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Characterization of the role of corticotrophin-releasing hormone and opioid peptides on hippocampal neurodegeneration and functional recovery following global cerebral ischemia in rats

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Characterization of the role of corticotropin-releasing hormone and opioid peptides on hippocampal neurodegeneration and functional recovery following global cerebral ischemia in rats.

A Doctoral Dissertation

by

Charlaine Charron

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ABSTRACT

The present thesis aimed to further characterize the role of CRH and opioid peptides and receptors in the modulation of neuronal damage induced by an ischemic insult. This thesis also evaluated the possibility that CRH effects may be partly opioid-mediated. Finally, behavioral assessment were performed in order to determine whether neuronal survival was accompanied by an improvement of behavioral deficits.

First, we determined whether administration of CRH prior to global cerebral ischemia in rats altered CA1 neuronal degeneration *in vivo* and open field activity. The impact of the selective blockade of CRH receptors, using the antagonist α-helical CRH (9-41), was also investigated. Having shown CRH-induced neuronal protection when administered prior to ischemia, a second objective was to determine whether administration of CRH at specific post-ischemic intervals could also induce protection, further elucidating the time window of its action. CRH was effective to reduce ischemia-induced neuronal damage and significantly improved spatial memory impairments in a radial maze when administered 8h following ischemia in rats. Then, we examined whether the selective blockade of kappa- and delta-opioid receptors (KOR and DOR), using nor-binaltrophimine and naltrindole, respectively, prior CRH administration had an impact on CRH-induced neuronal protection against global ischemic damage, showing interaction between these two peptidergic systems. Our findings revealed neuronal protection conferred by the opioid kappa and delta receptor agonists, and a reduction of CRH-induced neuronal protection when these opioidergic systems were blocked prior CRH administration. We also confirmed the neuroprotective actions of CRH *in vitro* and determined the impact of pre- and post-CRH administration on cortical neurons injury

following a potassium cyanide (KCN)-induced insult in rat primary cortical neurons. We also examined whether CRH neuroprotective effects on KCN-induced injury were altered by prior blockade of either CRH receptors, DOR or KOR. Finally, mRNA expression of enkephalin, dynorphin and DOR and KOR in CRH-treated primary cortical neurons was assessed using reverse transcription polymerase-chain reaction. Together, this series of studies suggest an important role of CRH as a modulator of neuronal survival, and propose this action to be induced via interaction of CRH with opioidergic systems.

GENERAL INTRODUCTION

1. Cerebral ischemia

1.1 Background

Stroke is the fourth leading cause of death in Canada and costs the canadian economy $2.7 billions a year (www.heartandstroke.ca). Each year, around 40,000 to 50,000 new cases of stroke will be detected in Canada and around 16,000 Canadians will die from this disease. A stroke is a sudden loss of brain function caused by the interruption of blood flow (i.e., ischemic stroke, ≈80%) or the rupture of blood vessels (i.e., hemorrhagic stroke, ≈20%) in the brain, leading to neuronal death. Depending on the specific brain structures affected, stroke can cause numerous functional impairments, including behavioral and memory deficits, which can significantly affect stroke patient's quality of life. Current clinical management of stroke is very limited. Tissue plasminogen activator (t-PA) may be given to ischemic stroke patients to help break up blood clots. However, to be effective in minimizing sequelae, t-PA must be administered within 3h of symptom onset. This therapeutic window of opportunity is often too narrow, as the reported median time between admission to the emergency department and examination by a physician is 9.7 hours [216]. Even when t-PA is administered in time, more that two thirds of patients still have an unfavorable neurological outcome [1]. Thus, identification of pharmacological compounds targeting downstream physiological processes that contribute to cellular changes and/or functional impairments following vascular accidents remains essential. Other palliative treatments include antiplatelets (eg aspirin, clopidogrel, dipyrdamole, and ticlopidine) and anticoagulants (eg heparin, warfarin) aimed at decreasing platelet aggregation and preventing blood clotting following an ischemic

stroke. In some cases, a carotid endarterectomy may be performed to remove plaque from inside the carotid artery. Medications may also be prescribed to treat risk factors for stroke, including hypertension and cholesterol. In the case of hemorrhagic strokes, surgery may be needed to repair the ruptured blood vessels.

Over the last decade, research has led to significant progress in the understanding of the cascade of physiological events leading to neuronal death following vascular accidents. To date, however, no treatment has demonstrated clinical efficacy in humans, despite repeated success in animal experimentation [351]. One of the reasons explaining this discrepancy is improper timing of treatment delivery. Thus, most clinical trials for novel stroke therapies have used treatment windows in the range of 6 to 12h, although prior animal studies suggest that the key treatment window to reverse the ischemia-induced neuronal alterations is in the range a few minutes to a few hours [519]. Important undesirable side effects in humans have also prevented the use of treatment. For example, hallucinations, psychosis and delirium were observed following glutamate antagonists administration [443]. Thus, current stroke research attempts to identify endogenous processes capable of inhibiting cellular damage and enhancing functional recovery. Hopefully, drugs that act on these endogenous processes and can be administered at clinically relevant time intervals following a stroke will be developed. In this quest, animal models of stroke remain an essential tool.

1.2 Global and focal models of cerebral ischemia

Global ischemic models mimic the effects of a cardiac arrest with its interruption of blood flow (CBF) over the entire brain, whereas focal ischemic models disrupt regional

CBF supplied by discrete arteries of the Willis circle. The middle cerebral artery is the most commonly blocked vessel in humans due to its direct connection with the carotid artery. Thromboembolic or atherothrombotic disease usually cause ischemic infarction [200]. In the early 1980s, three models of global cerebral ischemia in rodents were developed which remain widely used: (1) the four-vessel occlusion (4-VO) model in rats, in which the vertebral arteries are permanently occluded and the carotid arteries are clamped transiently [421], (2) the two-vessel occlusion plus hypotension (2-VO +hypo) model in rats, produced by transient occlusion of both common carotid arteries and simultaneous arterial hypotension (ca 50 mm HG) produced by withdrawal of blood [487], and (3) transient occlusion of both common carotid arteries (2-VO) in gerbils, sufficient to induce a global cerebral ischemia as these animals display an incomplete circle of Willis [251]. This last assumption is now being challenged and the validity of using such an ischemic model in gerbils questioned [266]. Other models also exist but they are less frequently used (for a review, see [220]).

The two most popular models of focal brain ischemia are the unilateral occlusion of the common carotid artery (CCA) in gerbils [273] and middle cerebral artery occlusion (MCAO) in rats [511]. Focal ischemia can either be permanent or transient, allowing reperfusion of cerebral blood flow. Focal cerebral ischemia results in a core of infarcted tissue, irreversibly damaged and for which no therapeutic intervention has been found. However, surrounding the core is the penumbral region, where cells can eventually die following ischemia but can still be rescued within a few hours of the ischemic event [418]. Thus, rescue of these viable neurons either by increasing blood flow and oxygen supply to the ischemic tissue or by altering the molecular events contributing to neuronal death

remain the main therapeutic goal. Although focal ischemia is most representative of cerebrovascular accidents observed in humans, there is a high variability of induced neuronal damage between animals upon occlusion of the middle cerebral arteries. Global ischemic models are more reproducible and widely used [484]. So far, studies have demonstrated similar cascade of physiological events involved in brain injury in these different models.

The four-vessel occlusion model represents the most commonly used to study the neuronal, physiological and behavioral consequences associated with a global ischemic insult in rats. Neurons vulnerable to global ischemic insult include the Purkinje cells of the cerebellum, as well as layers 3, 5 and 6 of the cerebral cortex [419]. The four-vessel occlusion model also produces selective degeneration of hippocampal CA1 pyramidal neurons in rats [251,419,421]. Small to medium-sized neurons of the striatum are damaged early (within 30 min) following ischemia. In contrast, a period of 48-72h following reperfusion will elapse before any evidence of neuronal injury is observed in CA1 hippocampal neurons, a phenomenon referred as «delayed neuronal death» [256,421]. Hippocampal CA1 pyramidal neurons can remain viable for up to 7 days before undergoing apoptotic cell death [302] : this delayed death opens a window opportunity for treatment. The next few paragraphs describe some of the known processes by which brain cells die as a result of ischemia. In order to guide the reader, a summary of the major processes leading to ischemic cell death is presented in Figure 1.

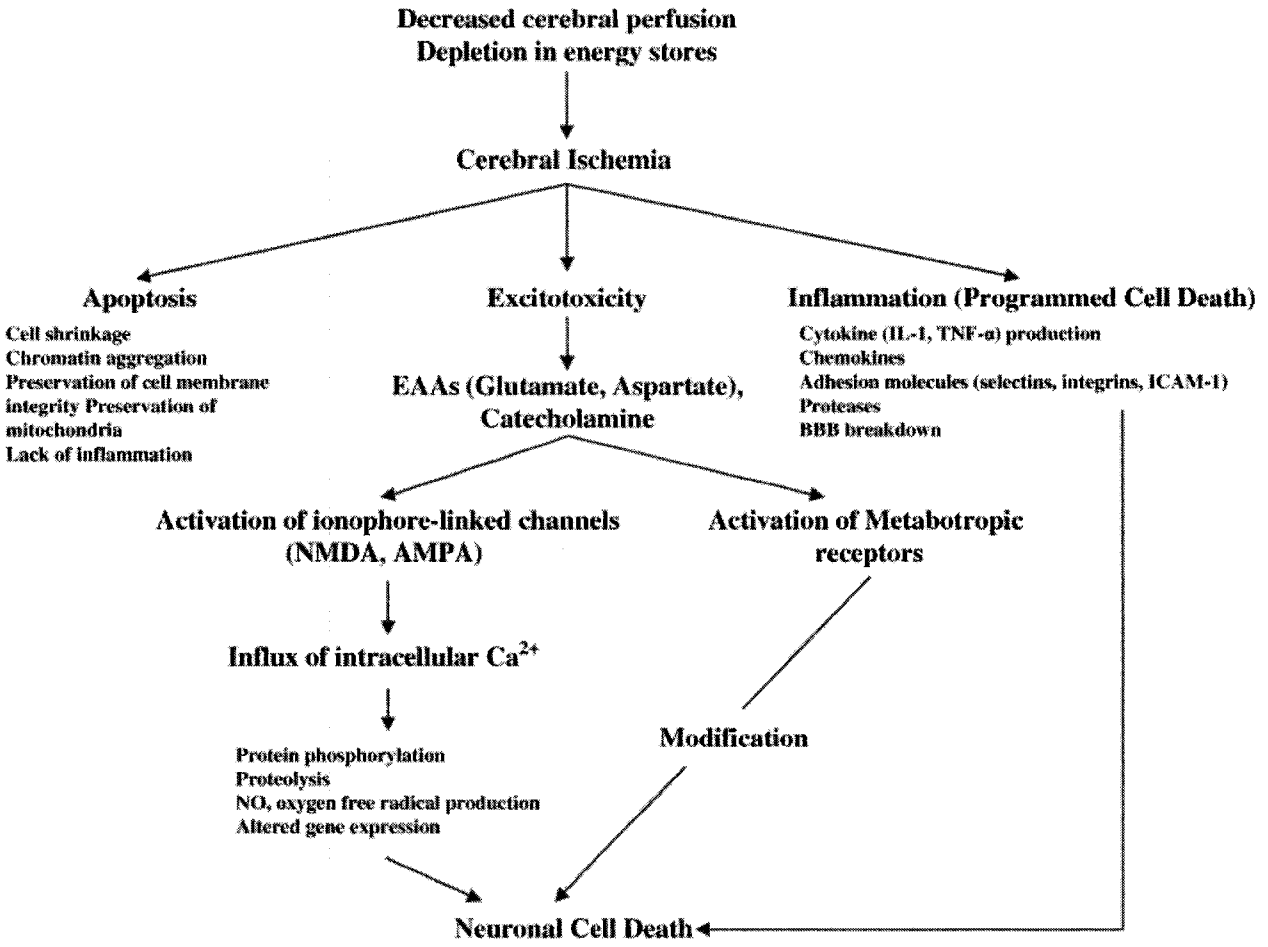


Figure 1 : Schematic diagram representing events leading to ischemic brain injury. [Harukuni, I. & Bhardwaj, A. (2006). Mechanisms of brain injury after global cerebral ischemia. Neurol Clin., 24(1), p.6.]

1.3 Neuronal ischemic injury

Cerebral ischemia results in two types of cell death, necrosis and apoptosis. They can be distinguished both morphologically and by their biochemical and molecular processes [361,489,525]. Necrosis is an uncontrolled/unregulated form of cell death characterized by disruption of cellular homeostasis following energy failure due to severe mitochondrial injury. Necrosis main features include initial cellular and organelle swelling, eventual loss of membrane integrity and cellular disintegration. Necrosis can damage surrounding cells by releasing cellular content into the extracellular space and triggering inflammation. In contrast, apoptosis is a controlled form of cell death characterized by DNA fragmentation, condensation and margination of the chromatin. It is accompanied by cellular shrinking, preservation of cell membrane integrity and mitochondria, lack of inflammation, cell budding (i.e., ruffling of the plasma membrane) and eventual breaking up of the cell into apoptotic bodies. Apoptosis is often referred synonymously with «programmed cell death», the latter being a type of cell death in which the cell uses specialized cellular mechanisms to kill itself, a phenomenon often seen during development. However, apoptosis can occur either as a result of programmed cell death or independently, without planned cellular programmation [525].

Different modulators of ischemia-induced cell death have been identified. The following paragraphs summarize the role of some of the main ones.

1.4 Tissue acidosis

Among critical physiological changes following an ischemic insult is the drastic reduction in cellular energetic fuel via massive breakdown of adenosine triphosphate

(ATP). This leads to compensatory activation of anaerobic glycolysis, which in turn increases levels of inorganic phosphate, lactate, and hydrogen (H^+) formation causing cellular acidification [483]. Increased acidosis is thought to lead to ischemic damage by one (or a combination) of the following three mechanisms: (1) acceleration of free radical production via H^+-dependent reactions, some of which are catalyzed by iron released from protein binding due to a lowering of the cellular pH, (2) perturbation of intracellular signal transduction pathways, leading to changes in gene expression or protein synthesis, and/or (3) activation of endonucleases which causes DNA fragmentation [476].

The worsening of ischemic damage in hyperglycemic animals and humans [538] has been associated with increased lactic acidosis. This phenomenon is at the origin of the «glucose paradox», because even though glucose represents the main modulator of ATP supply to oxygen-deprived tissue, hyperglycemia leads to a worse outcome. Recently, the «lactate-acidosis-hypothesis» has been challenged, and alternative hypotheses are now being proposed to explain the «glucose paradox» of cerebral ischemia. Among others explanations, it has been suggested that the hyperglycemia-induced negative impact on experimental stroke models results from the massive release of glucocorticoids following glucose administration [461].

1.5 Glutamate and the excitotoxic theory of ischemic brain injury

Disruption of cellular homeostasis is associated with excessive stimulation of post-synaptic excitatory amino acid (EAA) receptors, which is mainly attributable to the release of the excitatory neurotransmitters, glutamate and aspartate. Excitotoxicity represents a key phenomenon in the cascade of events mediating ischemia-induced

neuronal injury. Ischemic depolarization occurs when CBF decreases to levels of approximately 18 mL/100 g of brain tissue per minute. Neuronal cell death ensues if CBF reaches levels of 10 mL/100 g of brain tissue per minute [200], well below normal ranges (50 to 75 mL/100g of brain tissue per minute). The ischemia-induced inhibition of blood flow and oxygen supply leads to a decline of the high-energy metabolites ATP and phosphocreatine, resulting in a failure to maintain ionic gradients and a normal hyperpolarized membrane potential.

Two major stages have been identified following cerebral ischemia. They are characterized by differential alterations of ionic concentrations across the cell membrane [475]. During the first phase, lasting 1.5 to 2 min, a slow rise in extracellular potassium (K^+) is observed while extracellular calcium (Ca^{2+}), sodium (Na^+) and chloride (Cl^-) concentrations remain largely unaltered. This is followed by a second phase characterized by an abrupt increase in extracellular K^+ and accompanied with a precipitous decrease in extracellular Ca^{2+}, Na^+ and Cl^- ions. The critical loss of cellular Na^+ gradients in this phase, normally maintained by the ATP-dependent Na^+-K^+ pump, triggers cell depolarization. The resulting Ca^{2+} influx further depolarizes the membrane, stimulating excessive release of glutamate in the extracellular space. Subsequent activation of various endogenous signals shown to compromise cellular homeostasis ensues and these signals play a role in delayed neuronal damage.

1.6 Calcium

The disruption of Ca^{2+} homeostasis leading to massive Ca^{2+} entry into the cell plays a critical role in ischemic injury. Within 8 minutes of global ischemia, cytosolic

Ca^{2+} increases from a baseline of 60 to 90 nmol/L to values as high as 200 μmol/L in CA1 and cortical neurons [141,480]. A secondary increase in cytosolic Ca^{2+} is observed in CA1 pyramidal neurons approximately 4 to 6h following global ischemia in rats. This latter increase is presumably associated with irreversible cell damage [480]. Reperfusion leads to gradual recovery of the energy metabolites and subsequent restoration of ionic gradients [520].

Ischemia-induced Ca^{2+} influx is associated with the triggering of discrete catabolic processes, including activation of phospholipases, proteases, and endonucleases. Inhibition of protein synthesis and alteration of various gene expressions are intimately related to cell injury [403]. Thus, Ca^{2+} activates phospholipase A_2 (PLA_2), which in turn catalyzes the breakdown of membrane lipids forming fatty acids and arachidonic acid (AA). Further metabolism of AA by cyclooxygenase (COX) then leads to free radicals formation. Excessive Ca^{2+} release also promotes endonucleases expression within the cell, leading to DNA fragmentation and energy failure due to mitochondrial dysfunction.

Administration of calcium antagonists is associated with significant inhibition of ischemia-induced neuronal death. Among others, the conotoxin SNX-111, a specific N-type calcium channel blocker, is neuroprotective against both focal [64,399,510,575] and global [64,523,554] ischemia. These neuroprotective actions are mainly attributed to glutamate release inhibition [47,435,510].

1.7 Glutamate

Combined with a ubiquitous presence in the brain and its excitotoxic properties upon massive release, glutamate has been proposed as a critical modulator of ischemia-

induced neuronal death (for reviews see [33,93,325,445]). Increased glutamate levels have been reported following focal and global cerebral ischemia [10,34,67,188,286,321,334,518]. Several studies have also highlighted alterations of NMDA [158,166,210,499] and AMPA [102,158,177,379,396,397,408] glutamate receptors following focal and global ischemic insults in rodents. Ischemia-induced changes in kainate [384], as well as metabotropic glutamate receptor mRNAs [237,442] are also reported.

