprotection conferred by ischemic preconditioning or calcium channel blockers failed to reverse ischemia-induced hyperactivity [95,127,424]. Piera et al. [418] also reported no correlation between the amount of neuroprotection conferred by

serotonin antagonists at the hippocampus and the level of post-ischemic hyperactivity. Moreover, environmental manipulations, such as post-ischemic enriched housing environments [434], and certain pharmacological manipulations [424] inhibit ischemia-induced hyperactivity, while having little or no effect on CA1 hippocampal cell death. Another factor that has limited the association of hyperactivity and hippocampal cell death is its rapid occurrence within 24 h of the ischemic insult [90,234], prior to onset of neuronal degeneration.

Alternative hypotheses have thus been proposed to explain ischemia-induced hyperactivity. In this context, increases in neuronal excitability following ischemia have been proposed to account for enhanced locomotor activity [160]. Suzuki et al. [524] reported increased hippocampal neural activity 1 day following two vessel occlusion in gerbils while Chang et al. [80] observed the same phenomenon 2-3 days following focal ischemia in rats. Of interest, CRH has been shown to reliably increase forebrain and hippocampal EEG activity [492], and Lyons et al. [311] were able to reverse ischemia-induced increases in forebrain EEG activity with CRH antagonists. Furthermore, the ability of CRH to increase spontaneous electrical activity in the hippocampus has long been established [5] and CRH antagonists reduce the excitability of CRH interneurons in the hippocampus [439] as well as significantly attenuate norepinephrine-induced excitability in this region [177,221].

CRH activity may also have other neurophysiological effects at the hippocampus that contribute to locomotor activity. Pieretti et al [419] demonstrated that CRH injected into the cerebral ventricles of rabbits enhanced hippocampal theta voltage and frequency, a phenomenon related to initiation of locomotor activity and enhanced hippocampal EEG

activity [496]. In contrast, urocortin, which has potent effects at the CRH2 receptor, does not influence hippocampal EEG upon central administration [500].

Given these findings, one can postulate that administration of CRH1 antagonists prior to or just following the onset of ischemia may significantly reduce hippocampal excitability, which subsequently inhibits hyperactivity. This remains, at present, speculative as no definitive link has been demonstrated between ischemia-induced neuronal and locomotor activity and the physiological mediators of this excitability remain largely uncharacterized.

Interestingly, recent data from our lab [422] demonstrates that ischemia-induced hyperactivity is characterized by a disinhibition to explore anxiogenic environments. Thus, in the elevated plus maze, a well-validated model of exploratory anxiety, ischemic animals displayed significantly more open arm activity than sham-operated controls, suggesting a decreased level of anxiety in these animals. This anxiolytic effect appeared limited to exploratory models, as no such effect was demonstrated in a conflict-based anxiety model (the Vogel test) involving conditioned suppression of drinking. These findings suggested that neural processes mediating unlearned behavioural responses to naturalistic stressors (such as open space or an elevated plus maze) are affected by the ischemic insult, one of the consequences involving a disinhibition of exploratory behaviours in anxiogenic environments. Although the physiological mechanisms mediating these effects are unclear, the current study suggests a possible participation of CRH. Indeed, pretreatment of ischemic animals with CP-154,526 resulted in decreased exploratory activity in the open field accompanied by lower open arm activity in the EPM. Given a well-established role for CRH antagonists in conferring anxiolytic effects

in various behavioural tasks, these anxiogenic responses may appear paradoxical. However, the extensive time window between drug treatment and behavioral testing (ranging from 4 to 8 days) suggest that these behavioral effects are attributable to secondary effects of CRH1 receptor blockade. Interestingly, over the years many studies have shown mixed effects of CRH antagonists on anxiety in exploratory models, such as the elevated plus maze [22,43,170,193,194,380,510]. Considering the absence of effects of CP-154,526 administration on locomotion and anxiety in sham animals in the present study, distinct endogenous mechanisms are likely activated by global ischemia that subsequently lead to behavioural alterations several days later. In this context, early CRH activation has been demonstrated following ischemia in brain structures linked to mediation of emotional responses such as the amygdala [248,571]. Whether or not blockade of the effects of this initial amygdalar CRH rise through antagonist administration is involved in long-term behavioral alterations remains unknown. Inhibition of ischemia-induced hippocampal release of excitatory neurotransmitters by CP-154,526 treatment could also possibly play a role in these behavioral effects.

Together, findings from the present study failed to demonstrate neuronal protection and/or behavioural recovery of ischemia-induced functional impairments by selective or non-selective CRH antagonists in a model of global ischemia. This is consistent with growing evidence suggesting that CRH activation following ischemia may not necessarily exert neurotoxic actions. Although CRH1 antagonists influenced ischemia-induced behavioural activity in the novel open field and elevated plus maze, these alterations occurred in the absence of neuroprotection, suggesting that these deficits are

not closely related to hippocampal cell loss, contrary to ischemia-induced spatial memory deficits in the radial maze, which remained unaffected by CRH blockade.

General Discussion

The objective of the current thesis was to investigate the mediating role of CRH in the behavioural expression and physiological correlates of anxiety-related states and global ischemia. To reach these objectives the current thesis 1) characterized behavioural attributes and/or deficits associated with anxious and ischemic conditions, 2) documented endogenous fluctuations in CRH expression in distinct regions of the brain associated with anxiety or with a prolonged global ischemic insult, 3) attempted to elucidate physiological interactions between CRH and other molecular signals relevant to anxiety and ischemia and, finally 4) using CRH antagonists, assessed the impact of modulating CRH neurotransmission on behavioural expression, physiological events or neurodegeneration associated with anxious or ischemic states.

Since its characterization in 1981, CRH (primarily of hypothalamic origin) has gained the reputation of being the quintessential neuropeptide involved in the mediation and/or modulation of the stress response. As suggested on several occasions in this thesis, this reputation stems from its important mediating role in stress-induced HPA activation, and its ability to produce behavioural, autonomic and endocrine responses analogous to natural stressors. The amygdaloid complex, particularly the central nucleus (CeA) is also considered fundamental in the mediation or coordination of the stress response. Functional alteration of this structure influences the emotional and HPA response to psychological or processive-type stressors [9,20,130,198,232,522] while modulation of CRH peptide within the CeA influences the expression of anxiety-like behaviours [130]. Various stressors have also been shown to enhance amygdala CRH release [93,338,449].

These findings have strongly emphasized a role for CRH within the amygdala in the promotion and/or expression of anxiety-related states [113,277,501].