The initial search for therapeutic agents targeted glutamatergic systems. Despite the high number of distinct receptor subtypes, ionotropic glutamate receptors have been the main focus of previous studies. These receptors are categorized into three main classes: N-methyl-d-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionate acid (AMPA) and kainate (KA) receptors [132]. Neuroprotective actions of a large number of competitive (AP-7, dCPP-ene, CGS 19755) and non-competitive (MK-801, ketamine, CNS 1102, dextorphan, memantine, remacemide) NMDA receptor antagonists have been investigated [484]. In general, *in vivo* studies with NMDA antagonists have reported more consistent neuroprotective actions in focal ischemic models (for a review, see [63]), while mixed results are observed using models of global ischemia. Thus, although some studies reported hippocampal protection following administration of the NMDA antagonist MK-801 [172 151,173], others observed no effects on global ischemic damage in gerbils [62,465] and rats [61,357]. In addition, Ishimaru et al. [233] reported that MK-801-induced neuroprotective effects were transient. Neuroprotection against global ischemia-induced CA1 neuronal degeneration was observed at day 4, but not day 7 or 14 following vessel occlusion. In contrast, findings with AMPA/kainate antagonists are more consistent. Thus, treatment with NBQX, a selective AMPA/kainate antagonist, generally

confers neuroprotection against focal and global ischemia, with a few notable exceptions (for a review, see [171]).

Over the last decades, novel hypotheses have been proposed for the neuroprotective actions of NMDA antagonists. Among others, blockade of NMDA receptors could protect against cerebral ischemia by blocking cortical spreading depression or peri-infarct depolarizations (PIDs) [371,372]. Some NMDA receptor antagonists, including MK-801, also induce hypothermia [62], a phenomenon associated with neuroprotection and functional sparing [103]. In addition, some NMDA antagonists like dextromethorphan [285], CGS-19755 [509] and MK-801 [65] enhance regional cerebral blood flow. These side effects of glutamatergic drugs could account for their neuroprotective actions.

Recently, studies have focused on the role of discrete metabotropic glutamate receptors (mGluRs) in the modulation of ischemic injury. Among Group I mGluRs, evidence suggests that activation of mGluR1 but not mGluR5 contributes to ischemic neuronal death both *in vivo* and *in vitro* [336,395]. One interesting observation with the metabotropic glutamate receptors is that, while activation of Group I increases neuronal damage, stimulation of Group II mGluRs appears to reduce ischemic injury. Thus, treatment with LY379268, a selective mGluR2/3 receptor agonist, confers neuronal protection against global ischemia *in vivo* [49,50] and protects against apoptotic cell death *in vitro* [94].

Up to date, glutamate receptor antagonists had limited clinical utility because of serious side effects compromising cell functions in humans and a limited therapeutic window [335]. Recently, more selective antagonists such as CGX-1007, a potent NMDA

receptor antagonist targeting the NR2B subunit, have been shown to confer protection when administered up to 8h following focal cerebral ischemia [199,292,547], keeping this area of research alive.

1.8 Other important endogenous mediators of ischemic injury

1.8.1 Potassium channels

Potassium channels contribute to the maintenance of hyperpolarized membrane potentials. This inhibitory role suggests that the disruption of potassium channel activity could facilitate the ischemia-induced neurotoxic cascade while an activation of potassium channels could be protective. Activation of ATP-sensitive potassium channels (K^+(ATP)) has been reported to be neuroprotective in focal [322,470,506] and global [209] cerebral ischemia, and to be an important modulator of preconditioning-induced protection [42,405]. Such demonstration of increased tolerance to cardiac and cerebral ischemic insults in rats preconditioned to sublethal insults represents a good model to study the endogenous phenomenon mediating neuronal survival. Potassium channel openers also inhibit ischemia-induced release of glutamate *in vitro* [578] and *in vivo* [161], while potassium channel blockers increase glutamate release [578]. Hyperpolarization of synaptic terminals preventing calcium entry and glutamate release is one of the main mechanism by which they inhibit cellular death [209]. Opening of K^+(ATP) channels also blocks global ischemia-induced expression of c-fos, c-jun, heat shock protein, and amyloid beta-protein precursor genes [209]. It also inhibits ischemia-induced apoptosis via an increase of the anti-apoptotic protein Bcl-2 and suppression of Bax translocation and cytochrome c release [283]. In contrast, attenuation of outward potassium current through blockade of delayed rectifier

potassium channels (i.e., which are opened 1ms following cell membrane depolarization) reduces ischemia- [224,573], and glutamate-induced [576] apoptosis. This suggests that distinct potassium channel subtypes play different roles in the modulation of ischemia-induced neuronal death. In summary, activation of K^+(ATP) channels confers neuroprotection by hyperpolarizing the cell membrane and by limiting calcium influx and glutamate release while blockade of delayed rectifier potassium channels acts on mechanisms mediating apoptotic cell death.

1.8.2 Adenosine

Increased cellular depolarization and ATP breakdown during ischemia lead to an important increase in extracellular adenosine concentrations [154,187]. To date, four different subforms of adenosine purinergic receptors have been identified (A_1, A_{2A}, A_{2B} and A_3). They belong to the family of G-protein coupled receptors [155]. Adenosine appears to modulate neuroprotection by decreasing excitatory amino acid release, hyperpolarizing cell membrane, decreasing Ca^{2+} influx, and reducing free radicals formation and the loss of ATP [154,449]. Adenosine A_1 receptor density and mRNA expression are significantly reduced following stroke [2,119,356,378]. Stimulation of A_1 receptors produces neuroprotection in both focal and global experimental models of stroke (for a review, see [38]). In contrast, the role of A_{2A} receptors in the modulation of ischemic damage remains controversial because both agonists and antagonists confer protection in various models of cerebral ischemia (for a review, see [392]). The contribution of adenosine A_{2B} receptors in cerebral ischemia remains unknown. Finally, A_3 receptor agonists increase ischemia-induced neuronal damage and limit the beneficial effects of A_1 receptor stimulation [530]. Hippocampal neuroprotection induced by cerebral ischemic preconditioning appears

mediated through adenosine A_1 receptor activation and the opening of K^+(ATP) channels [211].

1.8.3 Sodium channel blockers

As one of the main modulator of synaptic transmission and induction of action potential, voltage-gated sodium channels represent another primary target to control ischemic damage. *In vitro*, treatment with tetrodotoxin (TTX), which blocks action potentials in nerves by binding to the pores of voltage-gated fast Na^+ channels, protects CA1 pyramidal neurons against glutamate- [265], hypoglycemia- [513] and potassium cyanide-induced toxicity [532]. TTX is also neuroprotective in global ischemia *in vivo* [296,555]. TTX delays tissue acidification and anoxic depolarization in global ischemia in rats [553] and hastens the recovery of evoked population spikes in anoxic rat hippocampal slices [45], potentially through the inhibition of glutamate release [514]. In addition, riluzole, a blocker of persistent Na^+ channels, is neuroprotective in rodent models of cerebral ischemia [19,307,413,534] presumably by blocking glutamatergic transmission in the CNS [133].

1.8.4 GABA

Because gamma aminobutyric acid (GABA) is the primary inhibitory neurotransmitter of the nervous system, it has readily been identified as an important modulator of ischemic injury. Two general classes of GABA receptors are known, $GABA_A$ ionotropic receptors and $GABA_B$ metabotropic receptors, coupled to a second messenger G-protein system. The majority of interneurons in the hippocampus are GABAergic and they appear largely spared from ischemia. Therefore, they could play a determinant role in the rescue of the most vulnerable neighbouring CA1 cells. Indeed, GABA uptake inhibitors attenuate ischemic damage [240] [230,402]. Administration of

muscimol [474] and chlormethiazole [78,111,112,473,488,502-504], two $GABA_A$ agonists which facilitate chloride influx through GABA receptors, are also neuroprotective in animal models of stroke. In addition, treatment with diazepam, a benzodiazepine facilitating $GABA_A$ receptor activity, protects against *in vivo* ischemia-induced hippocampal degeneration [240,462,463]. Diazepan also promotes ATP recovery and prevents cytochrome c release in hippocampal slices *in vitro* [162]. Finally, administration of the selective $GABA_B$ agonist baclofen inhibits ischemia-induced glutamate release and neuronal degeneration [17,267].

1.9 Nitric oxide and free radicals

In recent years, free radicals have been identified as a cause of cellular degeneration under normal and pathological states. Free radicals are highly reactive molecules composed of one or more unpaired electrons. They are known to react with DNA, proteins and lipids and cause varying degree of cellular damage and dysfunction. The cascade of metabolic events induced by ischemia stimulates the generation of oxygen free radicals (for a review, see [289]). A related endogenous phenomenon involves small second messenger molecules referred as nitric oxide (NO) synthesized from the amino acid L-arginine by the Ca^{2+}/calmodulin-dependent enzyme NO synthase (NOS) in mammalian tissues. Many studies identify NO as a critical mediator of neuronal ischemic damage because of a dramatic increase of NO following cerebral ischemia [308 125,226,227]. At least three isoforms of NO have been identified, neuronal, endothelial and inducible, which are thought to play different roles in ischemic neuropathology [36]. Endothelial NO is a potent vasodilator and plays a beneficial role by increasing cerebral blood flow and

decreasing platelet aggregation and adhesion, and also by reducing NMDA current. In contrast, neuronal and inducible NO play a direct deleterious role by reducing neuronal energy production through the inhibition of glycolytic and mitochondrial enzymes and by increasing DNA damage. Neuronal and inducible NO also indirectly trigger cytotoxicity by increasing the formation of oxygen free radicals. Thus, NO reacts with superoxide to produce peroxynitrite (ONOO-), another highly reactive free radical mainly produced via the metabolism of oxygen through the mitochondrial electron transport chain. However, superoxide can also be generated by xanthine oxidase or as a result of arachidonic acid metabolism. Peroxynitrite is then converted to hydroxyl radical (OH-) and nitrogen dioxide (NO_2), which then causes damage by producing DNA, protein and lipid oxidation or tyrosine nitrosylation. Inhibition of NOS reduces ischemic neuronal damage both in focal [228,560] and global [452] ischemic models. Although inhibition of NOS production is neuroprotective, the exact mechanism by which free radicals cause post-ischemic damage remains to be determined.

1.10 Apoptotic cell death : Differential mechanisms

1.10.1 Intrinsic and extrinsic pathways

The endogenous modulators discussed so far have mainly been studied in the context of the characterization of endogenous systems involved in necrotic cell death. However, ischemia-induced neuronal death is also due to the induction of apoptotic processes. Apoptosis can occur either through mitochondrial-dependent intrinsic pathway or receptor-mediated extrinsic pathway, both of which lead to caspase-3 activation [179,498,525]. Numerous caspases have been identified in the last decade. They represent a

family of proteases mediating apoptosis and can be categorized in three enzyme groups based on their relative role in the apoptotic pathway [179]. Group I caspases (1, 4, 5, 11, 12, 13, and 14) mainly function as cytokine processors, while Group III caspases (2, 8, 9, and 10) are initiator caspases activated upon the reception of death signals and which subsequently activates effector caspases. Group II caspases (3, 6, and 7) include effector caspases, which cleave discrete cellular substrates and influence cell survival.

In the intrinsic pathway, cytotoxic events including elevated intracellular calcium concentration and reactive oxygen species (ROS) lead to the release of the mitochondrial protein cytochrome c within the cytosol. Cytochrome c is involved in mitochondrial respiration and regulates the intracellular electron transport chain reaction for the production of ATP. This latter molecule is responsible for the transport of chemical energy within cells. Although the exact mechanism remains unclear, the release of cytochrome c into the cytosol appears to occur via a megachannel called the mitochondrial permeability transition pore (MPTP), which is regulated by the Bcl-2 family [468]. The Bcl-2 family contains both anti-apoptotic (Bcl-2, Ced-9, Bcl-X_L, Mcl-l, A1, Bcl-W, Bfl-1, Brag-1), as well as pro-apoptotic (Bax, Bad, Bak, Bik, Bcl-X_S, Bid, Hrk) proteins, which can either block or facilitate the release of cytochrome c. Once released within the cytosol, cytochrome c binds to the apoptosis protease activating factor (Apaf-1), forming a complex with dATP and activating caspase-9. Caspase-9 then cleaves and activates caspase-3. The resulting activation of caspase-3 then cleaves poly(ADP-ribose) polymerase (PARP) and activates caspase-activated Dnase (CAD), leading to DNA damage and apoptotic death.

In addition to these intracellular changes, an extrinsic pathway involves the activation of the Fas receptor or receptor 1 subtype of tumor necrosis factor ($TNFR_1$), through the binding of either the Fas ligand or tumor necrosis factor-α (TNF-α), respectively. The stimulation of these death receptor pathways leads to caspase-8 activation, which subsequently cleaves and activates caspase-3. Similar to the intrinsic pathway, activation of caspase-3 cleaves PARP and activates CAD, leading to DNA damage and apoptosis.

1.10.2 Evidence supporting ischemia-induced apoptotic cell death

The relative contribution of apoptosis and necrosis to ischemia-induced neuronal death remains controversial [361]. Ultrastructural studies have reported rare apoptotic cells in the CA1 subfield of the hippocampus following global [104,279] and focal [127] cerebral ischemia. However, other biochemical markers suggest that ischemia-induced neuronal death can occur through apoptosis. Among others, DNA fragmentation has been reported in the CA1 subfield of the hippocampus and the striatum following global cerebral ischemia [302]. DNA fragmentation is also observed in the cerebral cortex and other selective vulnerable neurons following focal cerebral ischemia [182,278,282]. In addition, stroke is associated with an up-regulation of pro-apoptotic factors and a down-regulation of anti-apoptotic factors. Thus, a decrease in Bcl-2 and Bcl-X, together with an increase of Bax has been reported following focal [175,231] and global cerebral ischemia [262]. Some evidence suggests that the anti-apoptotic proteins Bcl-2 and $Bcl\text{-}X_L$ are expressed in neurons that survive global cerebral ischemia in the CA3 and dentate gyrus regions [81,82,84]. The pro-apoptotic protein Bax is expressed in vulnerable CA1 neurons destined to die

[84,196]. The expression of caspase-3 is also upregulated in CA1 pyramidal neurons following global cerebral ischemia [83,174,362].

Other evidence supporting neuronal apoptosis comes from studies that show neuroprotection after discrete anti-apoptotic interventions. Thus, overexpression of Bcl-2 [254,319] and Bcl-X_L [545] in transgenic mice has been shown to be neuroprotective against stroke. Caspase inhibition has also been demonstrated to be protective against focal [277] [91,140,150,546] and global [214] cerebral ischemia. More specifically, inhibition of caspase-3 activity significantly confers protection against CA1 neuronal death following global ischemic insult [83,174]. Caspase-3 deficient mice are more resistant to ischemic stress *in vivo* and *in vitro* [269]. Inhibition of interleukin-1β-converting enzyme (ICE, now called caspase-1) family proteases also reduces ischemic damage [198,287]. Similarly, attenuation of focal cerebral ischemic injury is found in transgenic mice expressing a mutant ICE inhibitory protein [197]. Together, these findings suggest that ischemia-induced neuronal death likely occurs through pathways regulating either necrotic and apoptotic processes.

1.11 Role of inflammation

Cerebral ischemia is accompanied by a marked inflammatory reaction involving enhanced cytokines expression. Cytokines-like interleukin-1 and TNF-α regulate immunity, inflammation and hematopoiesis, [148,149,446,569]. Chemokines (CINC, MCP-1, and MIP-1) and adhesion molecules (selectins, intergrins, intercellular adhesion molecule 1) are expressed in ischemic brain tissue [159,250,383]. Increased release of these inflammatory molecules results in the breakdown of the blood-brain-barrier (BBB), culminating in cerebral oedema [118,191]. Pharmacological compounds that block the activity of

inflammation-related processes attenuate ischemic damage and improve neurological outcome [226,415].

Of all interleukins identified, interleukin-1 (IL-1) has been the most investigated for its role in the modulation of ischemic damage. The IL-1 family comprises three variants, IL-1α, IL-1β and IL-1ra. Among them, IL-1β appears to contribute the most to ischemic brain damage. Thus, IL-1β mRNA is increased in the rat brain following transient forebrain ischemia [343]. IL-1β administration in *in vivo* stroke models increases ischemic infarct volume [288,556]. In contrast, administration of the non-specific IL-1 receptor antagonist (IL-1ra) results in an inhibition of ischemia-induced neuronal damage [431,165,288,352]. Also, injection of specific anti-IL-1β antibodies reduces infarct volume caused by focal ischemia, which suggests that the beta form of Il-1 is the primary modulator of neuronal death [556]. IL-1β also exacerbates, while IL-1β antagonist attenuates neuronal injury induced by NMDA excitotoxic insult in hippocampal neurons *in vitro* [189]. Other interleukins, including IL-6 and IL-8 may also play a role in ischemic damage [447].

2. Assessment of behavior following global ischemia

Despite the importance of a reduction of ischemia-induced neuronal damage, recovery of function remains the primary goal of any therapeutic action. Global ischemic models are often used to examine the ability of pharmacological strategies to limit or prevent the development of behavioral and cognitive deficits following stroke. The various measures used to assess behavioral and/or cognitive sparing and recovery in ischemic research are described in the following sections.

2.1 Locomotor activity

Cerebral ischemia has been shown to induce increased locomotion when animals are placed in a novel open field [76,406,557]. Most studies have linked ischemia-induced hyperactivity to memory impairments resulting from hippocampal cell death [9,72,169,263]. It has been argued, for instance, that memory deficits delay familiarity with a new environment because the reduced ability to detect and remember spatial cues prolongs exploration and leads to increased locomotor activity [16,100,535]. Evidence of a memory deficit also comes from observations that ischemic animals habituate more slowly to a novel environment when tested across days, which might be expected from a delayed establishment of familiarity [100,535].

However, there is a temporal mismatch between hippocampal degeneration and locomotor hyperactivity. Thus, whereas neuronal death develops over two days and reaches its maximum on the third day after ischemia, locomotor hyperactivity peaks on day 1 following ischemia [40,242]. These results suggest that other mechanisms may account for the observed increased behavioral activity. Some studies also reported no correlation between CA1 neuronal damage and locomotor activity. For example, despite significant hippocampal CA1 protection in preconditioned gerbils, the animals still displayed habituation deficits in the open field [107,134]. In addition, Gulinello et al. [183] recently reported that global ischemia did not affect locomotion in the open field, while other studies reported reduced locomotor activity following bilateral occlusion of the carotid arteries in rodents [164,558,559].

One possible explanation for these discordant results is suggested by the demonstration that no alteration of open field activity is observable in rats with ischemic

damage restricted to hippocampal CA1 neurons [176]. However, the more extensive damage following two consecutive 7-min occlusions which produces cell death in the cerebral cortex and caudate nucleus is accompanied with persistent locomotor hyperactivity [232]. Thus, intensity of the insult [72] and the presence of extra-hippocampal damage may play a determinant role in the expression of hyperactivity following ischemia. Also, differences between sham and ischemic animals are considerably reduced or absent following repeated exposure to the open field [16,134,145,242]. In addition, some evidence suggests that cerebral ischemia fails to alter open field activity in animals tested several weeks following the insult [294,424].

It is also possible that global ischemia influences behavioral activity through processes other than memory. By nature, unfamiliar environments tend to be threatening or anxiogenic to rodents. Thus, differences observed between ischemic and sham animals in the open field may represent differential level of adaptation to an anxiogenic environment. Indeed, increased exploratory activity in the center of the open field is considered an index of decreased anxiety [417,441,479,493]. Such observation is consistent with previous studies reporting reduced anxiety level in ischemic animals in the elevated plus maze [358,406,557].

Whether locomotor hyperactivity results from memory impairments or decreased level of anxiety, and despite of the divergent results observed in the different studies, the open field is a test which is still widely used today by researchers to assess ischemia-induced behavioral alterations.

2.2 Spatial memory deficits in ischemic animals: The radial arm and morris water maze

Cerebral ischemia typically produces spatial memory deficits. Spatial memory tasks assess the ability of animals to navigate in an environment defined by their position relative to distal extramaze cues [349]. Spatial memory deficits are measured in the radial maze by recording reference or working memory errors. A reference memory error is made when an animal enters an unbaited arm, while a working memory error is a re-entry of a previously chosen baited arm. Global ischemia alters both types of memory deficits in the radial arm maze [120,176,255]. Pre-ischemic training reduces the number of both types of errors [529]. A negative correlation between the number of reference or working memory errors and the number of CA1 pyramidal cells has been reported [255].

The morris water maze (MWM) is another widely used spatial memory test. The maze consists of a circular pool filled with water. A platform is submerged below the water surface and the rat can climb on it to get out of the water and escape the necessity of swimming. Rats are placed into the pool from a variety of starting locations and, because there are no cues within the pool to guide the rat to the platform, acquisition of the platform position is based on extramaze cues. Escape latency, swim speed and swim distance are the main parameters assessed. Ischemia-induced deficits in the MWM has been reported in several studies [41,186,376,377,544]. In contrast, others reported an absence of deficits in spatial learning following global ischemia [15,181,255,436]. In addition, unlike findings with the radial maze, some studies reported a correlation between CA1 neuronal death and memory deficits in the MWM [358,376], while others did not [181,370]. Thus, compared to the MWM, the radial maze provides more reliable results because

performance in this test is better correlated with CA1 neuronal loss, thus allowing more convincing conclusions about the impact of pharmacological strategies on recovery of spatial memory deficits.

2.3 Passive avoidance test

The passive avoidance test represents a memory paradigm designed to assess the ability of animals to learn and remember associations. The apparatus is composed of two compartments separated by a guillotine door. One compartment has a grid floor that is used to deliver footshocks. The other compartment is composed of a Plexiglas floor and is considered the safe compartment. Following a habituation period, the animal is placed into the safe compartment for an acquisition trial and a footshock is delivered as soon as the animal enters the grid compartment. In a retention trial (usually 24h later), the time taken to enter the grid compartment is taken as an index of the recall of the shock. Compared to sham-operated animals, ischemic animals enter the footshock compartment after a shorter latency indicating poorer memory [12,242,246].

2.4 Object recognition

Cerebral ischemia is also associated with impairments of object recognition. Among existing paradigms, the delayed non-matching to sample (DNMS) task is frequently used to test non-spatial memory deficits. The task involves the presentation of a sample object that the subject displaces to retrieve a food reward hidden beneath. Following a delay during which the sample object is hidden from view, it is presented again, along with a novel object. The subject is rewarded for selecting the novel object

from this pair. Forebrain ischemia has been shown to severely impair DNMS performance in rats [355,551] and monkeys [18,580]. Although the role of the hippocampus in object recognition has been proposed [98,551], various studies challenge an hippocampocentric view, and support the idea that damage in parahippocampal structures might more readily explain the ischemia-induced object recognition deficits (for a review, see [354]). Thus, hippocampal removal failed to impair object recognition, and ischemia-induced DNMS deficits were significantly attenuated by hippocampal ablation performed before or shortly after global ischemia [355].

2.5 Y-maze

The Y-maze (or T-maze) can be used to assess spontaneous alternation. Rodents display a tendency to spontaneously explore different parts of a maze in succession without returning to the same arm. Good working memory is inferred from a high number of "alternations" from one arm to the next. One advantage of the Y-maze compared to other memory tests such as the 8-arm radial maze is that it does not involve food deprivation or punishment. Several studies reported ischemia-induced deficits in spontaneous alternation [8,184,320 235,236].

Another use of the Y-maze is the recognition memory test. With one arm of the-Y maze blocked, the animal is placed in one of the arms and is allowed to explore the 2 arms for 15 minutes, with visual cues displayed within the maze. The subject is then returned to the maze 1-4h (or days) later with all arms opened and behavior is monitored for 5 minutes. Based on the natural drive of rodents to explore novel environments, the animal

should spend more time exploring the previously unexplored blocked arm. Deficits in this task have been reported in hypoxic animals [28].

3. Role of neuropeptides in ischemia-induced neuronal damage and behavioral sparing

A neuropeptide is a synthesized chain of amino acids released from a neuron as a signalling molecule [218]. Neuropeptides regulate a wide range of physiological processes. They act as neurohormones, neurotransmitters and/or neuromodulators, maintain homeostasis and influence cognitive, emotional and behavioral functions, as well as hunger, thirst, sex drive, pleasure and pain. Peptides larger than 50 amino acid residues are usually classified as proteins. Over the last decades, there has been an increased interest in the role of neuropeptides in various psychiatric disorders, particularly depression and anxiety-related disorders (for a review, see [31]).

Different classifications have been proposed for peptides according to their structure, function and/or brain localization. For example, there are opioid neuropeptides (enkephalin, dynorphin, endorphin), gut-brain peptides (i.e., substance P, galanin, bombesin, neurotensin, neuropeptide Y), hypothalamic releasing hormone (i.e., corticotropin-releasing hormone, hypocretin, etc.), pituitary hormones (AVP, ACTH), and miscellaneous peptides (eg bradykinin). Biosynthesis of neuropeptides follows the steps common to any protein synthesis involving gene transcription. The pre-propeptide within the neuron nucleus is transcripted and the complementary mRNA segment leaves the nucleus to be translated via the ribosomes of the rough endoplasmatic reticulum into the pre-propeptide signal, which represents the precursor of the active peptide. On the rough

endoplasmatic reticulum, a portion of the pre-propeptide is cleaved off by activation of a signal peptide, to form the propeptide. Finally, the propeptide is cleaved off by converting enzymes in the Golgi apparatus to form the active neuropeptide. Neuropeptides are stored in large vesicles in the cell body and reach nerve terminals via axonal transport where they are released via Ca^{2+} modulated exocytosis. Once in the synaptic cleft, neuropeptides interact with discrete receptors and second messenger systems and activate various intracellular pathways. Most neuropeptides receptors belong to the G protein-coupled receptor superfamily.

Neuropeptides are distributed throughout the brain and their participation is investigated in a wide range of physiological and pathological conditions. Corticotropin-releasing hormone is undoubtedly one of the most extensively studied peptide, notably for its role as the main modulator of the hypothalamo-pituitary-adrenal (HPA) axis mediating stress responses.

3.1 Corticotropin-releasing hormone

In 1981, Vale and colleagues [521] identified a novel 41-amino acid sequence (H-Ser-Gln-Glu-Pro-Pro-Ile-Ser-Leu-Asp-Leu-Thr-Phe-His-Leu-Leu-Arg-Glu-Val-Leu-Glu-Met-Thr-Lys-Ala-Asp-Gln-Leu-Ala-Gln-Gln-Ala-His-Ser-Asn-Arg-Lys-Leu-Leu-Asp-Ile-Ala-NH2) from the ovine hypothalamus, which they named corticotropin-releasing factor. Based on some of its functions, the peptide is now commonly referred as corticotropin-releasing hormone (CRH).

CRH is processed from the precursor prepro-CRH, a 21 kDa 196-amino acid neuropeptide [521]. Human and rodent CRH gene sequences are homologous, and differ

from ovine CRH in only seven of the 41 residues [495,516]. The human CRH gene is located on the long arm of chromosome 8 [13] and composed of two exons, separated by an intervening intron. The first exon encodes most of the 5'-untranslated region in the mRNA, while the second exon contains the prohormone sequence and the 3'-untranslated region [464,469,516]. Different specific and non-specific antagonists for CRH receptors have been developed enabling the study of the peptide's behavioral and neuronal effects (see Table 1).

Table 1: List of CRH receptor antagonists.

$CRHR_1$ antagonists	$CRHR_2$ antagonists	non-selective CRH antagonists
CP154,526	Antisauvagine-30	α-helical CRH (9-41)
Antalarmin	$Astressin_2$-B	$_D$-Phe-CRH (12-41)
NBI27914		Astressin
NBI-30775 (R121919)		
NBI-35965		
CRA1000 and CRA1001		
DMP696 and DMP904		
SSR125543A		
R27899/CRA0450		

[Martinez, V., et al. (2004). Central CRF, urocortins and stress increase colonic transit via CRF1 receptors while activation of CRF2 receptors delays gastric transit in mice. J Physiol., 556(Pt 1):221-34.; Smagin, G.N. & Dunn, A.J. (2000). The role of CRF receptor subtypes in stress-induced behavioural responses. Eur J Pharmacol., 405(1-3):199-206.; Valdez GR. (2006). Development of CRF1 receptor antagonists as antidepressants and anxiolytics: progress to date. CNS Drugs., 20(11):887-96.]

3.1.1 Gene expression and CRH secretion

The regulation of CRH gene expression involves the activation of a number of biological pathways including cAMP-dependent protein kinase A (PKA), calcium/calmodulin-dependent protein kinase and diacylglycerol-dependent protein kinase C (PKC) pathways [482]. CRH gene expression also occurs following the cAMP-induced phosphorylation of cAMP-regulatory element-binding (CREB) protein [306]. Many neurotransmitters have been implicated in the regulation of CRH release. Thus, acetylcholine, norepinephrine, epinephrine, histamine, and serotonin have been shown to promote hypothalamic CRH release, while gamma-aminobutyric acid inhibits secretion of the peptide from the same site [306,482]. Additional factors or hormones that modulate the regulation of CRH release include, angiotensin, vasopressin, neuropeptide-Y, substance P, atrial natriuretic peptide, cholecystokinin, activin, melanin-concentrating hormone, β-endorphin, enkephalin, as well as the cytokines IL-1, IL-6, and tumor necrosis factor-alpha [306,482].

3.1.2 Distribution of CRH

Among sites where CRH is most densely expressed are the nerve cell bodies within, and proximal, to the dorsomedial parvocellular division of the paraventricular nucleus (PVN) of the hypothalamus. These neurons send axons to the median eminence and other hypothalamic and midbrain targets. CRH-immunoreactive cell bodies are also abundant in a number of other hypothalamic nuclei, including the supraoptic, suprachiasmatic, preoptic, premammillary, periventricular, arcuate, and magnocellular paraventricular nuclei [11,116,117,248,404]. In the neocortex, high CRH expression is observed in the prefrontal and cingulate cortices [501]. Dense CRH-immunoreactivity is also present

in the central nucleus of the amygdala (CeA), the bed nucleus of the stria terminalis (BNST), the parabrachial nucleus, and moderate to low expression is observed in the hippocampus, nucleus accumbens, substantia nigra, locus coeruleus, thalamus, raphe nuclei, periaqueductal gray (PAG), olfactory bulbs, nucleus of the solitary tract and cerebellum [338-341,348,451,453,501,524]. Apart from its expression in brain structures, CRH is also present in peripheral tissue cells, including the adrenal medulla, lymphocytes, pancreas, lung, liver, the gastrointestinal tract and skin [380]. An illustration of CRH distribution in the rodent brain is shown in Figure 2a.

3.1.3 Distribution of CRH receptors

Two distinct CRH receptor subtypes, $CRHR_1$ and $CRHR_2$, have been cloned and characterized in rats [290,291,412] and humans [86,281]. Both $CRHR_1$ and $CRHR_2$ subtypes have seven transmembrane domains, and are metabotropic receptors coupled to G proteins linked to intracellular cAMP activation [123]. There is 70% amino acid homology between the two receptor subtypes. However, their brain distribution appears largely distinct. Thus, while $CRHR_1$ mRNA expression is high in regions processing sensory information and involved in motor control, such as the cerebral cortex, cerebellum, pituitary and olfactory bulb, high densities of $CRHR_2$ are found in subcortical structures, including the lateral septum, choroid plexus, olfactory bulb, enthorinal cortex, amygdala and hypothalamus [75,401,412,416]. Four splice variants of $CRHR_2$ subtype have been identified: $CRHR_{2\alpha}$, $CRHR_{2\alpha\text{-tr}}$, $CRHR_{2\beta}$, $CRHR_{\gamma}$ [253,261,291,344,400]. $CRHR_{2\alpha}$ appears mainly localized in the brain, while expression of $CRHR_{2\beta}$ is predominant in the periphery [290]. CRH receptors, principally $CRHR_1$, are found in CA1 and CA3 pyramidal neurons [75,90,526]. The distinct

distribution pattern of both receptor subtypes suggests that they exert different functional roles. Figure 2b illustrates the distribution of $CRHR_1$ and $CRHR_2$ in the rat brain.

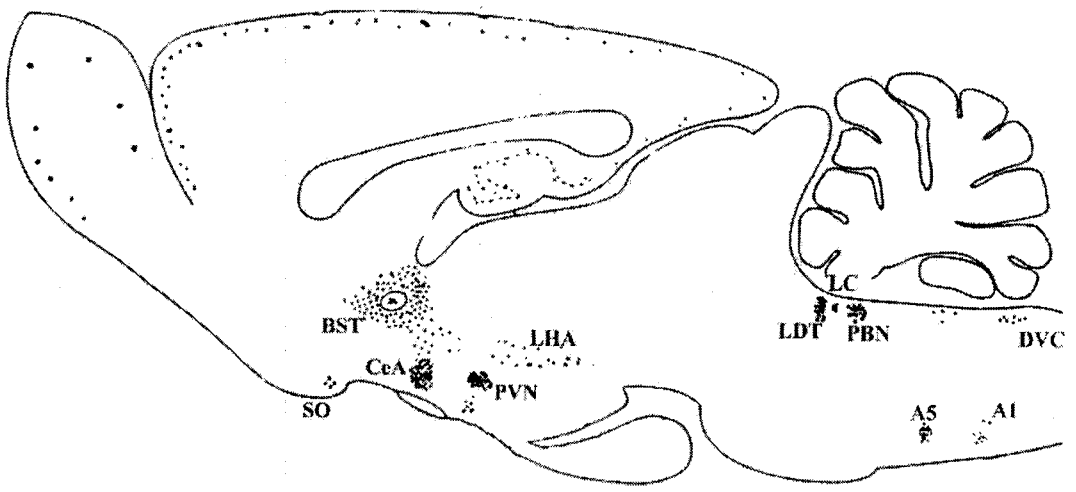


Figure 2a: Distribution of CRH in the rodent brain. [Smagin, G.N., et al. (2001). The role of CRH in behavioral responses to stress. Peptides, 22(5), p.714.]

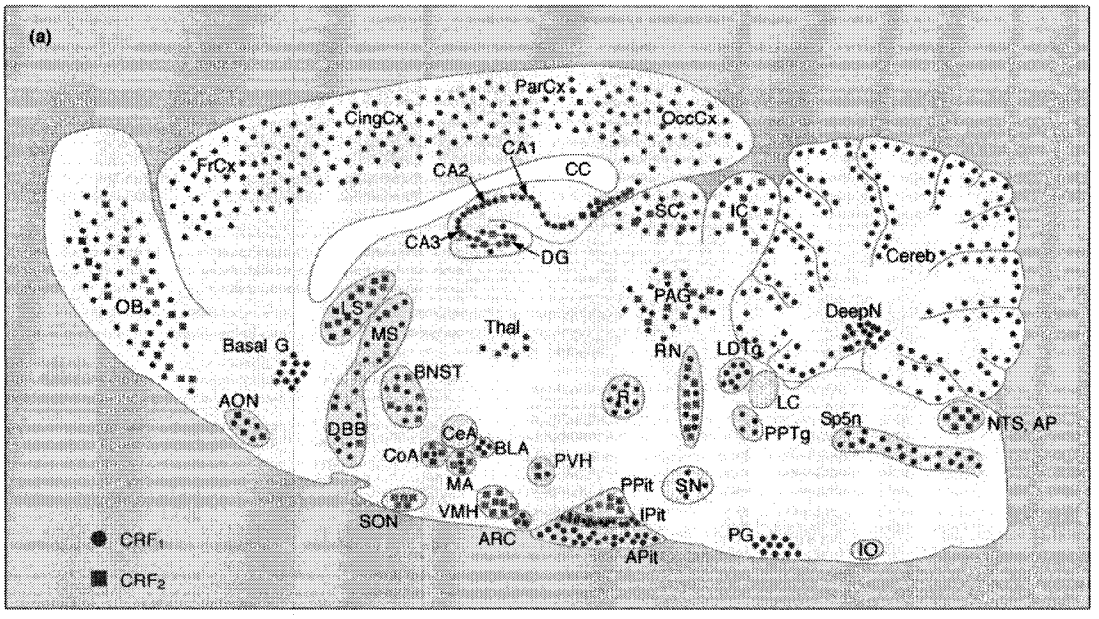


Figure 2b : Distribution of $CRHR_1$ and $CRHR_2$ in the rat brain. [Reul, J.M. & Holsboer, F. (2002). Corticotropin-releasing factor receptors 1 and 2 in anxiety and depression. Curr Opin Pharmacol., 2(1), p.26.]