Nonetheless, a number of findings have cast doubt regarding the scope of CRH's role in mediating certain stressors. Thus, inhibition of CRH binding protein (CRH-BP) in animals, which theoretically increases the bioavailability of CRH, is associated with increased locomotion, but fails to alter anxiety-related behavioural responses [193].

Moreover, CRH antagonists do not appear as efficacious as better established anxiolytic drugs such as benzodiazepines in altering anxious behaviours and/or states [37].

Although CRH antagonists are effective in modulating the behavioural manifestations of stimulus-specific fear [200,224,528], they have been largely ineffective in other anxiety paradigms such as the elevated plus maze or light/dark exploration task [170,380]. It is also interesting to note that the ability of CRH antagonists to produce anxiolytic effects in a number of behavioural tasks also appears dependent on the level of arousal prior to the test. Thus, CRH antagonists attenuate anxiety in instances when the behavioural tests are preceded by exposure to another stress [22,43,194] or by exogenous CRH infusion [510].

A second issue in investigations of the role of CRH in anxiety has concerned the degree to which activation of CRH in the amygdala is anxiety-specific. Thus, although CRH in this region responds to anxiogenic stimuli, it is also activated by stimuli that are merely arousing but not sufficient to stimulate an HPA axis response [317]. In fact, stimuli that have appetitive or rewarding properties also demonstrate the ability to increase amygdala CRH release [338]. Many of the experimental approaches assessing amygdala CRH changes thus far have relied on indirect post-mortem or static measurements of CRH synthesis and/or utilization, such as *in situ* hybridization,

immunohistochemistry and/or tissue content assessment obtained from brain micropunch [265,464,535]. Thus, one contribution of the current thesis aimed to apply a direct *in vivo* assessment of amygdala CRH release in the coordination of the stress response, using a more dynamic measure of peptide release before, during and after exposure to anxiogenic stimuli.

Another important issue raised over the years has concerned the development of ethologically valid tests for assessing putative anxiolytic compounds, as many animal models of anxiety have relied on the assessment of stimulus-avoidance or punished behaviours. This appears to be the case, for instance, in behavioural tests such as open-field exploration, the light/dark transition test, the elevated plus maze, and in tasks involving punished feeding or drinking. However, conditions of general anxiety disorder, panic disorder, stimulus-specific or social phobias in human populations are generally characterized by their ability to interfere with behaviours that individuals would normally engage in. Animal models that have aimed to reproduce these conditions are few and have not readily lent themselves to dynamic neurochemical assessment, using techniques such as *in vivo* microdialysis.

In the current thesis, we standardized a neophobia-based anxiety paradigm that closely fits the definition of a processive stressor and that allowed us to directly assess the release of CRH during behavioral responses to a naturalistic stressor. In this particular study, we exposed animals to a novel environment while monitoring the release of CRH at the CeA. The transfer of the animals from the home cage (the familiar environment) to a novel cage (the unfamiliar environment) was associated with a robust elevation in the release of CRH at the CeA, supporting the view that processive stressors activate CRH at

this area. In addition, relative to the home cage “stress-free” condition, placing animals in a novel environment was accompanied by a significant decrease in the consumption of a palatable snack. These behavioral effects were completely reversed by diazepam, a prototypical anxiolytic. Of particular interest however was the observation that the anxiolytic effects of diazepam pretreatment occurred in spite of a concurrent rise in CRH release at the CeA. This supports the contention that CRH at the CeA is not involved in the genesis of the behavioral responses to certain anxiogenic stimuli. Furthermore, central and systemic pretreatment with various CRH receptor antagonists failed to alter the behavioral effects of the novel cage, suggesting that CRH activity within and outside the amygdala does not appear to be involved in the behavioral changes associated with stressor exposure in these conditions.

Together, these findings have contributed to broaden the knowledge on the endogenous processes mediating the role of CRH in anxiety in specific ways: 1) Through the characterization of novelty-induced suppression of palatable snack intake as an ethologically valid model of anxiety which allows for the monitoring of CRH release, as well as other molecules in freely behaving rats, before, during and after exposure to anxiogenic challenges, 2) through demonstration that the release of CRH at the CeA, while activated by naturalistic stressors, does not fluctuate with changes in the anxiety state, and 3) via demonstration that inhibition of amygdala or extra-amygdala CRH release is not associated with anxiolytic responses as assessed by the behavioural response to certain processive stressors.

It should be added that exposure of animals to a novel environments has reliably produced autonomic and neuroendocrine changes similar to that produced by natural

stressors, as well as increases in anxiety-like behaviours such as defecation, defensive withdrawal and grooming. Thus, given the results of the current thesis, a change in the animals' anxiety state produced by the novel cage appears to be the most likely explanation for the subsequent suppression of palatable food intake. However, at this point, the contribution of processes other than anxiety cannot be definitively ruled out. Thus, changes in an animal's state of curiosity produced by a novel environment, could conceivably influence exploratory behaviours to the point of disrupting feeding behaviour.

This possibility notwithstanding, the findings from the current thesis contrast with widely held assertions that CRH is a primary regulator of the behavioral response to stress and anxiety. However, the ambiguity regarding the specific role of CRH in anxiety has recently been supported by other research findings. Using transgenic mice, Weninger et al. [562] demonstrated that although stressor-induced behavioral changes required CRH receptors, they could be produced in mice completely devoid of CRH. Similarly, Muglia and colleagues [360] noted that mice without a CRH-producing gene displayed a behavioral response to stressors that was similar to that observed in wild-type cohorts. Interestingly, anxious behavioural manifestations in these CRH-deficient mice were obtained despite blunted neuroendocrine responses to stress. Concordant with this observation, Yagi & Onaka [578] reported that while novelty was capable of inducing HPA activation, as indicated by increased ACTH and corticosterone release, this activation was unaffected by pretreatment with the benzodiazepine chlordiazepoxide. Thus, in line with Muglia et al.'s [360] findings, a dissociation appears to exist between activation of the HPA axis and the behavioural response to anxiogenic stimuli. Given its

central role in HPA activation, a similar dissociation may exist between behaviour and CRH activation under certain anxiogenic conditions.

That CRH activation occurs following anxiogenic stimuli is well established. However, if it is not mediating the behavioural responses to anxiety, then its actual function needs to be reconsidered. One possibility is that CRH is involved in learning or memory processes perhaps involved in adaptation or accommodation to threatening situations. For instance, it has been observed that acute stressors have the capability of enhancing memory consolidation and recall [489] possibly as part of an adaptive response. Interestingly, centrally injected CRH and/or CRH administration into the dentate gyrus of the hippocampus has been shown to enhance retention and other memory processes [101,281,282,437]. Considering the presence of important bi-directional pathways between the hippocampus and amygdala, the sustained rise in amygdala CRH release might be related to memory processes heightened or enhanced following exposure to an unfamiliar and potentially threatening environment. Future studies might examine this possibility by comparing the release of peptides in subjects with and without a history of exposure to the stressful stimulus. If processing and storing cues associated with the environmental stimuli involves a sustained release of CRH, then in animals with prior stress exposure, this release might return to baseline more quickly. Another potential study could involve assessment of the rate of habituation to a novel environment in rats chronically infused with CRH antagonists at the amygdala. If memory consolidation processes are mediated by amygdala CRH activation, then inhibition of these processes might interfere with the normal rate of habituation or familiarization to a novel environment.