3.1.4 CRH-related peptides

In addition to ovine [521] and rat/human CRH [434], the CRH family of peptides includes mammalian urocortin (Ucn, [527], UcnII (also called stresscopin-related peptide) [432], UcnIII (also called stresscopin) [275]), as well as amphibian sauvagine [345] and fish urotensin [270]. Ucn share 63% and 45% sequence homology with urotensin and CRH, respectively. In comparison to CRH distribution, Ucn mRNA expression in the rat brain is mainly found in the Edinger-Westphal (EW) nucleus, and distributed in lesser amounts in the lateral superior olive, hippocampus, basal ganglia, medial septum, PVN, and lateral hypothalamus [527,549]. CRH and Ucn both bind $CRHR_1$ and $CRHR_2$, although Ucn binds $CRHR_2$ with 40-fold greater affinity than $CRHR_1$ [527]. On the other hand, UcnII and UcnIII bind selectively to $CRHR_2$ [223,275,290,432]. CRH and Ucn can further bind to a non-receptor corticotropin releasing factor-binding protein (CRF-binding protein) [411,527], whereas UcnII and UcnIII do not [275].

3.1.5 CRH and the HPA axis

The role of CRH as a prime modulator of the hypothalamic-pituitary-adrenal (HPA) axis is widely accepted. Following stress exposure, CRH is synthesized in highest levels in the medial parvocellular part of the paraventricular nucleus (PVN) of the hypothalamus, leading to enhanced release of the peptide from the median eminence. CRH is then transported to the anterior pituitary through the portal vascular system, where it stimulates the secretion of adrenocorticotropin (ACTH) and β-endorphins from corticotropes. In response to ACTH stimulation, the adrenal cortex produces glucocorticoids, mainly cortisol in humans and corticosterone in animals. Glucocorticoids in turn act back on the hypothalamus and pituitary in a negative feedback cycle to

suppress CRH and ACTH production. An illustration of the HPA axis is shown in Figure 3.

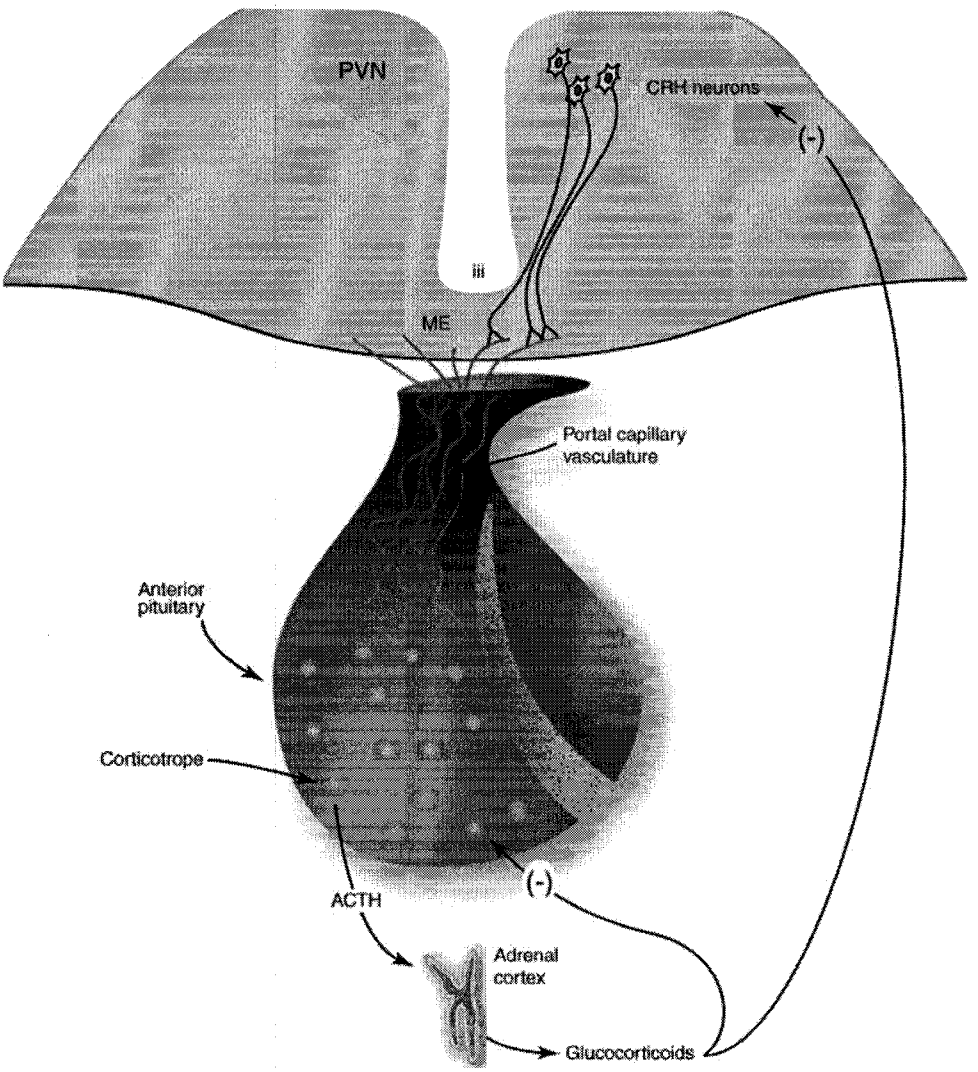


Figure 3 : Illustration of the hypothalamic-pituitary-adrenal (HPA) axis. [Bloom, F.E., McConnell, S.K. & Squire, L.R. (2002). *Fundamental Neuroscience*, Academic Press, p.1044.]

3.1.6 Impact of CRH on behavior

Numerous studies have demonstrated the role of CRH in a wide range of behaviors including arousal, attention, motor function, feeding and reproduction [243,496,522]. To date, the contribution of CRH to the modulation of stress and anxiety has been the most studied. In this context, the ability of CRH to alter activity of the serotoninergic (for a review, see [272]) and noradrenergic [14] systems is noteworthy.

A role for both $CRHR_1$ and $CRHR_2$ in the modulation of anxiety has been inferred from studies using CRH antagonists, antisense oligodeoxynucleotides (ODNs) and knockout gene targeting approaches (for reviews, see [21,496]). Strong evidence suggests that CRH induces its anxiogenic effects by acting primarily at $CRHR_1$, whereas conflicting interpretations exist for the role played by $CRHR_2$ in mediating stress-induced behaviors [21]. Administration of CRH has also been shown to alter locomotion in a dichotomic manner, depending on the baseline state of arousal of the animal. Thus, CRH increases locomotor activity when animals are tested in a familiar environment [136,241,260,466,500], but suppresses exploration in an unfamiliar one [20,35,60,500,507]. In addition, CRH modulates learning and memory in site- and receptor-specific manner. Thus, direct activation of hippocampal $CRHR_1$ enhances learning in response to fear conditioning, while activation of $CRHR_2$ into the lateral intermediate septum impairs learning in the same memory model [425]. CRH has also been shown to enhance spatial memory in the Morris Water Maze [27], to improve acquisition of a visual discrimination task [259] and to facilitate passive avoidance learning [85,130,271,280]. Others reported that CRH receptor agonists can suppress food intake [204], enhance acoustic startle reactivity [121] and facilitate defensive burying [128].

CRH activation contributes to metabolic, neuroendocrine and autonomic changes and represents an adaptive response following acute stress that contributes to the restoration of homeostasis. CRH hyperdrive and/or hypofunction has been demonstrated under chronic stress conditions as well as in a number of mood, stress-related and other disorders, including major depression, dysthymia, atypical depression/seasonal affective disorder, posttraumatic stress disorder, chronic fatigue/fibromyalgia, eating disorders, alcohol dependence and personality disorders (for a review, see [95]). Recently, dysfunction of the CRH system has been associated with the progression of Alzheimer's disease [25].

3.1.7 Participation of CRH in cerebrovascular disease

CRH involvement in various stress-related disorders has led researchers to investigate its participation in cerebrovascular accidents as they represent one of the most invasive physiological stress associated with critical homeostatic perturbations. In recent years, numerous studies have shown endogenous alterations of CRH gene and receptor expression in distinct brain regions in rodents following ischemia and neurodegenerative diseases[202,249,550].

Characterization of region-specific changes in CRH immunoreactivity and/or endogenous release following cerebral ischemia suggests alterations in CRH level at various extrahippocampal loci (including the lateral, ventromedial and paraventricular nucleus of the hypothalamus, the amygdala and the piriform cortex), but no significant endogenous CRH fluctuations appear initiated at short reperfusion intervals in the most vulnerable brain region, the hippocampal CA1 layer. Recent work in our laboratory failed to observe significant alterations in post-mortem CRH concentration in the CA1 region of the hippocampus 4, 24, and 72h following global cerebral ischemia [249]. Similarly, *in vivo*

CA1-CRH release remained unaltered during the initial 3h sample period following the ischemia insult. Consistent with these results, Choi et al. [92] reported no change in CRH immunoreactivity in the CA1 region of the hippocampus 3, 12, 24, 48 and 72h post-ischemia. However, a transient increase was found 96h following ischemia. These findings suggest that alteration of CRH levels in the CA1 pyramidal cell layer is secondary to other physiological processes taking place during ischemia, and that CRH may be involved in late physiological processes mediating neuronal death or survival.

3.1.8 CRH and cerebral ischemia

Initial studies showed reduction of ischemia-induced cell death in rats pre-treated with CRH antagonists [295,497], suggesting a deleterious role of CRH in the modulation of ischemic damage. These findings were controversial, since Fox et al. [152] reported that both CRH and its antagonist α-helical (9-41) provided protection against hypoxic insult in hippocampal cultures. Interestingly, α-helical CRH (9-41) has been shown to act as a partial agonist of $CRHR_1$ at certain doses [485], raising the possibility that its neuroprotective action was achieved through agonist activity at this receptor. In addition, a recent study by our lab using either CP154,526, a selective non-peptide $CRHR_1$ antagonist or the non-selective antagonist α-helical CRH (9-41), failed to alter ischemia-induced CA1 neuronal damage or improve spatial memory following global ischemia in rats [407].

In recent years, *in vitro* studies have demonstrated the neuroprotective actions CRH against various neurotoxic insults. For example, CRH prevents glutamate-induced neurotoxicity in rat hippocampal cultures [138] and its maximum neuroprotective effects were observed when the peptide was administered between 8-12 h following glutamate

challenge [138]. This result suggests that CRH depletion during the late reperfusion period may contribute to cellular damage. In addition, CRH has been shown to protect against amyloid β (Aβ) and various oxidative insults in hippocampal, cerebellar and cortical cultures [26,142,276,393], as well as reduce apoptotic cell death through the modulation of pro- and anti-apoptotic processes [142,276,426].

3.1.9 CRH-related peptides and protection

Consistent with the results obtained with CRH, recent evidence suggests that other CRH-related peptides could exert neuroprotective effects. Urotensin I, urocortin and sauvagine, but not urocortin II significantly inhibit oxidative- and excitotoxic-induced cell death *in vitro* [142,394]. Urocortin also protects primary cardiac myocytes from hypoxic/ischemic injury when applied before or after the ischemic insult [59,374], and reduces infarct size in isolated rat hearts when administered before or after the ischemic injury [58]. Recent evidence also suggests that urotensin I may reduce infarct size in experimental cerebral ischemia *in vivo* (Strijbos, Skaper and Parson, unpublished observations).

3.2 Opioid peptides

3.2.1 Endogenous opioid ligands and receptors

Although early studies have focused on their analgesic properties, opioid peptides are also known to contribute to the regulation of multiple physiological functions, including endocrine, cognitive, affective, cardiovascular, gastrointestinal, immune, respiratory and autonomic (for a review, see [44]). Recently, the neuroprotective role of opioid peptides were tested in ischemic injury models [23].

Endorphins, enkephalins ([Met5]enkephalin, [Leu5]enkephalin) and dynorphins (dynorphin A (1-17), dynorphin A (1-8), dynorphin B(1-13), α- and β-neo-endorphin) represent the three classes of endogenous opioid peptides. They are synthesized from different precursor proteins: pro-opiomelanocortin (POMC), pro-enkephalin (PENK) and pro-dynorphin (PDYN) [43,109,429]. The opioid peptides can bind to three distinct receptor groups referred as mu(μ), delta(δ), and kappa(κ), which mediate various functional effects in both the peripheral and central nervous system. Opioid receptors are coupled to G proteins, which interact with a variety of potassium and calcium channels and inhibit adenylate cyclase [106]. Proenkephalin-derived peptides have the highest affinity for δ receptors, but can also bind to μ receptors [122]. On the other hand, endorphin binds equally to μ and δ receptors [472]. Finally, prodynorphin-derived peptides have highest affinity for κ receptors [79], but can also bind to μ and δ receptors [423,460]. All three primary groups of opioid receptors can be further subdivided. Two delta receptors, δ1 and δ2, have been proposed based on pharmacological profile and interactions with various endogenous ligands [410,490]. Similarly, kappa receptors include κ1, κ2 and κ3 variants [581 96,548], while mu receptors include μ1- and μ2-opioid receptors [385,386]. The most recent addition to the endogenous opioid peptide family is nociceptin/orphanin-FQ, which is derived from a fourth opioid precursor, pronociceptin/orphanin-FQ and is the endogenous ligand for the opioid receptor like 1 (ORL_1) [205]. A summary of the existing endogenous opioid peptides and their precursors, as well as their opioid receptor affinity is presented in Table 2. In the study of the various behavioral and neuronal effects induced by opioid peptides, many selective and non-selective agonists and antagonists for the three main classes of opioid receptors have been developed (see Table 3).

Table 2 Endogenous opioid peptides, their precursors, and their relative receptor affinity.

Precursors	Opioid peptides	Receptors
Pro-enkephalin	[Met]-enkephalin [Leu]-enkephalin	$\delta > \mu >>> \kappa$
Pro-opiomelanocortin	β-Endorphin	$\mu \approx \delta >> \kappa$
Pro-dynorphin	Dynorphin A Dynorphin $A_{(1-8)}$ Dynorphin B	$\kappa >>> \mu > \delta$ $\kappa >> \mu > \delta$ $\kappa >>> \mu > \delta$
Pro-nociceptin/ orphanin-FQ	Nociceptin/orphanin-FQ	ORL_1

Table 3 Agonists and antagonists for, μ-, κ- and δ-opioid receptors.

Opioid receptors	Selective ligands		Non-selective ligands	
	Agonists	Antagonists	Agonists	Antagonists
μ	Morphine Fentanyl Methadone DAMGO Dermorphin	CTOP	Levorphanol Etorphine	Naloxone Naltrexone β-Funaltrexamine
κ	U50,488H Dynorphin A U62,066E (spiradoline) U69,593	nor-binaltorphimine MR-2266	Levorphanol Etorphine EKC	Naloxone Naltrexone
δ	DPDPE DADLE (-)Tan-67 STOM SNC 80 Deltorphin DSLET	Naltrindole BNTX Naltriben NTB	Levorphanol Etorphine	Naloxone Naltrexone

3.2.2 Distribution of opioid peptides

Both prodynorphin- and proenkephalin-related peptides are widely but unevenly distributed in the rat brain [568]. The highest concentration of prodynorphin-derived peptides are observed in the substantia nigra [α-neoendorphin, dynorphin B, and dynorphin A-(1-8)] and median eminence (dynorphin A and β-neoendorphin) [568]. High concentrations of dynorphin A (1-8) are also reported in the lateral preoptic area, ventral premamillary nucleus, anterior hypothalamic nucleus, dorsomedial nucleus, arcuate nucleus, and medullary reticular nuclei [565], while dynorphin B is found in the lateral preoptic area, parabrachial nuclei, globus pallidus, the periaqueductal gray matter, anterior hypothalamic nucleus, nucleus accumbens and hippocampus [566].

On the other hand, the highest concentrations of Leu-enkephalin and Met-enkephalin-Arg-Gly-Leu are observed in the globus pallidus, followed by the central amygdaloid nucleus [568]. Other regions showing high enkephalin levels include the anterior hypothalamic nucleus, lateral preoptic area, nucleus of the solitary tract (medial and commissural parts), bed nucleus of stria terminalis, dorsomedial nucleus, parabrachial nuclei, periaqueductal gray and motor hypoglossal nucleus [563]. Both prodynorphin- and proenkephalin-derived peptides are minimally expressed in the frontal cortex [568].

Of interest to the current thesis, endogenous opioid peptides are present within the most vulnerable region to global ischemia, the hippocampus [313,316,326-329,331,563-568]. Dynorphin appears to be more concentrated in the mossy fibers projecting from the dentate granule cells to the apical dendrites of the CA3/CA4 pyramidal cells [80,217,327,328,331,369,528,540,541]. In contrast, enkephalin is predominantly localized on the

hippocampal perforant pathway [80,153,326-329], which fibers relay signals from the enthorinal cortex to the dentate gyrus.

3.2.3 Distribution of opioid receptors

The three opioid receptor subtypes are widely expressed and form a distinct pattern of distribution within the rat brain, sometimes overlapping with one another [311,312,314,315]. Thus, high level of μ binding is observed in the anterior cingulate cortex, neocortex, amygdala, hippocampus, ventral dentate gyrus, presubiculum, nucleus accumbens, caudate putamen, thalamus, habenula, interpeduncular nucleus, pars compacta of the substantia nigra, superior and inferior colliculi, and raphe nuclei. In contrast, δ binding is restricted to a few brain areas, including the anterior cingulate cortex, neocortex, amygdala, olfactory tubercle, nucleus accumbens, and caudate putamen, while κ binding is densely distributed in the amygdala, olfactory tubercle, nucleus accumbens, caudate putamen, medial preoptic area, hypothalamus, median eminence, periventricular thalamus, and interpeduncular nucleus [314].

There are a number of discrepancies between the distribution of receptor mRNA and binding sites, suggesting receptor transport [311]. Saggital brain sections showing the localization of opioid-receptor mRNAs and μ-, δ-, and κ-receptor binding sites in the CNS are presented in Figure 4. A high correlation between μ-receptor mRNA expression and binding is observed in the striatal patches of the nucleus accumbens and caudate-putamen, diagonal band of Broca, globus pallidus and ventral pallidum, bed nucleus stria terminalis, most thalamic nuclei, medial and cortical amygdala, mammillary nuclei, presubiculum, interpeduncular nucleus, median raphé, raphé magnus, parabrachial nucleus, locus coerulus, nucleus ambiguus and nucleus of the solitary tract. In contrast,

discrepancies between μ-receptor mRNA and binding are found in the neocortex, olfactory bulb, superior colliculus, spinal trigeminal nucleus and spinal cord.

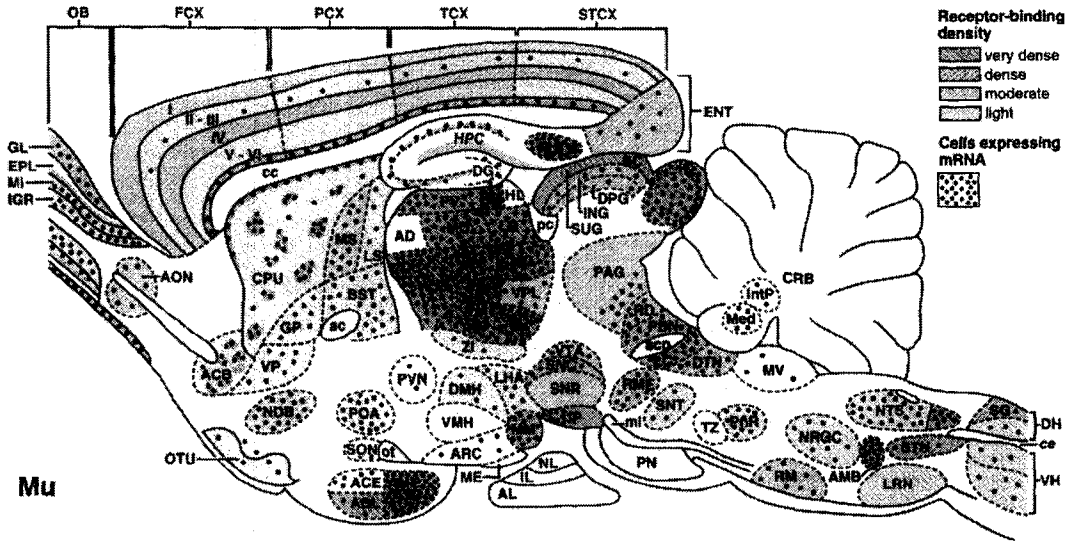
On the other hand, a high degree of correlation between δ-receptor mRNA expression and binding is observed in the anterior olfactory nucleus, neocortex, caudate-putamen, nucleus accumbens, olfactory tubercule, diagonal band of Broca, globus pallidus and ventral pallidum, septal nuclei, amygdala and pontine nuclei. Regions expressing high levels of δ-receptor mRNA and low receptor binding include the internal granular layer of the olfactory bulb and ventromedial nucleus of the hypothalamus. Discrepancies are also observed in several brainstem nuclei, the cerebellum, spinal cord, external plexiform layer of the olfactory bulb and the superficial layer of the superior colliculus.

Finally, a high degree of concordance between κ-receptor mRNA expression and binding is observed in the nucleus accumbens, caudate-putamen, olfactory tubercule, bed nucleus stria terminalis, medial preoptic area, paraventricular, supraoptic, dorsomedial and ventromedial hypothalamus, amygdala, midline thalamic nuclei, periaqueductal gray, raphé nuclei, parabrachial nucleus, locus coeruleus, spinal trigeminal nucleus, and the nucleus of the solitary tract, while differences are found in the substancia nigra, pars compacta, ventral tegmental area and the neural lobe of the pituitary.

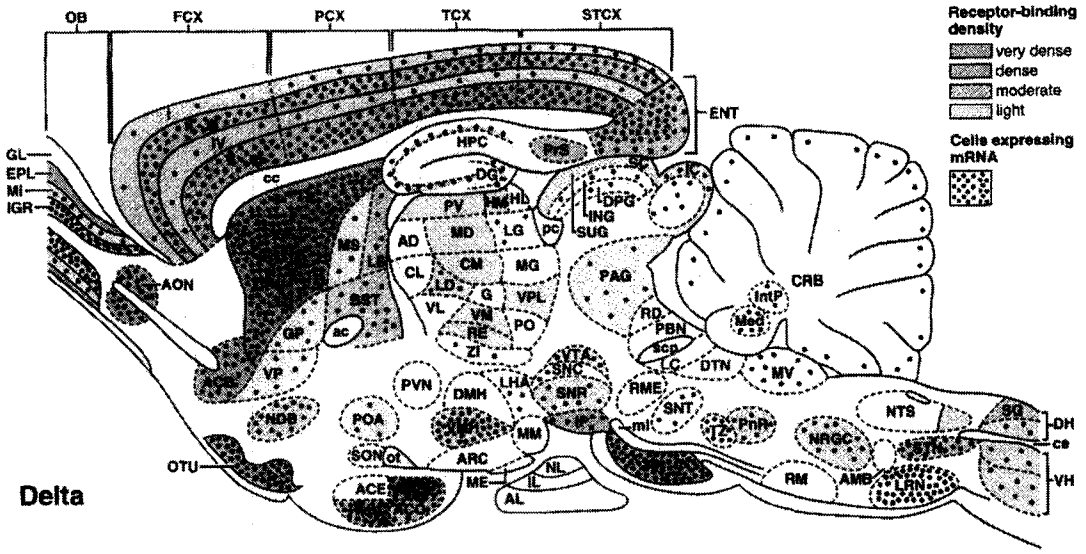
Several studies demonstrated the presence of opioid receptors in the rat hippocampus [310,312-316,330,331,515]. The presence of both the opioid peptides and receptors in the hippocampus suggest that opioidergic stimulation and/or blockade prior or following cerebral ischemia using distinct opioid agonists and antagonists could affect ischemic

neuronal damage, largely taking place at that brain locus, possibly via indirect actions on CA1 pyramidal neurons. Such possibility will be discussed in following sections.

a) Mu-receptor distribution



b) Delta-receptor distribution



c) Kappa-receptor distribution

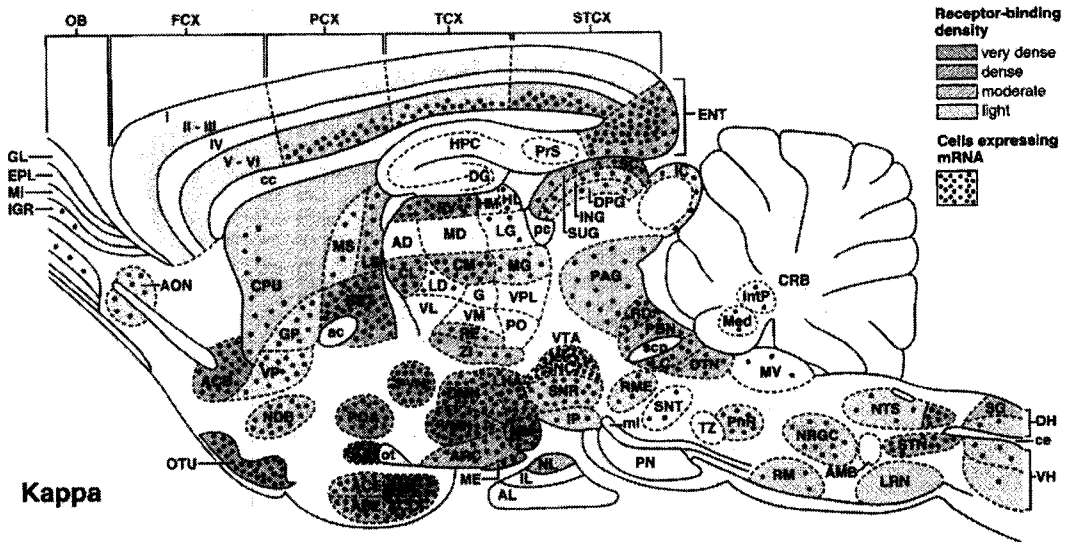


Figure 4 : Localization of opioid-receptor mRNAs and μ-, δ-, and κ-receptor binding sites in the CNS [Mansour et al. (1995). Opioid-receptor mRNA expression in the rat CNS: anatomical and functional implications. Trends Neurosci., 18(1), pp.24-25].

3.2.4 Opioid systems and cerebral ischemia

Multiple studies have demonstrated the participation of endogenous opioids in various physiological and pathological states, including stroke. Among the most attractive opioid targets are kappa- and delta-opioid receptors as they exert protective actions against ischemia-induced neuronal degeneration. The kappa-opioid receptor (KOR) agonists U50,488H, U62,066E and enadoline (CI-977) have all been shown to confer significant CA1 neuronal protection against forebrain ischemia in gerbils [190,512,552]. Many U50,488H analogs, including BD-449, BD-737 and BD-738, have also been shown to significantly protect against ischemia-induced neuronal damage in gerbils [105]. Similarly, the KOR agonists enadoline [201,264,299,300,427], BRL 52537 [178,573] and GR-89696 [22] reduce focal ischemic damage in rats or cats. Significant neuronal protection is reported when KOR agonists are administered between 4 and 6h post-ischemia [24,37,87], suggesting a prolonged post-ischemia treatment window with KOR agonists.

Separate studies have examined whether KOR agonists-induced cellular protection does translate into improved functional recovery. Handa et al. [192] reported significant improvement of motor deficits induced by focal ischemia in rats treated with dynorphin. Shortly following ischemia, U50,488H and dynorphin also prevent impairment of spontaneous alternation in the Y maze and inhibit the lengthening of transfer latency in the elevated-plus maze. U50,488H and dynorphin also decrease step-through latency in the passive avoidance test [235,236,512] and improve working memory performance in a three-panel runway task [373].

Although many researchers have investigated the impact of KOR agonists administration on ischemia-induced behavioral and memory deficits, one important limit

of existing studies is the short reperfusion intervals (8 days or less) at which the effect of kappa agonists were tested, precluding firm conclusions about the efficacy of such treatment to induce long-term post-ischemic neuronal protection and functional recovery.

In contrast to KOR, the role of delta-opioid receptors (DOR) in the cascade of events following cerebral ischemia remains largely unexplored. The cardioprotective properties of DOR are, however, well established. Thus, administration of DOR agonists have been shown to increase myocardial [48,439], and hypoxic [46] tolerance, a phenomenon reversed by pre-treatment with DOR antagonist [440]. A number of studies have also demonstrated the participation of DOR in the cellular protection conferred by cardiac ischemic preconditioning [375,457-459,542]. Similarly, morphine-induced cardioprotection has been shown to be mediated through DOR [375,391,459]. DOR stimulation also enhances cardiomyocyte protection in chicks [225,284,332,333] and rabbits [71,508]. Recently, Watson et al. [539] reported a significant reduction of infarct size in rats treated with ARD-353, a non-peptide DOR agonist, either before or after acute myocardial infarction. *In vitro*, stimulation of DOR protects cortical neurons against glutamate-induced injury [571]. Some evidence suggests that the cardioprotective effects induced by DOR is mediated through delta1- rather than delta2-opioid receptors [323,324,391,457,542].

Despite an acknowledged role of DOR in cardiac ischemia, the contribution of these opioid receptors to cerebral ischemia remains controversial. Borlongan et al. [51] demonstrated significant reduction of ischemia-induced striatal infarction in rats pre-treated with delta opioid (D-ala 2, D-Leu5) enkephalin (DADLE: 4mg/kg, 4 injections at 2h intervals), prior to middle cerebral artery occlusion. However, Iwata et al. [238] failed to observe inhibition of hippocampal damage in rats treated with DADLE (0.25, 1, 4, or 16

mg/kg) prior to a 10 min global ischemic insult. Recently, Zhao et al. [574] reported that opioid preconditioning with Tan-67 (3mg/kg), a selective delta1-opioid receptor agonist (injected 24h prior focal cerebral ischemia), reduced infarct size and improved neurologic function in rats. However, no improvement was found when administered 12 or 48h before middle cerebral arteries occlusion. Thus, while the cardioprotective effects of DOR are well established, the role of these opioid receptors in the modulation of global ischemic injury and functional recovery remains to be determined.

Controversial results are also found with mu-opioid receptors (MOR) both within cardiac and cerebral ischemic models. Thus, while some studies reported cardioprotection [342,456,537 467], others observed no difference [167,438] or detrimental effects [317] following MOR agonist administration. Recent evidence also suggests that MOR agonists exert their cardioprotective actions through the activation of delta-opioid receptors [245,375,391,459]. In the brain, one study reported increased ischemic injury following administration of the selective MOR agonist fentanyl [257], while others reported no deleterious effect [77,347,494]. In addition, some studies showed improvement of stroke outcome following administration of the non-selective MOR antagonist naloxone [143,219,579], while others have not [244,274]. Considering these divergent results, it is difficult to draw firm conclusions as to the role of MOR in modulation of ischemic processes.

3.2.5 Ischemia-induced changes in endogenous opioid peptides and their receptors

Studies showing endogenous changes of DOR and KOR and their respective endogenous ligands, enkephalin (ENK) and dynorphin (DYN) during and after ischemia represent additional evidence supporting a role for these peptides in cerebral ischemia. Thus, Friedman et al. [157] have demonstrated a rapid inhibition of ENK precursor

preproenkephalin mRNA levels in striatal neurons 1 and 3.5h following focal ischemia in rats. At short-term intervals (1-6h), significant reduction of DOR has also been reported in infarcted and penumbral cortices of mice, while more delayed (24h) inhibition of KOR was observed following transient [55] and permanent [53,54] focal cerebral ischemia. On the other hand, Benfenati et al. [32] found reduced striatal DOR 7 days following forebrain ischemia in rats. Consistent with a relationship between enkephalin depletion and ischemia-induced neuronal death, Popovici et al. [409] found that the downregulation of striatal preoproenkephalin preceded DNA fragmentation. Lasting reductions of hippocampal DYN levels have also been reported following global cerebral ischemia in gerbils [156].

The dynamic changes in KOR and DOR expression following ischemia suggest that alterations of these receptors and/or endogenous ligand concentrations could contribute to physiological processes involved in neuronal death or survival. Assuming that opioid peptides activation can be associated with a neuroprotective action and that endogenous peptides are depleted during and/or following ischemia, pre-treatment with opioid agonists could possibly counteract the deleterious impact of post-ischemic reductions of ENK and DYN secretion and/or DOR and KOR expression. Opioid agonists could represent an interesting therapeutic strategies for stroke. However, when considering this possibility, a caveat is that all kinds of receptors located on dying hippocampal neurons are probably decreased following ischemia but their preservation do not necessarily imply that they would play a role in neuroprotection.

3.3 Interaction between CRH and the opioidergic system

Interactions between CRH and opioidergic systems have been demonstrated in both central and peripheral nervous systems. In the brain, CRH stimulates the release of endorphin (END), DYN [7,366], and ENK [367] from the rat hypothalamus *in vitro.* Consistent with these findings, administration of the antagonist α-helical CRH (9-41) reduces the release of END, ENK and DYN from the hypothalamus both *in vivo* and *in vitro* [368]. CRH also produces the release of Met-ENK and DYN from the neostriatum *in vitro,* and the globus pallidus and caudate nucleus *in vivo,* in a dose-related and receptor-specific manner [481]. Conversely, opioid peptides can stimulate the synthesis and release of CRH. Thus, stimulation of KOR induces hypothalamic CRH release *in vitro* and *in vivo*, an effect reversed by the administration of KOR antagonist [70,365].

In the periphery, CRH-induced antinociception and inflammation is opioid-mediated [297,298,350]. In a rat model of localized hindpaw inflammation, CRH stimulates the release of END [68], as well as met-ENK and DYN A [69] from immune cells, an effect reversed by administration of α-helical CRH (9-41). Furthermore, CRH-induced antinociception is inhibited by local application of specific antibody to END and met-ENK [68,69,454]. Conversely, CRH-induced analgesia is reversed by administration of MOR and DOR antagonists [454].

Together, these results suggest close links between these peptidergic systems in the CNS. In light of their respective role in modulation of ischemia-induced cell death, possible interactions may exist between specific ligands and/or receptors of these peptide families, which may impact on neuronal or functional recovery following ischemia.

4. Thesis research objectives

The overall objective of the current thesis is to further characterize the contribution of CRH and opioid peptides to hippocampal degeneration and functional recovery following global cerebral ischemia. It also examines the possibility that CRH neuroprotective effects may partly be attributable to the release of opioid peptides and/or an indirect activation of opioid receptors in rats.

This current thesis is presented as a series of three articles, each of which addresses specific research objectives. Together, this thesis contains six main objectives:

1) Objective 1: Characterization of the role of corticotropin-releasing hormone on *in vivo* global ischemia-induced hippocampal neuronal death and behavioral activity of rats.

The first manuscript determines whether administration of CRH prior to global cerebral ischemia in rats alters CA1 neuronal degeneration and changes behavioral activity in the open field. It represents an initial step toward the characterization of the role CRH in neuronal protection *in vivo*. The impact of the selective blockade of CRH receptors was also investigated.

Objective 2: Characterization of the effect of post-ischemic administration of corticotropin-releasing hormone on CA1 neuronal death and memory impairments following *in vivo* global cerebral ischemia in rats.

Having shown in the previous experiment that CRH was neuroprotective when administered prior the ischemic insult, a second objective of the first manuscript was to

determine whether administration of CRH at specific post-ischemic intervals could induce hippocampal protection, further elucidating the possible time window of its action. Another goal of that study was to characterize the impact of a CRH post-treatment on behavioral activity and spatial memory in rats using the open field test and 8-arm radial maze, respectively.

Objective 3: Assessment of the neuroprotective properties of the selective kappa-opioid receptor agonist U50,488H on hippocampal neurodegeneration, behavioral activity and working memory deficits at long interval following global ischemia in rats.

Various studies have shown that the administration of kappa-opioid receptors can protect against ischemic damage in rodents. In manuscript 2, the impact of kappa-opioid receptor stimulation using a global ischemic model is determined. That study also characterized the long-term impact of such a treatment on behavioral activity and working memory deficits in rats, using the open field and the 8-arm radial maze, respectively. In light of interactions between opioid and CRH peptide systems, this study also represents an initial step to identify possible mechanisms by which CRH exerts its actions.

Objective 4: Impact of the selective delta1-opioid receptor agonist, DPDPE, on ischemia-induced hippocampal neuronal death, and behavioral profile in rats.

Numerous *in vivo* and *in vitro* evidences have supported a protective role of delta-opioid receptors against cell death following various ischemic cardiac insults. Although cardiac and cerebral ischemia share some endogenous mechanisms associated with enhanced cellular protection, no studies have assessed the participation of these receptors in cerebral ischemia. Thus, another goal of manuscript 2 was to determine whether delta1-opioid receptors may play a similar role in cerebral ischemia in rats and modulate functional recovery.

Objective 5: Assessment of kappa- and delta-opioid receptors as potential mediators of CRH-induced neuronal protection against global ischemia in rats.