It has also been suggested that, like stress exposure itself, central CRH activation might sensitize certain brain mechanisms to subsequent stressors [105,494]. Central administration of CRH prior to stress exposure has been shown to increase the electrophysiological output of locus coeruleus neurons during stress exposure [104,494], in essence preparing the brain for a more rapid response to external stimuli. If endogenous CRH acts in a similar manner, then the sustained release of CRH at the CeA observed in the current thesis might serve to sensitize the brain to future stressors, as the amygdala shares anatomical links with the locus coeruleus. One of the benefits of such a system could be to allow for a more efficient biological and/or neuroendocrine response upon subsequent exposures to a particular stressor.

Another possibility is that CRH increases as a means to prepare the organism to adequately respond to novel encounters or situations. One effect of stress-induced CRH activation is an increased release of glucocorticoid, such as cortisol or corticosterone, from the adrenals. An unexpected move to an unfamiliar environment may require an adjustment involving a change in homeostasis. Thus, it is possible that increasing energy stores through the release of glucocorticoids or other substances represents part of an instinctive preparatory response to this adjustment. Such a release would not necessarily occur only under stressful conditions, as homeostatic changes following onset of positive (rewarding) stimuli have also been shown to stimulate glucocorticoid secretion [412]. As well, other systems such as catecholaminergic processes at the prefrontal cortex or nucleus accumbens also respond to both stressful and rewarding stimuli such as food [532], reinforcing drugs [569] and sex [563].

An additional possibility is that amygdala CRH may respond to features of salience or emotional significance, regardless of whether they are positive or negative. Amygdala neurons are known to mediate processes by which sensory stimuli gain emotional and motivational significance [9,277]. Furthermore, electrophysiological studies have shown that the degree to which amygdala neurons respond to cues associated with food depend on the apparent affective significance of the stimuli [156,593,595]. A change from a familiar to unfamiliar cage represents a salient change in the rodent's environment, and the degree of emotional significance may be reflected by activation of amygdala CRH. Such a hypothesis may underlie observations by Merali et al. [338] demonstrating a similar increase in amygdala CRH release induced by aversive and pleasurable events, namely 20 min of restraint and the consumption of a palatable snack. Further research examining the response of amygdala CRH to other rewarding stimuli such as sexual contact or reinforcing drugs could test this possibility.

Finally, the possibility remains that CRH increases are attributable to a general arousal response. Transfer to a novel environment requires adjustment to different stimuli, necessitating a degree of alertness and vigilance as the rodent explores the new situation. It is well documented that CRH enhances vigilance [513] and impairs sleep in part as a consequence of this effect. As well, Ehlers et al. [138] showed that central injections of CRH produced increased EEG activity at the level of the frontal cortex in rats, an effect they state is consistent with a role for CRH in hyperarousal. Indeed, one of the consequences of sympathetic arousal produced by central CRH administration is a sharpened focus or attention. Furthermore, as indicated earlier, Makino et al. [317] found that manipulating rats' social environment produced an activation of the amygdala CRH

system without simultaneous activation of hypothalamic CRH system or HPA activation. The authors concluded that amygdala CRH may be more sensitive than hypothalamic CRH since it responds to the initial arousal produced by an aversive event. As the aversive stimuli gains in intensity or proximity, other systems become activated which initiate physiological coping responses such as HPA activation.

Although numerous possibilities exist regarding the functional role of CRH activation following novelty exposure, the present findings suggest that its precise role in mediating anxiety may need to be reconsidered. The effectiveness of CRH antagonists in producing anxiolytic effects clearly depends on the behavioural model being tested. It may thus be necessary to identify important defining features of the various stress models that may determine the functional importance of CRH. As indicated in chapter 2, CRH antagonists are particularly effective in attenuating stimulus-specific fear, for instance the defensive response to a predator [224] or in anxiety tests that are preceded either by exposure to another stress [22,43,194] or exogenous CRH infusion [510]. In contrast, in situations that represent a more ambiguous threat, such as an open field or a novel environment, CRH antagonists less reliably attenuate the behavioural response.

Another useful distinction might be the use of conditioned and unconditioned stressors. The presence of distinct neural circuits which are differentially activated by conditioned and unconditioned stressors has some support in studies investigating stress and monoamines [191,356]. The currently available evidence also suggests that CRH antagonists are particularly effective in conferring anxiolytic activity in models involving a learned fear response or the conditioned association of stimuli with stressful events. These include models of conditioned defeat [170,193,224] and conditioned fear

[200,252]. In contrast, unlearned or naturalistic stressors may activate pre-wired neural circuits that include the activation of CRH, although the behavioural response may involve the recruitment of additional substrates or neurocircuitry, which as yet remain undetermined. Thus, while CRH activation in these situations may potentially reflect a state of anxiety, it does not mediate the behavioural response. In this context, attempts to modulate behavioural responses, using CRH antagonists, have been less effective in models such as the elevated plus-maze (assessing unlearned aversion to heights), light/dark transition (aversion to bright lights) and neophobia (aversion to new environments) [29,170,380,597].

Thus, in elucidating the specific role of CRH in mediating anxiety, the possibility is introduced that different neural circuitry (CRH-dependent and CRH-independent) mediate behavioural manifestations of stress/anxiety depending on features of the stressor. This might include the use of learned vs unlearned stressors, or stimulus-specific vs ambiguous stimuli. Ultimately, it may be necessary to distinguish between different forms of stressor-related anxiety, each of which may recruit different neural substrates and require distinct treatment strategies. Characterization of the precise circuitry and the contribution of various neurotransmitters and/or neuropeptides would be an important focus for future research.

CRH and ischemia

In addition to its role in stress and anxiety, CRH has more recently been implicated in neurodegeneration in models of kainate-induced neurotoxicity [417], seizure activity [314,416], traumatic brain injury [457] and cerebral ischemia [311,516].