Considering the protection observed with both peptidergic systems and the existence of many interactions both within the central and the peripheral nervous system, another goal of this thesis was to elucidate possible interactions between CRH and opioid peptides. We examined whether the selective blockade of kappa- and delta-opioid receptors prior CRH administration has an impact on CRH-induced neuronal protection against global ischemic damage in rats *in vivo*.

Objective 6: Impact of pre- and post-CRH administration in rat primary cortical neurons against chemical ischemia *in vitro*, and assessment of possible mediation of CRH-induced effects by opioid receptor activation.

Manuscript 3 evaluated the impact of pre- and post-CRH treatments against chemical ischemia in rat primary cortical neurons *in vitro*. The study determined whether the protection conferred by CRH was receptor-dependent, as well as opioid-mediated.

The current thesis is presented as a serie of three articles. The order in which each of the objectives are addressed is not necessarily in a sequential order of presentation. Thus, the first article covers the first, second and fifth objective. On the other hand, the second manuscript addresses the third and fourth objective, while the third article targets the sixth objective.

5. Scientific contribution

The experiments reported in the present thesis demonstrate for the first time the role of CRH and its interaction with opioid peptides in the modulation of endogenous processes associated with an ischemic insult. They demonstrate that CRH confers protection against ischemia-induced CA1 neuronal degeneration and functional sparing when administered both prior and following vessel occlusion. This thesis elucidates potential mechanisms mediating this effect, i.e., through the release of opioid peptides and/or activation of opioid receptors.

MANUSCRIPT 1

Pre- and post-ischemic administration of Corticotropin-releasing hormone alters CA1 neuronal degeneration and functional recovery following *in vivo* global cerebral ischemia in rats

Abstract

The contribution of Corticotropin-releasing hormone (CRH) in the modulation of ischemia-induced cell death remains unclear. We characterized the impact of pre-ischemic intracerebroventricular (i.c.v.) injections of CRH administration (0, 0.1, 1, 5μg, 15 min prior to vessel occlusion) on neuronal damage following global ischemia in rats. The injection of 5μg of CRH led to a 37% increase in CA1 neuron survival compared to vehicle-treated ischemic animals and significantly reversed the ischemia-induced increase of rearing behavior made in the center of the open field. Pre-treatment with α-helical CRH (9-41) abolished this neuronal protection. We also assessed the participation of discrete opioid receptors to CRH-induced neuronal protection. Our findings showed that the selective blockade of kappa- and delta-opioid receptors (using nor-binaltorphimine and naltrindole, respectively) prior to CRH administration significantly reduced CA1 neuronal protection. A second goal of this study was to determine whether CRH (5μg; i.c.v.) conferred protection when administered 3 or 8h post-ischemia. Post-treatment with CRH 8h following global cerebral ischemia led to a 61% increase in CA1 neuron survival compared to vehicle-treated ischemic animals. Neuronal protection translated into significant improvement of ischemia-induced spatial memory deficits assessed in the 8-arms radial maze although no difference in behavioral activity in the open field was observed between groups. Together, these findings represent the first demonstration of enhanced neuronal survival following *in vivo* CRH administration in a global model of ischemia in rats. They suggest the possibility that CRH-induced neuroprotection involves opioid receptor activation.

Keywords: Corticotropin-releasing hormone, CRH receptors, α-helical CRH (9-41), kappa-opioid receptors, nor-binaltorphimine, delta-opioid receptors, naltrindole, neuronal protection, hippocampus, CA1 pyramidal neurons, behavioral activity, working memory, rats.

1. Introduction

The 41 amino acid polypeptide Corticotropin-releasing hormone (CRH) is widely distributed within the central nervous system [115]. Its role as a primary mediator of the stress response is well characterized and linked to direct activation of the hypothalamic-pituitary-adrenal (HPA) axis, and its ability to produce behavioral, autonomic and endocrine responses analogous to natural stressors upon central administration [381].

Recently, studies have suggested the possible involvement of CRH in the cascade of physiological events mediating ischemia-induced neuronal degeneration. A neurotoxic role for CRH has initially been inferred from studies that demonstrated neuroprotection in rats treated with the non-selective antagonist α-helical CRH (9-41) prior to global or focal ischemic insults [295,497]. Since then, numerous *in vitro* findings suggested an opposite conclusion, namely, that CRH administration confers neuroprotective effects in various oxidative and excitotoxic lesion paradigms. For example, Elliott-Hunt et al [138] recently demonstrated significant *in vitro* protection of hippocampal CA1 neurons against glutamate-induced neurotoxicity following CRH administration. Pedersen et al. [393] also reported a significant decrease of amyloid-beta toxicity in cortical and hippocampal neurons following CRH administration. In that experiment, they also showed that CRH could inhibit glutamate- or oxidative stress-induced neurotoxicity, effects blocked by pre-treatment with a CRH antagonist, suggesting receptor-mediated actions. *In vivo*, Craighead et al. [110] reported that CRH infusion into either the hypothalamus or amygdala failed to cause cell death or modify the damage caused by striatal injections of the excitotoxin S-AMPA. In a recent study, we failed to demonstrate reduced ischemia-induced CA1 neuronal damage and alterations of spatial memory impairments in rats

treated either with α-helical CRH (9-41) or the selective non-peptide $CRHR_1$ antagonist CP154,526 [407]. To date, there is no direct *in vivo* assessment of CRH effects on ischemia-induced neuronal degeneration. The present experiment thus examined the impact of CRH pre-treatment on neuronal damage following global ischemia in rats *in vivo*, and determined whether the neuroprotection translated into improved behavioral performance. The possibility that theses effects were receptor-dependent was also investigated, using α-helical CRH (9-41).

The second experiment is based on the observation that opioid peptides have been shown to regulate the stress response (for a review, see [135]) and that different interactions between the CRH and opioidergic systems have been found in the peripheral and central nervous system (CNS). Among others, CRH-induced antinociceptive effect has been shown to be opioid-mediated [68,69,454,455]. In the rat brain, CRH is co-localized with dynorphin (DYN) and enkephalin (ENK) in various brain areas [215 74,414,444,450,517]. The synthesis and release of CRH is also modulated by opioid peptides [52,70,505]. Finally, several studies have demonstrated enhanced cellular survival in animal models of cardiac and cerebral ischemia associated with stimulation of kappa (KOR) and delta (DOR) opioid receptors [190,264,375,459]. We therefore examined whether the neuroprotection granted by CRH following ischemia was mediated through an interaction with opioid peptides. More specifically, we determined whether the selective blockade of KOR or DOR (using nor-binaltorphimine (BIN) and naltrindole (NAL), respectively) prior to CRH administration had an impact on ischemia-induced neuronal degeneration and behavioral deficits.

Although the first goal of this study was to provide important clarifications as for the role played by CRH in the modulation of ischemia-induced neuronal degeneration, the clinical impact of this finding remains limited since the peptide was administered prior to the ischemic insult. Thus, in the second part of this report, we also examined the impact of CRH administration at distinct post-ischemic time intervals (3h and 8h) on CA1 neuronal degeneration, and determined whether the neuroprotection translated into behavioral changes.

2. Materials and methods

2.1 Animals

Male Wistar rats weighing between 225-250g at time of arrival in our facility were obtained from Charles River Laboratories (Rochefort, Québec, Canada). They were individually housed and maintained on a 12 h light/dark cycle (lights on at 7:00 AM) with free access to water and standard rat chow. The room temperature was maintained at 21-23°C with 60% relative humidity. Upon arrival, animals were acclimatized 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 were approved by the University of Ottawa Animal Care Committee.

2.2 Surgical Procedures

Intracerebral cannula

Rats were implanted with a cannula aimed at the third ventricle for subsequent intracerebroventricular administration of CRH. Animals were placed in a stereotaxic

frame with a horizontal skull position. A guide cannula (22 gauge) was 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 and Watson [390]. The guide cannula was anchored to the skull using 4 stainless steel screws and acrylic dental cement. After one week of recovery, rats underwent 4-vessel occlusion, as described below.

Global ischemia

Rats underwent surgery for forebrain ischemia using the four-vessel occlusion procedure (4-VO), as described by Pulsinelli and Brierley [420]. Rats were deeply anesthetized by inhalation of 2% halothane in oxygen. Core temperature was controlled throughout the surgery using a temperature-regulated heating blanket connected to a rectal thermometer (Homeothermic Blanket Control Unit, Harvard Instruments, Natick, MA), and rats kept warm using a heating pad during vessel occlusion and for 12-16h following surgery and reperfusion. The vertebral arteries were irreversibly occluded by electrocoagulation, and a small-diameter silk thread was placed 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. 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, the control animals' carotid arteries were exposed but not clamped.

2.3 Drug treatments

CRH pre-treatment

Corticotropin-releasing hormone (purchased from American Peptide Company) was diluted in a vehicle consisting of 0.9% saline solution immediately before use. The 3rd ventricular microinjections were made through a stainless steel injection cannula that extended 0.5 mm below the tip of the guide cannula and into the third ventricle. The injection cannula was connected by a polyethylene tubing to a Harvard infusion pump. The 5μl of peptide solution or saline were infused at a constant rate over a 1 min period. The cannula was left in place an additional 30 sec to minimize back flow along the guide cannula. To determine the neuroprotective action of pre-ischemic CRH, four separate groups of rats received either an i.c.v. injection of CRH (0.1, 1 or 5μg: n=7, 9 and 8, respectively) or saline (n=10) 15 min before ischemia. Two groups of sham-operated rats injected i.c.v. with either saline or CRH (5μg) were used as controls for the non-specific effects of CRH (n=8 in each group). To determine whether the CRH-induced hippocampal protection was receptor-dependent, a separate group of ischemic rats (n=8) received an i.c.v. injection of 25μg of the antagonist α-helical CRH (9-41) (Phoenix pharmaceuticals) 15 min prior the i.c.v. administration of CRH (5μg). The antagonist was administered 30 min prior vessel occlusion. The CRH doses were selected on the basis of their ability to produce behavioral, neurochemical and/or immunological responses [129,203,491,492], as no studies have tested CRH neuroprotective effects *in vivo*. The α-helical CRH (9-41) dose was selected on the basis of its efficacy to block brain CRH receptors without exerting agonistic effects [337,433].

Blockade of delta- and kappa-opioid receptors

Naltrindole (NAL) and nor-binaltorphimine (BIN) (Sigma-Aldrich) were dissolved in 0.9% saline immediately before administration. Both were injected into the third ventricle in a volume of 5μl. To determine whether the CRH-induced hippocampal protection was mediated via release of opioid peptides, DOR and KOR were blocked using NAL (20μg, n=8) or BIN (30μg, n=10), respectively in two separate groups of ischemic rats, 15 min prior to CRH administration (5μg; i.c.v. injected 15 min prior vessel occlusion). Two groups of ischemic rats were injected i.c.v. with either antagonist alone [NAL (20μg, n=10) or BIN (30μg, n=9)] fifteen minutes prior to vessel occlusion and acted as control to determine intrinsic effects of these antagonists on neuronal density. The NAL and BIN doses were selected based on their ability to block cocaine-induced sensitization to seizures and death [57] or reverse KOR-induced analgesia [144], respectively.

CRH post-treatment

To test the effect of CRH post-treatment, 5μg of CRH was injected i.c.v. either 3h (n=8) or 8h (n=9) following vessel occlusion. The 8h time interval chosen for CRH administration was based on Elliott-Hunt et al. [138] results showing optimal protection at this time interval against glutamate-induced neuronal death in hippocampal cultures *in vitro*. On the other hand, the 3h time interval was selected based on the contention that most pharmacological therapies used in experimental models of stroke have been shown effective when administered within 3h of symptom onset. Control groups included one group of ischemic rats that was injected i.c.v. with saline (n=8) and two groups of sham animals that were treated either with saline (n=8) or CRH (5μg; n=7) 3h post-ischemia.

2.4. Apparatus and procedures for behavioral testing

Open Field

Five days following reperfusion, rats were tested in an open field and their behavior was monitored for 5 min using a computer and data logging software. The observation arena was made of gray Plexiglas (LWH: 75 X 75 X 30 cm), with a gray opaque floor. The floor was painted with a grid of 25 10X10 cm squares. The entire arena was kept on a table 90cm above the floor. Rats were brought in the testing room at least 30 min prior testing to minimize the impact of anxiety and/or stress on behavioral performance. Black curtains surrounded the open field, and behavior was monitored with an overhead camera. Frequency of line-crossing and rearing in the center and the periphery of the open field was counted, and the time during which animals groomed or were inactive was recorded. Behaviors displayed within the first row of squares adjacent to the walls of the apparatus were recorded as activity in the periphery of the open field.

8-arm radial maze

Two days following the open field test, animals that received CRH post-treatment were also tested in the radial maze. The maze consisted of eight arms (60 X 12 cm, with a 5 cm high lip around each arm) extending from a central octagonal area (32 cm in diameter with a 30-cm high clear Plexiglas wall). Plexiglas sliding doors permitted entry into each arm. The floor of the arms and central area was 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 sidewalls. At the end of each of the maze's arm was a food cup (recessed 1 cm into the floor) that held a piece of Fruit Loop

cereal. The animals only received 4-5 rat chow pellets/day to motivate them to eat the food reward.

During habituation, rats were placed in the radial arm maze for 3 daily 10-min exploration periods. Baits were initially available throughout the maze to encourage exploration, but was gradually restricted to the food cup. Following this habituation period, each animal was placed individually in the center of the maze with doors to all arms closed. Upon the opening of all doors, the rat could enter any of the eight arms. When the rat had consumed the reward 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. Doors were then reopened, and the procedure repeated. A trial ended when all baits were consumed or when 15 min had elapsed. An arm entry was recorded when all four limbs were inside one of the maze arm. Number of working memory errors (visiting an arm that was previously entered) and the time taken to consume all baits were recorded. Before the doors were opened for each trial, the experimenter ensured that the orientation of the animal's head was different from one trial to the other in order to minimize the development of a positional habit. The maze floor was cleaned after each trial in order to minimize olfactory cues. Animals were tested every day for 12 days.

2.5 Analysis of neuronal density on thionin-stained sections

Upon completion of behavioral testing, and 30 (CRH post-treatment) or 7 days (CRH pre-treatment and blockade of DOR or KOR) following reperfusion, rats were euthanized and their brain removed, frozen on dry ice and stored at -80°C. Serial coronal frozen sections (14 μm) of the hippocampal region were obtained and stained with

thionin. Neuronal density of the hippocampal CA1 subfield was determined using the method of Kirino et al.[252]. Analysis of neuronal density was performed on coronal sections located between 3.14 and 4.16 mm posterior to bregma [390]. Total linear length of the CA1 was measured by means of a digitizer. Neurons in the stratum pyramidale within the CA1 subfield were observed using a LEICA DAS microscope attached to a SONY digital camera and their number was estimated using a computer-assisted cell counting procedure (Norton Eclipse software, v 6.0). Neurons with shrunken cell bodies or surrounding empty space were excluded. Neuronal density of the CA1 region was quantified by taking the number of visually intact pyramidal cells per 1 mm linear length of the CA1 stratum pyramidale. The neuronal density was estimated by the average number of neurons counted from six sections on the right and left hippocampus.

3. Results

3.1 Effect of CRH pre-treatment and of the selective blockade of CRH receptors on ischemia-induced behavioral deficits and hippocampal degeneration.

3.1.1 CA1 neuronal density

All analyses were conducted using SPSS (V 15.0). The alpha level was set at 0.05 for all omnibus tests. Figure 1 shows the effect of a 10 min global ischemia with CRH pre-treatment on hippocampal CA1 neuronal density. Results are expressed as means ± SEM. A two-way randomized group ANOVA was performed on the data from vehicle- and 5μg CRH-treated animals to determine the effect of surgery (ischemic vs. sham) and drug (0 vs. 5μg of CRH). Statistical analysis revealed a significant effect of both factors (surgery, $F_{(1,30)}=89.362$, $p<.001$; drug, $F_{(1,30)}=10.334$, $p=.003$) and their interaction

($F_{(1,30)}$=4.367, p=.045) on cell density. Bonferroni-adjusted pair-wise comparisons indicated significantly lower CA1 neuronal density in ischemic rats compared to sham animals (57 % reduction; $p < .001$). In addition, there were more CA1 neurons in ischemic animals pre-treated with the highest dose of CRH (5μg: $p < .001$; ≈ 37% more neurons compared to vehicle-treated ischemic animals in the CA1). A one-way ANOVA performed on all ischemic groups revealed no overall difference. Thus, the two lower doses of CRH (0.1 and 1μg) did not modulate the impact of ischemia on CA1 neuronal density. Although not significant, the data suggest that pre-treatment with the 0.1μg dose also conferred a modest hippocampal protection (see Figure 1). These findings provide evidence that CRH alters hippocampal degeneration in a global model of cerebral ischemia.

The effect of the selective blockade of CRH receptors with the antagonist α-helical CRH (9-41) prior to CRH (5μg) administration on hippocampal CA1 neuronal density is shown in Figure 1. A planned comparison revealed significantly decreased neuronal density (≈ 80% CA1 neuronal loss) in ischemic rats treated with α-helical CRH (9-41) prior to a 5μg CRH injection, compared to 5μg-CRH treated ischemic rats ($t_{(14)}$=7.229, $p<.001$). Thus, the blockade of CRH receptors before CRH administration abolished the beneficial actions of CRH on hippocampal pyramidal neurons, suggesting a receptor-dependent effect.

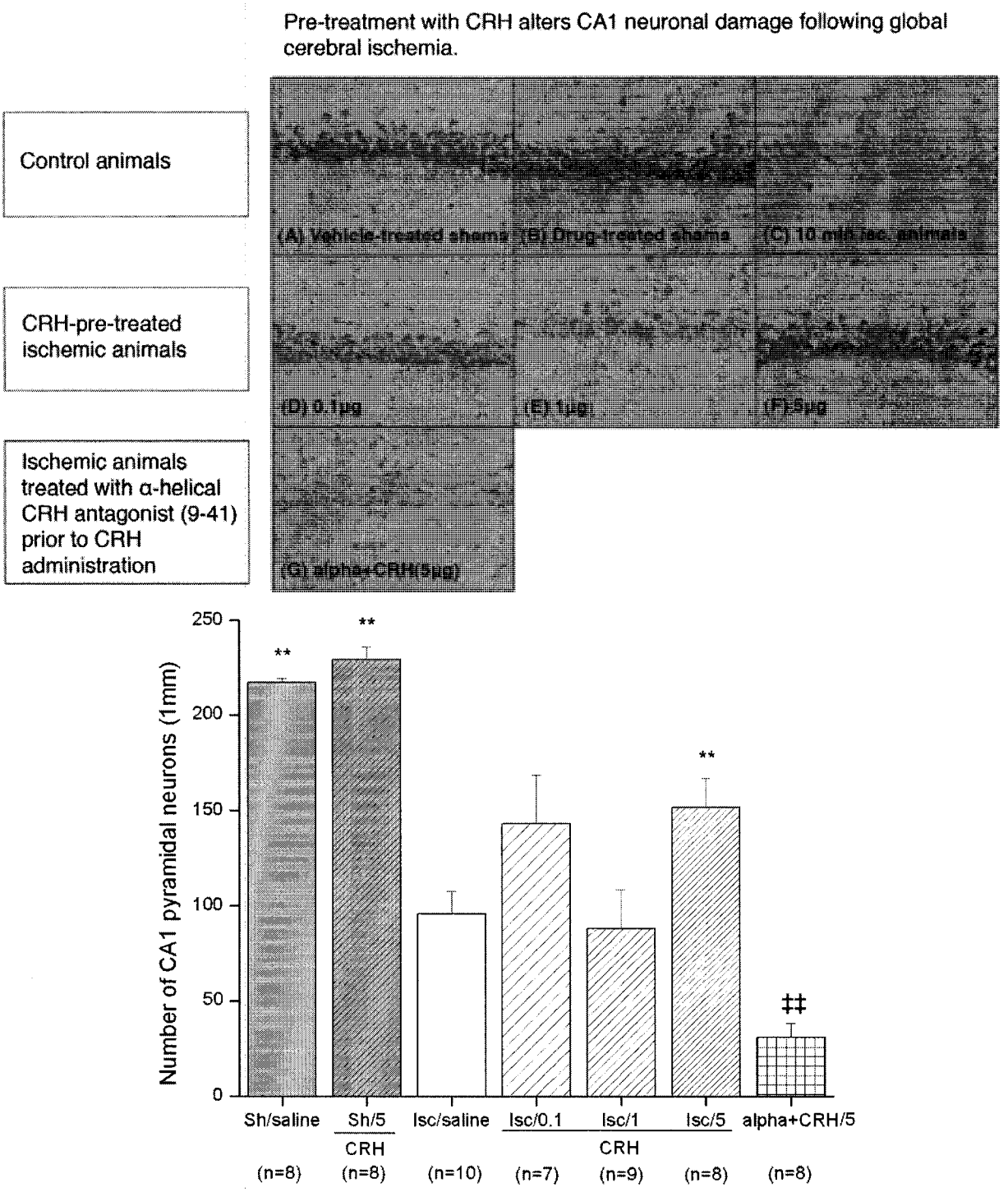


Figure 1: Representative photomicrographs (Thionin stain, Top panel) and histogram (bottom panel) representing the mean number of CA1 pyramidal neurons for the different experimental groups. Pre-treatment with CRH(5µg) induced ≈ 37% increase in CA1 neuron survival in ischemic rats, an effect reversed in rats pre-treated with α-helical CRH(9-41). **, ‡‡, Indicates significantly different from vehicle- and CRH(5µg)-treated ischemic animals, respectively ($p < .001$).

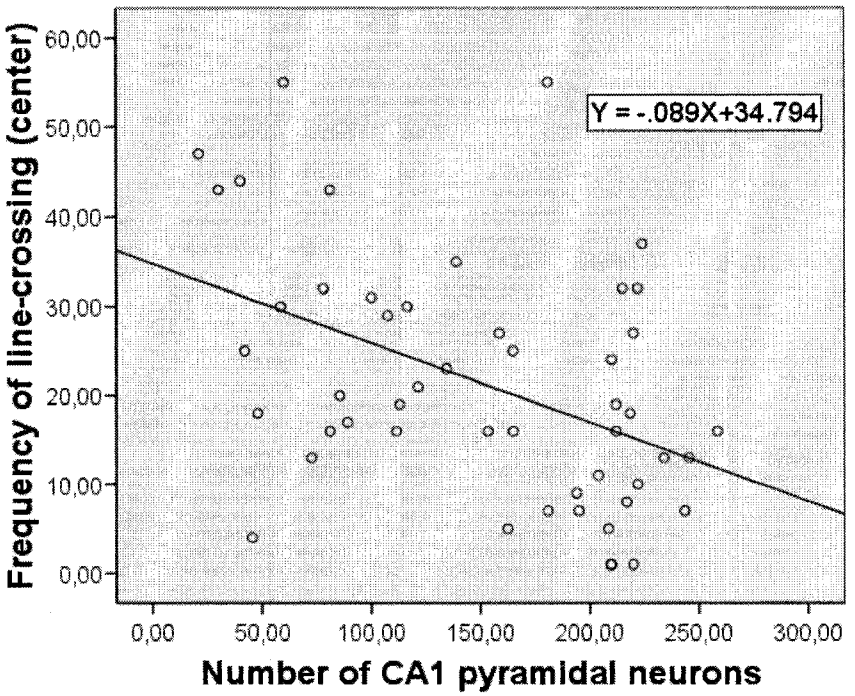
3.1.2 Open Field

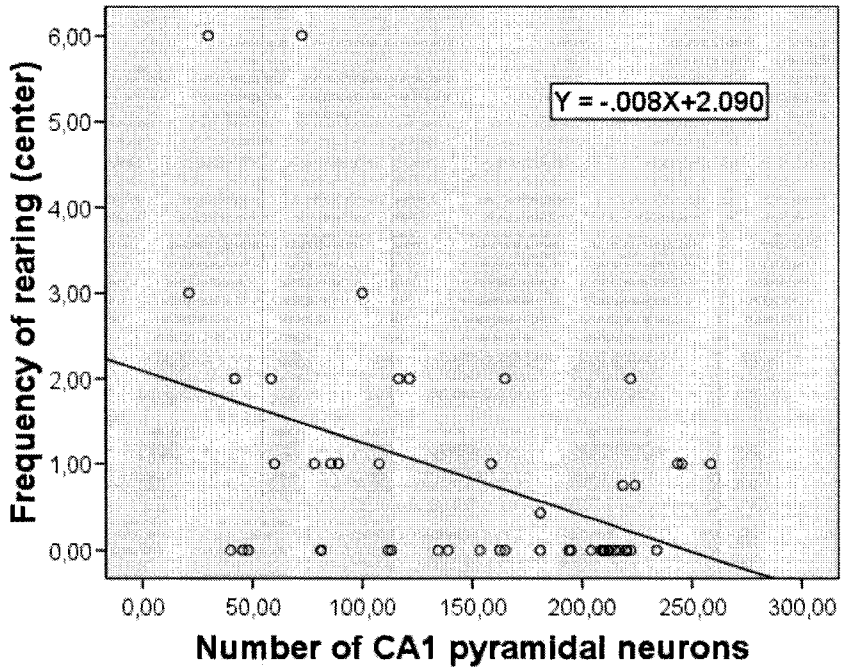
Correlational analysis revealed a negative relationship between neuronal density and the frequency of line-crossing ($F_{(1,48)}$=12.273, r = -.451, p<.001) and rearing ($F_{(1,48)}$=10.986, r = -.432, p=.002) occurring in the center of the open field (Figure 2a), while no correlation was found with neuronal density for both behaviors performed in the periphery of the apparatus. Figure 2b shows the impact of CRH pre-treatment on the behavioral activity profile of rats in the open field for the 5 min monitoring period. The time spent either grooming or inactive is shown in Table 1. A two-way ANOVA performed on the data from vehicle- and 5μg CRH-treated animals revealed a significant effect of surgery for the number of line-crossing ($F_{(1,30)}$=5.033, p=.032) and rearing ($F_{(1,30)}$=5.313, p=.028) in the center of the apparatus. Bonferroni-adjusted pair-wise comparisons indicated that this difference was attributable to increase in these behaviors in ischemic animals compared to shams (p<.05). A significant effect of drug ($F_{(1,30)}$=6.228, p=.018) and surgery X drug interaction ($F_{(1,30)}$=14.661, p=.001) was also found for the frequency of center rearing. Bonferroni-adjusted pair-wise comparisons revealed that vehicle-treated ischemic rats reared more often in the center of the open field compared to ischemic animals pre-treated with 5μg of CRH and shams (p < .05). A one-way ANOVA performed on all ischemic groups also produced a significant group difference with respect to center rearing ($F_{(3,30)}$=4.455, p=.011) and time grooming ($F_{(3,30)}$=3.732, p=.022). Bonferroni-adjusted post-hoc analysis revealed that 0.1μg CRH-treated ischemic rats reared in the center of the apparatus less often compared to vehicle-treated ischemic rats (p=.036). Thus, treatment with either 0.1 or 5μg of CRH reversed the ischemia-induced increase in center rearing behavior. On the other hand, rats pre-treated

with 1μg of CRH spent significantly more time grooming than ischemic animals pre-treated with 0.1 or 5μg of CRH ($p < .05$).

The impact of CRH receptor blockade on behavioral activity in the open field is shown in Figure 2b. Statistical analysis revealed that that ischemic animals pre-treated with α-helical CRH (9-41) prior to CRH administration reared in the center of the apparatus more often than ischemic rats pre-treated with CRH ($t_{(14)}=2.255$, $p=.041$).

a)


Frequency of line-crossing (center)
60,00
50,00
40,00
30,00
20,00
10,00
0,00
Y = -.089X+34.794
0,00
50,00
100,00
150,00
200,00
250,00
300,00
Number of CA1 pyramidal neurons


Frequency of rearing (center)
6,00
5,00
4,00
3,00
2,00
1,00
0,00
Y = -.008X+2.090
0,00
50,00
100,00
150,00
200,00
250,00
300,00
Number of CA1 pyramidal neurons

b)

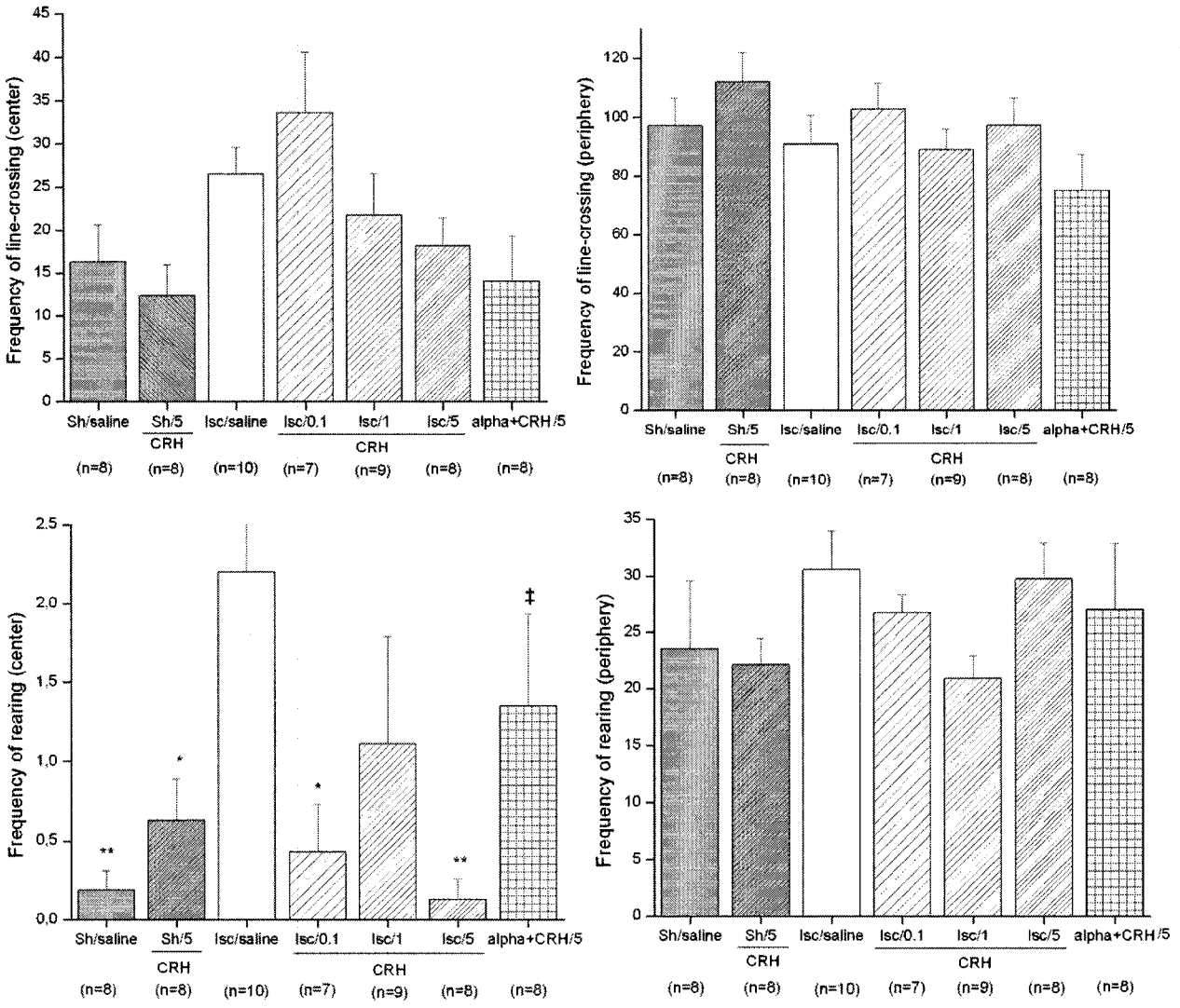


Figure 2: (a) Relationship between neuronal density and behavior performed in the center of the open field. The degree of CA1 neuronal damage is negatively correlated with behavior performed in the center but not the periphery of the apparatus. (b) Activity profile of rats in the open field over the 5 min monitoring period. *,**; ‡, significantly different from vehicle- and CRH(5μg)-treated ischemic animals, respectively ($p < .05$ and $p < .001$).

Table 1
Effect of the various drug treatments on time spent either grooming or inactive.

Behavior	Grooming	Inactivity
Treatment		
CRH pre-treatment		
Sham/saline	34.46±11.05	5.48±4.33
Sham/5	33.16±6.51	0.49±0.32
Isc/sal	27.20±7.36	3.30±2.84
Isc/0.1	16.30±5.55‡	5.16±3.38
Isc/1	47.44±9.35	4.19±2.20
Isc/5	16.43±6.13‡	1.25±1.25
alpha+CRH/5	20.53±8.19	5.76±4.00
Blockade of DOR and KOR		
NAL	16.20±4.69	0.00±0.00
BIN	22.56±7.03	4.91±2.95
NAL+CRH	12.54±5.37	0.85±0.57
BIN+CRH	9.96±3.03	8.25±6.03
CRH post-treatment		
Sham/saline	16.56±4.75	0.00±0.00
Sham/CRH	17.01±3.44	0.70±0.70
Isc/saline	14.31±3.55	0.00±0.00
Isc/CRH(3H)	17.06±3.09	5.50±4.71
Isc/CRH(8H)	18.83±4.65	12.24±9.60

Each cell represents the mean ± S.E.M. of time (sec) spent either grooming or inactive at the end of the 5 min monitoring period.
‡, significantly different from Isc/1 ($p<0.05$).

3.2 Impact of the selective blockade of DOR and KOR prior CRH (5μg) administration on ischemia-induced hippocampal degeneration and behavioral deficits.

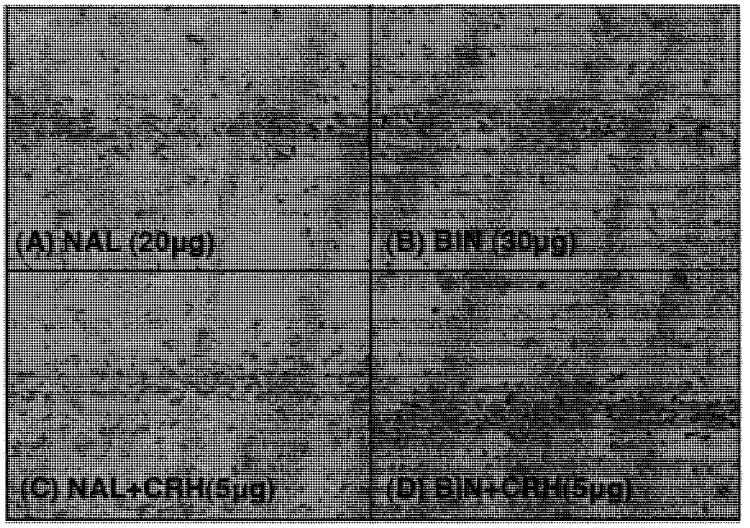
3.2.1 CA1 neuronal density

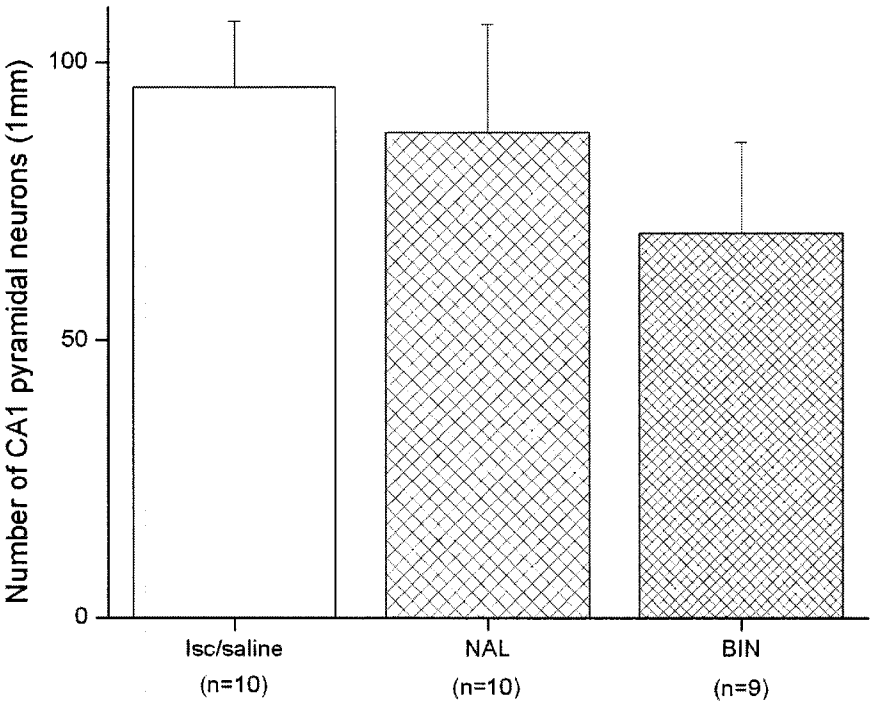
Figure 3 shows the effect of the selective blockade of DOR and KOR prior CRH (5μg) administration on hippocampal CA1 neuronal density. In this figure, as well as in the following one (figure 4), the Isc/saline and Isc/5 groups of animals are the same ones used for the preceding experiments and are solely presented to allow a direct visual comparison. To determine whether BIN or NAL had an intrinsic effect on hippocampal degeneration, we injected i.c.v. two distinct groups of ischemic animals with these antagonists. Planned comparisons performed between ischemic rats treated with NAL or BIN alone and vehicle-treated ischemic animals revealed no difference between groups in the mean number of cells ($t_{(18)}$=.360, p=.722 and $t_{(17)}$=1.324, p=.203, respectively). In addition, statistical analysis conducted between CRH-treated ischemic rats and ischemic animals pre-treated with NAL or BIN prior to CRH administration revealed a significant effect of group for the mean number of cells ($t_{(14)}$=2.369, p=.033 and $t_{(16)}$=2.386, p=.030, respectively). Thus, ischemic rats pre-treated with NAL or BIN before central administration of CRH showed decreased neuronal density compared to CRH(5μg)-treated ischemic rats (≈ 49% and 38% CA1 neuronal loss, respectively). These findings suggest that the CRH-induced neuroprotection against ischemic damage in a rat model of global cerebral ischemia can be modulated by opioid activity.