Evidence of a direct neurotoxic role for CRH has come from models of global and focal ischemia in which CRH antagonists were able to confer significant neuroprotection when administered before or shortly after the ischemic insult [311,516]. Models of ischemia attempt to reproduce physiological conditions analogous to conditions such as cardiac arrest or stroke in humans that produce a temporary reduction in cerebral blood flow.

As mentioned in the introduction of this thesis, ischemia produces a delayed cell death containing features of both necrosis and apoptosis. That CRH activation may contribute to ischemia-mediated cell death is suggested by a number of physiological interactions with components of both necrotic and apoptotic pathways. For instance, glutamate enhances CRH release both *in vivo* and *in vitro* [99,226]. Conversely, administration of CRH stimulates glutamate release at the locus cereleus [494] and NMDA receptors at the hippocampus [281]. Exogenously administered glutamate is potently activated within 15 min of a global ischemic insult and reliably induces neurotoxicity in this context. In addition to its impact on glutamate, CRH stimulates intracellular Ca^{2+} , enhances concentrations of pro-inflammatory cytokines, such as interleukin-1 and $\text{TNF}\alpha$, and promotes phospholipase activity, which contributes to the breakdown of cell membranes.

Among the first and most severely damaged areas of the brain in response to global ischemia are pyramidal CA1 neurons of the hippocampus. This vulnerable region contains interneurons that release CRH. The documented functions of CRH in this region include facilitation of excitatory activity through inhibition of slow afterhyperpolarizations (sAHP's). Thus, CRH has been shown in a number of instances

to significantly enhance the firing rate of hippocampal CA1 pyramidal neurons [5,203,492]. Combined with its interactions with other physiological mediators of the ischemic cascade, and the neuroprotective actions of CRH antagonists, some investigators have proposed that CRH represents an endogenous signal in the global ischemic cascade which contributes to cell death.

However, a number of aspects regarding its participation in global ischemia require clarification. Thus far, it has been unclear whether a global ischemic insult actually provokes an increase in CRH activity, particularly within vulnerable neurons. Moreover, such an increase has yet to be directly linked to neurotoxicity, as exogenously administered CRH fails to produce neurotoxic effects or exacerbate glutamate-induced neurotoxicity [98]. In addition, the CRH antagonists that thus far have demonstrated neuroprotective capacity have a number of limitations. These include non-selective blockade of CRH receptors and the demonstration of agonistic properties for these compounds at high doses. Furthermore, the ability of CRH antagonists to attenuate ischemia-induced behavioural impairments remains unexamined.

In addition to neuronal damage, global ischemic insults produce a number of functional deficits of which the most reliably reproduced are hyperactivity and spatial memory impairments. However, research into ischemia-induced behavioural change lags considerably behind characterization of cellular and molecular changes. For instance, although deficits in ischemia-induced hyperactivity have been widely reported and open field activity is commonly used to assess the efficacy of neuroprotective compounds, a characterization of the nature and extent of this behaviour has yet to be conducted. Future evaluation of putative neuroprotective compounds would benefit from a better

understanding of the nature of these behavioural impairments in part to further elucidate their relationship to hippocampal damage.

The current thesis attempted to address these issues by 1) providing a characterization and elaboration of ischemia-induced behavioural impairments, such as novelty-induced hyperactivity, as well as determine the behavioral profile of ischemic animals in novel anxiety measures, as well as tests of habituation, 2) characterizing region and time-dependent changes in CRH throughout the brain in response to a global ischemic insult, and 3) assessing the effect of specific and non-specific CRH antagonists in conferring neuroprotection and reversing ischemia-induced behavioural changes.

The findings from these investigations have contributed novel information in specific ways. First, these studies have demonstrated that ischemia-induced hyperactivity is characterized by a disinhibition to explore anxiogenic environments (such as an open field or an open elevated arm). This hyperactivity failed to induce significant deficits in habituation to novel environments using a paradigm distinct to the novel open field (i.e., novelty-induced feeding suppression paradigm). A second important contribution of this thesis has been to demonstrate region specific CRH activation and structure-dependent alterations of CRH following global ischemia. Our findings suggested that no significant immediate or long-term (up to 72 h post-ischemia) endogenous CRH fluctuations might be initiated in the most vulnerable brain region to ischemic damage, the hippocampal CA1 layer. Interestingly however, transient CRH activation in extra-hippocampal regions such as the piriform cortex, amygdala and hypothalamus were observed 4 and 24 h post-ischemia. CRH release at the central nucleus of the amygdala but not the hippocampal CA1 pyramidal layer was also rapidly enhanced following global ischemia.

Finally, our findings failed to support any neuroprotective effects of CRH blockade against ischemic damage. In our hands, specific and non-specific CRH antagonists failed to reverse ischemia-induced cell death or spatial memory impairments, although CRH1 antagonism proved able to attenuate ischemia-induced hyperactivity without concomitant neuroprotection.

Together, findings from the current thesis suggest the involvement of CRH peptide as an endogenous mediator of some physiological processes associated with a global ischemic insult. However, our results failed to support a neurotoxic role for CRH peptide in global ischemia. This conclusion is principally driven from the absence of neuroprotection conferred by CRH antagonists administered before or after ischemia. Previous demonstrations of the effectiveness of CRH antagonists in preventing hippocampal damage in global ischemia have been obtained using alpha-helical CRF, a peptide-based antagonist that has a relatively short duration of action believed to be 60 min [56]. This suggests that if its neuroprotective properties were attributable to mediation of CRH at the hippocampus, increase in CRH at this site should be observed shortly following ischemia. CRH mRNA expression is known to change quite rapidly in the hippocampus (15 min - 1h) in other interventions such as stress or direct electrical and/or pharmacological stimulation [162], suggesting that changes initiated by a global ischemic insult, if they did occur, would be fairly rapid.

Nonetheless, it remains possible that despite the absence of changes at the hippocampal CA1 layer at the tested intervals, CRH may exert indirect effects on these neurons via CRH ascending pathways from other brain structures such as the piriform cortex and amygdala where endogenous peptide changes have been observed. It should

also be noted that more retracted changes in CRH immunoreactivity at the CA1 have been observed [85], suggesting secondary activation of the peptide at that brain locus and a potential participation in late physiological processes.

It is therefore possible that the neuroprotection reported with CRH antagonists in prior studies was mediated via inhibition of other potentially neurotoxic signals, which are dependent on CRH release. For instance, norepinephrine and serotonin release at the CA1 of the hippocampus, which produces excitatory effects in these regions, is inhibited by CRH antagonism [221], and noradrenergic antagonists have been shown to confer limited neuroprotection in models of global ischemia [267]. However, in the current thesis, using both selective and non-selective CRH antagonists, we were unable to demonstrate neuroprotection when the antagonists were administered either before or shortly after global ischemia. Of interest, this finding remained unaffected even using a dose previously shown to inhibit norepinephrine and serotonin release at the CA1 [221]. In addition, blockade of CRH failed to reverse behavioural deficits associated with hippocampal CA1 cellular damage, such as spatial memory impairments as assessed using the 8-arm radial maze. However, CRH1 antagonism using CP-154,526 administered before or shortly after ischemia reversed ischemia-induced hyperactivity in the novel open field, as well as ischemia-induced anxiolytic effects in the elevated plus maze. These behavioural changes occurred despite the absence of hippocampal neuroprotection, suggesting that these particular behaviours are likely linked to components of the ischemic process independent of CA1 neuronal damage.

Furthermore, our findings revealed that pretreatment with a neuroprotective dose of the NMDA antagonist MK-801 failed to alter CRH concentrations in hippocampal or

parahippocampal brain regions. This suggests that the effectiveness of an established neuroprotectant in rodent models of global ischemia may not depend on modulation of CRH activity in these regions. However, our assessment was limited to 24 h post ischemic changes and it remains possible that the drug may exert a significant impact on CRH concentrations at these loci at other time intervals following ischemia. In contrast, ischemia-induced CRH fluctuations at the hypothalamus, and to a lesser extent at the amygdala, were affected by NMDA antagonism. These findings are consistent with *in vivo* and *in vitro* studies demonstrating glutamate-induced CRH activation in hypothalamic and amygdala neurons (e.g. [99,226]).

However, recent findings suggest that the relationship between glutamate and CRH with regards to hippocampal activity and/or neurotoxicity may be more complex than originally assumed. Thus, Tremblay et al. [537] found that MK-801 preconditioning was unable to influence NMDA-induced Ca^{2+} influx following ischemia. This is of particular interest as CRH excitatory effects, particularly at the hippocampus, are believed to be mediated by Ca^{2+} -dependent K^{+} conductance [4,492]. This further suggests that the neuroprotective effects of MK-801 may involve mechanisms unrelated to CRH activation. Interestingly, Givalois et al [162] found that MK-801 actually potentiated short-term increases in CRH mRNA expression at the CA1, CA3 and dentate gyrus following prolonged restraint stress. The authors argued that at the hippocampus, glutamate (at least through NMDA activation) may play an inhibitory role on CRH expression.

There is in fact evidence to suggest that rather than acting to enhance glutamate-mediated neurotoxicity, CRH may actually inhibit it. Thus, Elliott-Hunt et al. [140]

recently demonstrated neuroprotection to hippocampal CA1 neurons following CRH administration *in vitro* in a model of glutamate-induced neurotoxicity. This apparently was mediated through modulation of mitogen-activated protein kinases (MAPK) including stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK). In their study, CRH suppressed glutamate-induced JNK/SAPK activation. These enzymes are part of a plethora of intracellular signals that become activated in response to various physiological stressors and, in turn, mediate various pro and anti-apoptotic mechanisms. For instance, SAPK/JNK, which belongs to the MAPK family of enzymes, promotes neuronal apoptosis [370]. Their accumulation in vulnerable neurons has been implicated as a causal factor in the neurodegeneration induced by Alzheimer's and Parkinson's diseases [470]. In addition, CRH has been shown to enhance another MAPK enzyme (p42/p44), potentially via stimulation of intracellular cAMP. This enzyme has been strongly linked to neuroprotective processes, as it appears to serve as a counterbalance to various pro-apoptotic signals [199]. The ability of CRH to exert neuroprotective actions appears to partly depend on the phosphorylation of neuroprotective MAPK's, since inhibition of these enzymes completely block CRH-induced neuroprotection.

An interesting aspect regarding these studies is that in the above experiments, the maximum neuroprotective effects of CRH were observed when the peptide was administered between 8-12 h following the glutamate challenge. This remote time window of opportunity may suggest that endogenous action of CRH within this time interval may contribute to secondary mechanisms involved in the rescue of vulnerable neuronal populations. The existence of a similar therapeutic window has yet to be demonstrated in global ischemic models. Nonetheless, caspase-dependent mechanisms

involved in cell death have been shown to reach their peak of action between 12-24 h in other models of brain injury, potentially allowing for a prolonged therapeutic window of some molecules targeting these endogenous changes [183]. One potential implication of these recent findings is that CRH depletion during reperfusion (principally at more critical time intervals) could contribute to cellular damage.

The present thesis did not measure CRH alterations at time intervals (8-12 h) where administration of CRH was shown to exert maximal neuroprotective effects against glutamate-induced neurotoxicity. Our findings suggest early increases in CRH concentrations (within the initial 4 h post-ischemia) at the piriform cortex, hypothalamic nuclei and increased release at the amygdala. This was followed by decreased intracellular CRH concentrations in various brain regions 4 and/or 24 h post-ischemia. Decreased CRH concentration can indicate that enhanced release has taken place in the time interval preceding these changes. Indeed, this seems to be the case for the hypothalamic nuclei and the amygdala where increased release and/or post-mortem concentrations had been observed at earlier intervals. Although post-mortem findings by themselves are sometimes difficult to interpret, it is however interesting to speculate on how these CRH alterations may modulate neurodegeneration and/or protection. Thus, according to findings showing neuroprotective actions of CRH, increased levels 4 h following ischemia in the piriform cortex, which sends projections to the hippocampus, may promote neuroprotection. Conversely, decreased CRH concentrations found in various brain regions at the 24 h post-ischemic intervals could be interpreted as a limiting factor of the action of the peptide on enzymatic reactions shown maximal between 12-24 h following brain injury, potentially compromising its ability to further promote cellular

repair and/or survival. The observation that ischemia-induced CRH depletion was reversed by pretreatment with the neuroprotectant drug MK-801 in some regions (such as the hypothalamus) may suggest a contribution of CRH to discrete endogenous mechanisms promoting optimal post-ischemic recovery (perhaps associated in the present case with improved regulation of homeostatic processes).

Future research may wish to document endogenous changes taking place in the described window of opportunity between 4 and 24 h following global ischemia, to help further elucidate fluctuations of CRH prior to the 24 h time interval. One also needs to specifically address the impact of CRH administration following global ischemia. In light of time-dependent neuroprotective actions of CRH *in vitro*, it may also be of interest to investigate the effectiveness of CRH agonists and/or antagonists administered at later time intervals following reperfusion, to determine if there is an optimal window whereby CRH modulation would be most effective in reversing cell death.

With regards to the role of CRH in excitotoxic processes, there is indeed accumulating evidence that endogenous CRH may serve neuroprotective functions. For instance, Pedersen et al. [401] found that amyloid-beta peptide, which represents a causal factor in neurodegeneration in Alzheimer's disease, could be reduced by CRH administration. Amyloid-beta exerts part of its neurotoxic effects via mechanisms, which not unlike ischemia, involve disruptions in Ca^{2+} homeostasis [175,328]. Pedersen et al. [401] determined that CRH was able to stabilize the effect of amyloid-beta on hippocampal neurons by preventing intracellular calcium increase. Although the mechanism for this stabilization is not certain, the effects on calcium channels may result from inhibition of the enzyme carbachol, which stimulates intracellular calcium at the

locus coeruleus, and was shown to be specifically inhibited by CRH [67]. These authors also showed that CRH could inhibit neurotoxicity produced by glutamate or oxidative stress (for instance, through administration of lipid peroxidation). Interestingly, in these studies, administration of a CRH antagonist actually exacerbated neurotoxicity, further suggesting that reduced CRH signaling could contribute to subsequent cell death.

Somewhat consistent with this, studies of post-mortem brain tissue of Alzheimer's patients showed decreased CRH concentrations and increased CRH-binding protein (CRH-BP) particularly in neurons undergoing degeneration [36]. In addition to suggesting that a decrease in the bioavailability of CRH in vulnerable regions may be a contributing factor in cell death, the authors eluded to a potential mechanism. Increases in CRH-BP may reduce the bioavailability of endogenous CRH by sequestering it within the synapse. Although this is an interesting possibility, thus far global ischemic insults have not proven to activate CRH-BP, at least at the CA1 of the hippocampus [85]. Hippocampal changes in CRH-BP have also remained unaffected by acute or chronic restraint stress [305]. Interestingly, however, increased CRH-BP mRNA expression was observed at the dentate gyrus and dentate hilus 30 min following amygdala-kindled seizures [504].

Other studies using *in vitro* models of oxidative stress also support neuroprotective actions of CRH. In these models, oxidative challenges were produced by administration of amyloid-beta, which promotes lipid peroxidation via activation of hydrogen peroxide (H_2O_2), or the administration of buthionine sulfoximine (BSO), which depletes the intracellular antioxidant glutathione, through inhibition of its synthesizing enzyme. Thus, Lezoualc'h et al [288] found that, in response to these conditions, hippocampal

neurons that overexpressed the CRH1 receptor received enhanced neuroprotection following CRH administration. Furthermore, CRH antagonists specifically blocked this protective effect. The protective activity of CRH was associated with increased release of a variant of amyloid-beta precursor protein (APP) called non-amyloidogenic APP (nAPP). nAPP is a neurotrophic factor shown to prevent neurotoxicity in cellular models of oxidative stress [327] or glutamate toxicity [476] and also provide resistance against p53-mediated apoptosis [576]. It has also been shown to increase synaptic density in mouse brain [359]. It should be added, however, that the association of nAPP increases with CRH neuroprotection does not necessarily mean the two are mechanistically linked, just that the two processes appear to co-occur.

In addition to these effects, recent evidence has shown that CRH can strongly suppress pro-apoptotic pathways. Radulovic et al. [438] found that *in vitro* activation of caspases through stimulation of a number of death receptor complexes, was suppressed by CRH, primarily via stimulation of CRH1. This suppression appeared mediated by inhibition of proteolytic enzyme processing of the caspase pre-cursor pro-caspase 3. CRH inhibition of protein kinase A (PKA) was also involved in retarding the pro-apoptotic cascade.

In addition to direct pharmacological and physiological effects, evidence also suggests that CRH may indirectly contribute to ischemia-induced effects by altering the permeability of the blood-brain barrier (BBB). Damage to the BBB leading to increased permeability has been reported shortly following cerebral ischemia [585]. It has been suggested that this damage is a consequence of ischemia-induced inflammatory responses, leucocyte accumulation, oxidative metabolism and increases in arachidonic

acid and lipid peroxidation [205,428,451]. Enhanced permeability may allow for entry of a number of neurotoxic or inflammatory molecules. CRH has been shown to prevent alteration of the BBB induced by number of injuries[369,381,557] including cerebral ischemia [196]. Although the physiological mechanisms underlying this effect are unclear, evidence suggests that CRH may exert a direct stimulatory effect on cerebral endothelium cells which promotes conservation of the BBB [196].

Thus, the literature regarding CRH and neurotoxicity provides a somewhat conflicting picture. On one hand, previous studies have shown that centrally administered, non-specific CRH antagonists confer neuroprotection in models of global and focal ischemia [311,516] and of seizure-induced neurodegeneration [314]. This suggests that CRH directly or indirectly exerts neurotoxic effects under certain conditions. In contrast, other studies suggest that CRH interacts with various neuroprotective signals to confer neuroprotection in a number of models.

In the assessment of the contribution of CRH to ischemic damage, one might consider the physiological conditions that may underlie the obtained findings. Thus, the two studies demonstrating neuroprotective effects for CRH antagonists in global and focal ischemic models were conducted *in vivo* whereas studies suggesting neuroprotective actions of CRH have largely been conducted on post-mortem tissue or *in vitro*. Thus, to a certain extent, different physiological conditions may account for the divergent findings. Nonetheless, CRH administration at physiologically relevant and supra-physiological doses, failed to produce neurotoxic effects in both *in vivo* and *in vitro* models. Thus, Craighead et al. [98] found that 24 h exposure to CRH (10 pM-100 nM) had no deleterious impact on cortical neurons, nor did it influence NMDA-induced neurotoxicity

in rats. Furthermore, direct *in vivo* infusion of CRH (0.3 - 5 μ g) into discrete regions of the rat brain, including amygdalar, hypothalamic, striatal and cortical nuclei, also failed to induce cell death or potentiate glutamate-induced neurotoxicity.

The *in vivo* studies presented in the current thesis also failed to demonstrate neuroprotective actions following CRH blockade (using specific and non-specific antagonists) in global ischemia, nor did they reverse behavioural deficits specifically associated with hippocampal damage. Thus, the data obtained using *in vivo* models do not necessarily support a neurotoxic role for CRH. It should be noted that a 10 min global ischemia resulted in massive neuronal degeneration at the hippocampal CA1 layer (75-95% neuronal loss as compared to sham-operated rats) in our study. Although reported to be of similar magnitude (approximately 80%) the neuronal degeneration in the CA1 layer depicted in Lyons et al.'s study [311] appeared significantly reduced as compared to that observed in our animals. It thus remains legitimate to also consider the possibility that the intensity of the ischemia itself might significantly modulate the capacity of a single molecule to confer neuroprotection.

Despite neuroprotective actions of CRH blockade in some models, accumulating evidence has failed to support the contention that high CRH concentrations are neurotoxic, or that global ischemia is associated with significant endogenous CRH increases in vulnerable regions. Thus data from the current thesis and from other studies (e.g. Choi et al. [85]) have failed to demonstrate a rapid enhancement of CRH release or CRH immunoreactivity in hippocampal CA1 neurons in response to global ischemia. In contrast, the current thesis did identify significant CRH activation in regions devoid of neurodegeneration. Global ischemia produced a rapid and pronounced increase in

amygdala CRH release, with extracellular levels reaching 200% of baseline within 3 h. Similarly, within 4 h, CRH concentrations increased by approximately 50-100 % at the piriform cortex, as well as the lateral, ventromedial and paraventricular nucleus of the hypothalamus. These structures are not associated with neuronal degeneration suggesting that high increases in CRH in response to a global ischemic insult are not necessarily associated with concomitant cell death.

Given the absence of evidence directly implicating the CRH molecule in neurotoxic processes, another consideration may be stimulation of CRH receptors by other endogenous ligands. Thus, the CRH antagonists which thus far have conferred neuroprotection block both CRH1 and CRH2 receptors, and numerous peptides besides CRH stimulate these receptors including urocortin, urotensin and sauvagine [108,590]. Interestingly, urocortin has a higher affinity than CRH at both receptors, but especially at the CRH2 receptor subtype [126]. As with CRH, urocortin has also been shown to produce excitatory effects at the dorsal hippocampus [500]. Although data regarding the role of these molecules in neurotoxicity is limited, a number of studies have suggested that urocortin may exert protective actions. For instance, urocortin has been shown to reliably protect cells of the heart in models of myocardial ischemia [163,275] in part through activation of MAPK pathways [477], which as indicated earlier, serve to counteract some pro-apoptotic processes. Pedersen et al. [401] also found that urocortin was actually 10 times more potent than CRH in producing neuroprotective effects in *in vitro* models of oxidative stress and glutamate toxicity involving cultured hippocampal neurons. Interestingly, this neuroprotection appeared to be mediated via the CRH1 receptor. CRH2 receptors are much less prominent at the hippocampus and their

functional role in this region has not been well defined. Further research is warranted to determine the specific role of CRH2 receptors in ischemia and neurodegeneration and the possible role of other CRH-like molecules, which thus far have not been implicated. Nonetheless, it appears unlikely from these observations that the neuroprotective effects of CRH antagonists can be principally attributable to blockade of the urocortin peptide signal. It should however be added that in the current thesis, CRH1 and non-specific CRH antagonists (which simultaneously block CRH1 and CRH2) failed to confer neuroprotection suggesting that ligands which bind to either one or both these receptors subtypes do not play significant role in mediating cell death following global ischemia. The recent development of selective CRH2 antagonists may allow for a closer investigation of the possible role of this receptor.

Thus far, the discussion has mainly focussed on the potential contribution of CRH to ischemia-induced neuronal damage. The wide range of endogenous CRH functions, including homeostatic or regulatory effects, however, renders possible a multitude of different roles for the peptide in the ischemic process. Interpretations of the findings should consider some of these functions.

In this context, the rapid CRH activation at the amygdala and piriform cortex following global ischemia observed in the current thesis may be related to CRH participation in excitatory effects such as those induced by seizure and/or epileptiform behaviour. The fact that CRH antagonists are not neuroprotective does not discount the strong excitatory effects CRH may have in response to ischemia. Thus, the piriform cortex makes numerous bilateral connections with limbic structures, particularly the hippocampus and amygdala, and is involved in the spread of excitatory waves, to and

from these areas, in response to limbic seizures [332,333]. Thus, significant CRH increases at the piriform cortex may result from excitatory effects that propagate from the hippocampus, enhancing CRH release at that site. Conversely, excitatory activity may initiate from the piriform cortex to affect hippocampal CA1 neurons. The generalization of seizures to the dorsal hippocampus is indeed believed to be specifically mediated by the piriform cortex [244]. This parahippocampal site also represents one of the first activated locus in kindling-induced seizures, involved in the generalization and amplification of electrical signals following kindling [435].

Although it is not a commonly studied phenomenon, ischemic insults are associated with epileptic seizure induction in a subset of experimental subjects. These subjects are commonly removed from the study to eliminate physiological or neurotoxic effects that seizures may produce, confounding specific effects of the ischemia. Nonetheless, these phenomena observed following severe ischemia suggest that excitatory activity potentially mediated by CRH may also exist in ischemia despite a sub-threshold for epileptiform activity. To this effect, Lyons et al. [311] demonstrated a reversal of ischemia-induced increases in EEG activity at the forebrain in rats pretreated with CRH antagonists. Other observations support the possibility that CRH may act to promote, amplify or sustain excitatory activity in limbic regions following ischemia. Thus, central administration of ovine CRH to rodents can produce tonic/clonic seizures or generalized motor convulsions within 3–7 h of administration [137]. The electroencephalographic and behavioural profiles of CRH-induced seizure activity appear indistinguishable from that produced by electrical kindling of the amygdala [137], a region where CRH activity appears to be increased in response to global ischemia. More recently, Baram et al. [26]

indeed concluded that CRH epileptiform activity appears initiated at the amygdala before recruitment of the dorsal hippocampus and cerebral cortex.

If CRH can stimulate epileptiform activity, the opposite has also been demonstrated. Thus, kainic acid elicited seizures have been shown to produce a specific activation of CRH at the piriform cortex [415]. Smith et al. [504] further showed that amygdala-kindled seizures produce an increase in CRH immunoreactivity and CRH mRNA expression at the dentate gyrus of the hippocampus. Such CRH activation is similar to that observed following global ischemia in our study, suggesting that global ischemia could elicit discrete increases in CRH as part of an excitatory processes that may or may not be ultimately tied to neurodegenerative processes.

In addition to a participation in the propagation of excitatory activity through limbic regions, CRH may be involved in other physiological events following global ischemia. Thus, CRH plays an acknowledged role in thermogenesis. Central CRH administration has been shown to stimulate sympathetic outflow, metabolic rate and brown adipose tissue thermogenesis in rodents [59,60,285]. Rothwell [462] further demonstrated significant thermogenic changes in response to infection, injury, or inflammation, all of which are present following an ischemic insult. In this regard, existing relationships between CRH and some cytokines appear important. Interleukin-1beta (IL-1 β) exerts potent endogenous pyrogenic activities that stimulate increases in body temperature [106]. Activation of this cytokine has been reported in various brain regions following focal ischemia [345]. Of interest, the thermogenic effects of IL-1 β were shown highly dependent on the release of CRH from the hypothalamus [460,469]. Indeed, IL-1 β - induced increases in body temperature, metabolic rate and white cell count activity are all

prevented by pre-treatment with CRH antagonists or CRH antibodies [460]. Such physiological mechanisms may in part explain the rapid activation of CRH in hypothalamic nuclei observed in the present thesis following a global ischemic insult.

Related to hypothalamic functions, CRH may contribute to anti-inflammatory processes initiated in response to an ischemic insult. Thus, experimentally induced allergic encephalomyelitis or arthritis have both been shown to significantly alter hypothalamic CRH expression [185,186]. Furthermore, centrally administered CRH exerts potent anti-inflammatory actions partly through inhibition of natural killer (NK) cell cytotoxicity [220]. This effect is rapidly observed, within 10 min of a central CRH injection, and persists for approximately 60 minutes [218]. It appears specific to CRH actions since it is blocked by centrally administered CRH antagonists [220].

Interestingly, reductions in NK activity is observed following stressful exposures such as foot shock, an effect also antagonized by central administration of CRH antiserum [219]. This suggests that under certain stressful conditions, discrete CRH neurons activate the sympathetic outflow to the spleen resulting in reductions in the activity of NK cells [217,219]. The role of hypothalamic CRH in autonomic activation is well acknowledged [59,61]. Thus, it remains possible that some of the short-term increases in hypothalamic CRH observed following global ischemia in the current thesis, may also serve an anti-inflammatory role. Finally, CRH activation of the HPA axis lead to significant increases in circulating glucocorticoid levels, which also have powerful anti-inflammatory effects. Thus, adrenalectomy in animal models, which significantly reduces circulating corticosterone levels, has been shown to exacerbate arthritis-induced inflammation [187], a phenomenon significantly attenuated by dexamethasone (a glucocorticoid receptor

agonist) and corticosterone [511]. Given the disruption of numerous physiological actions occurring following global ischemia, some of which attempting to attenuate cerebral edema formation and infection, it is possible that CRH activation of the HPA axis represents one defensive mechanism of the organism designed to minimize immune or inflammatory reactions.

The spectrum of CRH actions also involves an acknowledged participation of the peptide in locomotor behaviour. Locomotor hyperactivity represents one of the most commonly observed behavioral consequence of an ischemic insult in rodents [79,160,270,424]. As mentioned in the introduction of this thesis, CRH induces a dose-related change in locomotor activity, whereby at low or moderate doses it stimulates locomotion although inhibits it at high doses. Studies have also demonstrated that while exogenously administered CRH stimulates locomotor activity in familiar environments, it inhibits exploration of unfamiliar (and presumably stressful) environments [130]. However, the data in this regard is not entirely uniform. For instance Lee & Tsai [283] found that CRH administered into the dentate gyrus and, to a lesser extent the amygdala, produces an increase in locomotor activity in the center area of a novel open field (normally an anxiogenic environment). This effect appears partly attributable to increases in hippocampal norepinephrine release [280]. In the present thesis, ischemia-induced increases in CRH immunoreactivity were observed at both the amygdala and dentate gyrus. Interestingly, our findings also demonstrated that pretreatment of ischemic animals with the CRH1 antagonist CP-154,526, using a dose shown to reduce hippocampal norepinephrine release [221] significantly attenuated ischemia-induced hyperactivity in a novel open field. Future research may wish to examine the ability of

CRH antagonism at discrete brain loci (such as the amygdala and dentate gyrus) to inhibit hippocampal norepinephrine release following ischemia to determine if these interventions concomitantly attenuate increases in locomotor behaviour.

Conclusions

When addressing the different actions of CRH, it readily becomes apparent that this endogenous signal may play a number of roles in the ischemic process. Findings from the current thesis did not directly support a neurotoxic role for CRH, although there is evidence to suggest that CRH may mediate these neurotoxic actions indirectly as well as participate in various other physiological effects including propagation of electrical activity in the brain, promotion of thermogenesis and anti-inflammatory effects and/or stimulation of locomotor activity. With regards to its effect on locomotor activity, examination of the impact of CRH antagonists on ischemia-induced behavioural changes in the current thesis raises one important question. Indeed, the ability of CRH1 receptor antagonism to attenuate ischemia-induced hyperactivity without concomitant neuroprotection of hippocampal CA1 neurons suggests that changes in activity levels represent a non-specific response to the ischemic process, independent of hippocampal neurotoxicity. The absence of ischemia-induced CRH changes in CA1 neurons following ischemia further indicate that the antagonism does not act on this neuronal population to exert its impact on behavior. Rather, this behavioral effect may result from ischemia-induced excitation and/or damage in extra-hippocampal regions. This is interesting as ischemia-induced hyperactivity has been specifically attributed to increased firing rate of hippocampal neurons although no correlation has yet been found [160]. The involvement

of the striatum in this effect has also been contemplated due to its participation in regulation of motor functions, but thus far evidence has not supported this hypothesis [343].

Recent findings (including the ones presented in this thesis) also support legitimate reinterpretation of earlier findings associated with ischemia. In this context, deficits in object recognition tasks following global ischemia have recently been shown independent of disruptions of hippocampal functions, and rather have been attributed to extra-hippocampal brain loci, including the rhinal cortex [132,364,366]. It thus becomes crucial for future research to address changes outside the hippocampus in the assessment of the physiological and functional consequences of global ischemia. So far, damage to extra-hippocampal areas and determination of the contribution of these sites in the ischemic process has largely been omitted, in part due to the difficulty of assessing damage in these extra-hippocampal brain loci. Advances in histological techniques and microscopy should allow for more complete identification and assessment of damaged areas.

In summary, the current thesis has attempted to expand the understanding of the role of CRH in two main areas of investigation. As for numerous neurotransmitters and neuromodulators, the assessment of the endogenous contribution of this peptidergic signal is complex and its functions mediated by multiple fiber pathways and receptor subtypes. Its physiological and behavioural functions largely depend on the characteristics of the behavioural modalities under investigation and the specific brain regions studied. The excitatory effects and the role of CRH in neurodegenerative processes no doubt require further characterization. These actions may be specific to

discrete excitatory and/or neurotoxic processes under study, rendering difficult comparisons of different pharmacological or physiological models. Through its contribution to the study of the role of CRH in anxiety and ischemia, the present thesis highlights the complexity of the role of this peptide and challenges some of the assumptions as to its participation in mediating these states. The present work also tried to further validate testing paradigms possibly enabling better characterization of the behavioral consequence of anxiety and global ischemia. Together, we hope that these studies helped to provide enlightenment into the endogenous role of CRH as well as identify potentially fruitful avenues for future research.

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