Blockade of delta and kappa opioid receptors prevents CRH-induced neuronal protection.

Ischemic animals treated with antagonists alone

Ischemic animals treated with antagonists prior to CRH administration





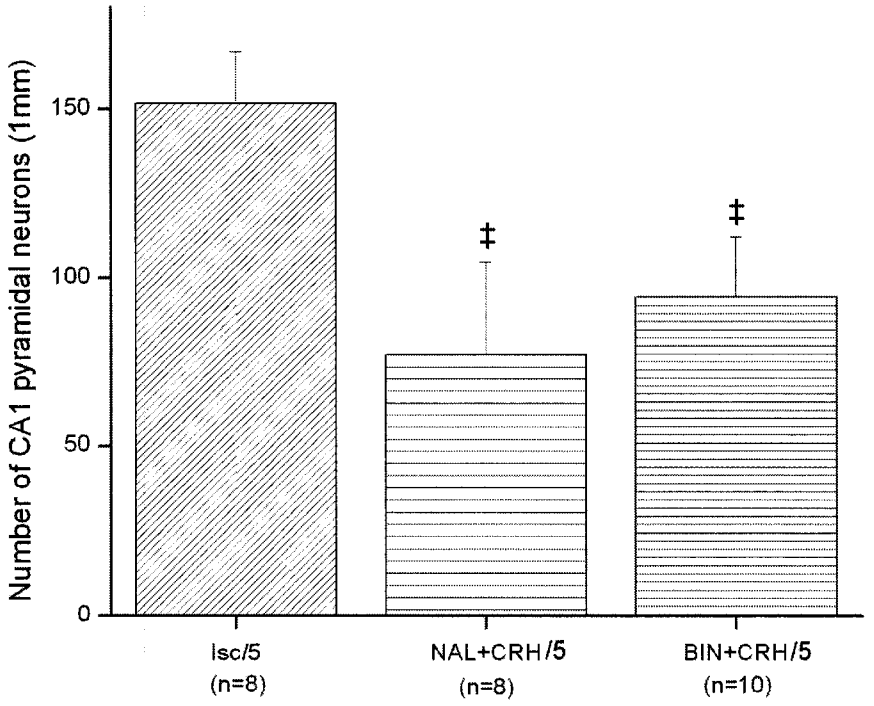


Figure 3: Representative photomicrographs (Thionin stain, Top panel) and histogram (bottom panel) representing the mean number of CA1 pyramidal neurons for the different experimental groups. Blockade of either KOR or DOR reversed CRH-induced neuronal protection. ‡, significantly different from CRH(5μg)-treated ischemic animals ($p < .05$).

3.2.2 Open Field

Figure 4 shows effects of the selective blockade of DOR and KOR prior to CRH administration on behavioral activity in the open field. The time spent either grooming or inactive is presented in Table 1. Ischemic rats pre-treated with NAL and BIN alone made significantly fewer line-crossing ($t_{(18)}$=3.045, p=.007 and $t_{(17)}$=4.019, p<.001, respectively) and rearing ($t_{(18)}$=2.381, p=.028 and $t_{(17)}$=3.688, p=.002, respectively) in the center of the open field compared to vehicle-treated ischemic rats. Thus, administration of the antagonists alone reversed the ischemia-induced increased level of behavioral activity in the center of the open field. In the first part of this study, we showed that pre-treatment with CRH reversed the ischemia-induced elevated number of rearing made in the center of the open field. Treatment with NAL or BIN prior to CRH administration did not reverse this effect; all groups displayed a similar level of rearing behavior in the center of the apparatus. However, compared to CRH-treated ischemic animals, ischemic rats pre-treated with NAL prior to CRH made significantly more line-crossing ($t_{(14)}$=3.284, p=.005) and rearing ($t_{(14)}$=2.119, p=.05) in the periphery of the open field. No other significant group difference was found.

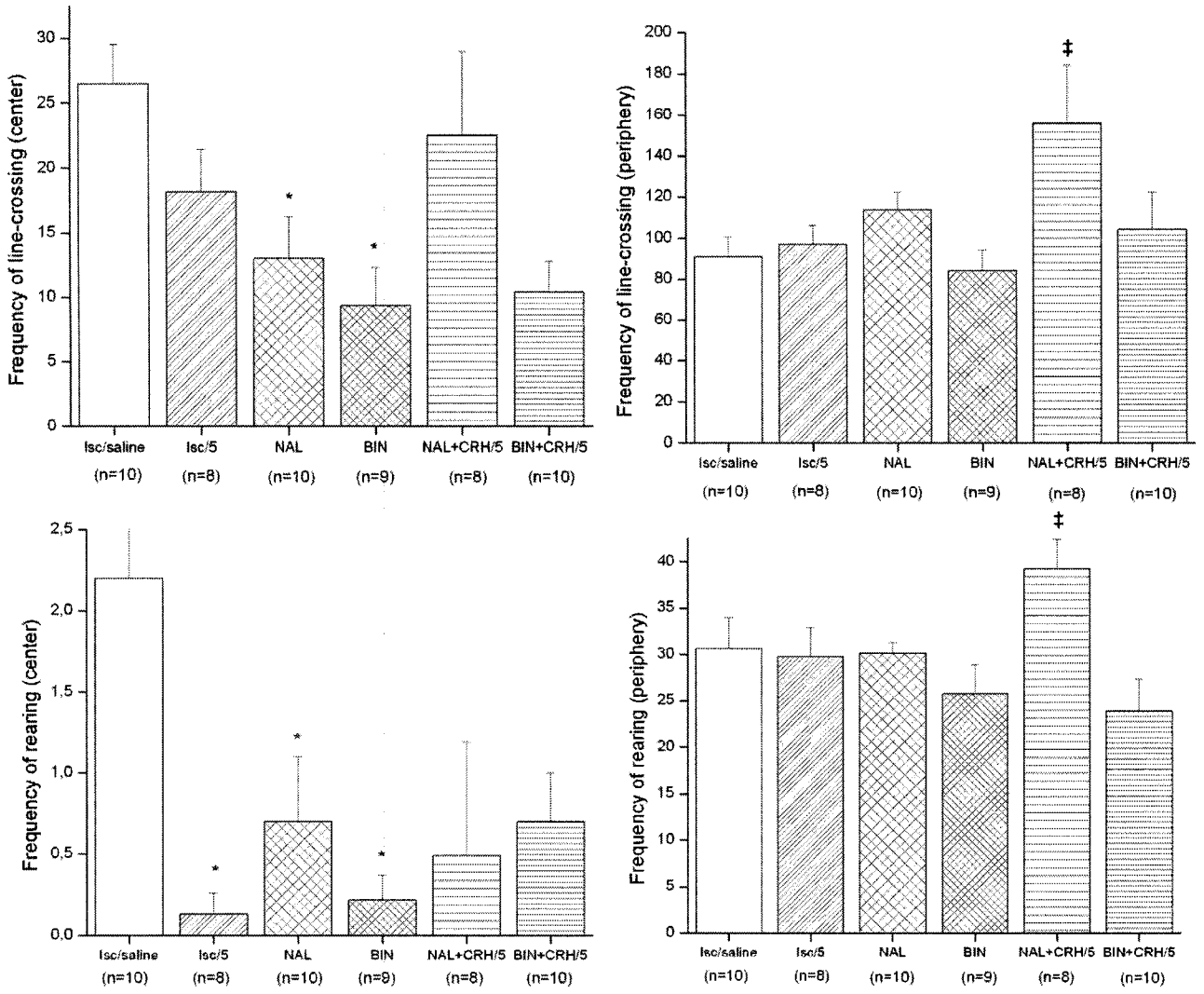


Figure 4: Activity profile of rats in the open field over the 5 min monitoring period. *, ‡, significantly different from vehicle- and CRH(5μg)-treated ischemic animals, respectively ($p < .05$).

3.3 Effect of CRH post-treatment on hippocampal degeneration and ischemia-induced memory and behavioral deficits.

3.3.1 CA1 neuronal density

Figure 5 shows the impact of post-treatment with 5μg CRH on hippocampal CA1 neuronal damage induced by a 10 min global ischemia. A two-way ANOVA performed on animals treated 3h post-ischemia revealed a significant effect of surgery ($F_{(1,27)}$=62.816, $p<.001$). Bonferroni-adjusted pair-wise comparisons revealed that this effect was due to significantly lower cell densities in ischemic animals as compared to sham rats (≈ 74.5% CA1 neuronal loss). No significant effect of drug or surgery X drug was found. A one-way ANOVA performed on all ischemic animals revealed a significant effect of group ($F_{(2,24)}$=24.915, $p<.001$). Bonferroni-adjusted post-hoc comparisons revealed a higher number of CA1 pyramidal cells in ischemic animals treated with CRH 8h following vessel occlusion compared to vehicle-treated ischemic rats (≈ 61% increase in neuron survival). Thus, treatment with CRH (5μg) 8h post-ischemia significantly enhanced neuronal survival in the CA1 subfield of the hippocampus, whereas it had no impact when administered 3h following vessel occlusion.

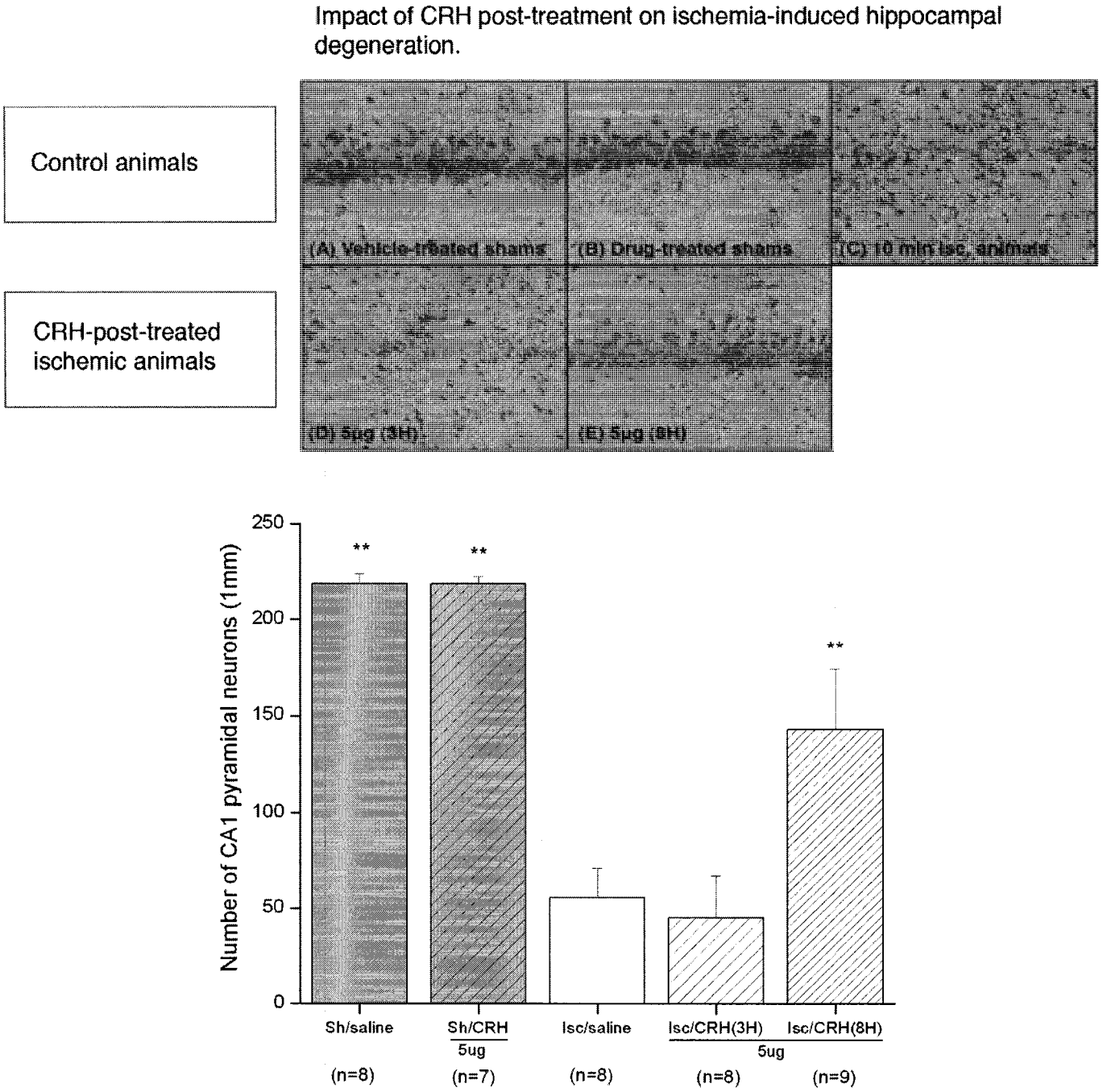


Figure 5: Representative photomicrographs (Thionin stain, Top panel) and histogram (bottom panel) representing the mean number of CA1 pyramidal neurons for the different experimental groups. CRH post-treatment 8h following ischemia significantly increased neuronal survival (≈61%). **, significantly different from vehicle-treated ischemic animals ($p < .001$).

3.3.2 8-arm radial maze

Figure 6a illustrates the negative relationship between hippocampal neuronal density and the total number of working memory errors made in the 8-arm radial maze, defined as the sum of errors made over the 12-day experimental trials ($F_{(1,36)}$=7.816, r = -.422; p=.008).

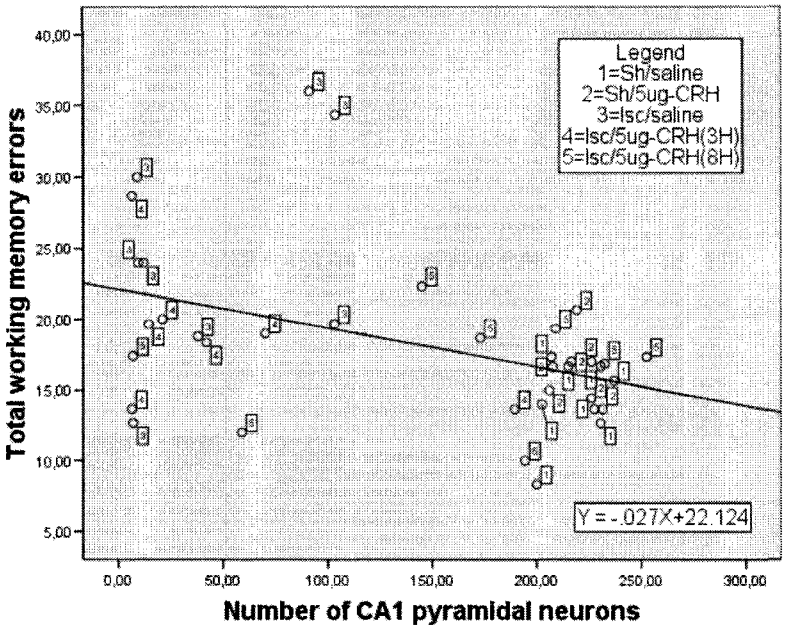
Figure 6b presents the performance of sham and ischemic animals in the radial maze and the impact of CRH post-treatment on working memory errors. Because of poor mobility in the 8-arm radial maze, one vehicle- and one CRH(8h)-treated ischemic rat were not included in the analysis. For this analysis, the 12 test days were grouped into 3-day blocks (i.e. days 1-3, 4-6, 7-9, 10-12) and mean errors within these 3-day blocks were analyzed. The number of working memory errors made in the 8-arm radial maze was analyzed using a 2 X 2 X 4 mixed ANOVA design on all animals treated at the 3h time interval, with surgery (ischemic vs. sham) and drug (0 vs. 5μg of CRH) as the independent factors and time (four blocks of 3-day trials) as the repeated factor. The Huyndt-Feldt correction for violations to the assumption of sphericity was applied and the degrees of freedom adjusted for all repeated variables having more than two levels [221]. Results revealed a significant effect of time ($F_{(2.69,69.8)}$=10.288, p<.001) and surgery ($F_{(1,26)}$=13.148, p<.001) but no significant interaction. Bonferroni-adjusted pair-wise comparisons revealed that overall, ischemic animals made significantly more working memory errors than sham subjects (p<.001). A 3 X4 mixed ANOVA design performed on all ischemic animal data also revealed a significant effect of group ($F_{(2,20)}$=3.582, p=.047). Bonferroni-adjusted pair-wise comparisons demonstrated that ischemic animals treated with CRH at the 8h time interval made significantly fewer working memory errors than

vehicle-treated ischemic rats (p=.046). An ANCOVA using CA1 neuronal density as the covariate removed the significant effect of group ($F_{(2,19)}$=2.777, p=.088) indicating that the changes in maze errors observed depended on neuronal density. A 5 X 4 mixed ANOVA design using all groups of animals performed on time (min) spent to complete the task revealed a significant effect of time ($F_{(2.77,85.75)}$=32.834, p<.001), but failed to show a significant effect of group ($F_{(4,31)}$=.128, p=.971) or time X group interaction ($F_{(11.07,85.75)}$=.764, p=.685).

3.3.3 Open Field

Figure 7 shows the impact of CRH administered post-ischemia on the behavioral activity profile of rats in the open field during a 5 min monitoring period. The time spent grooming or inactive is shown in Table 1. The analysis revealed no difference. Correlational analysis showed no relationship between activity in the open field and CA1 neuronal density.

a)



b)

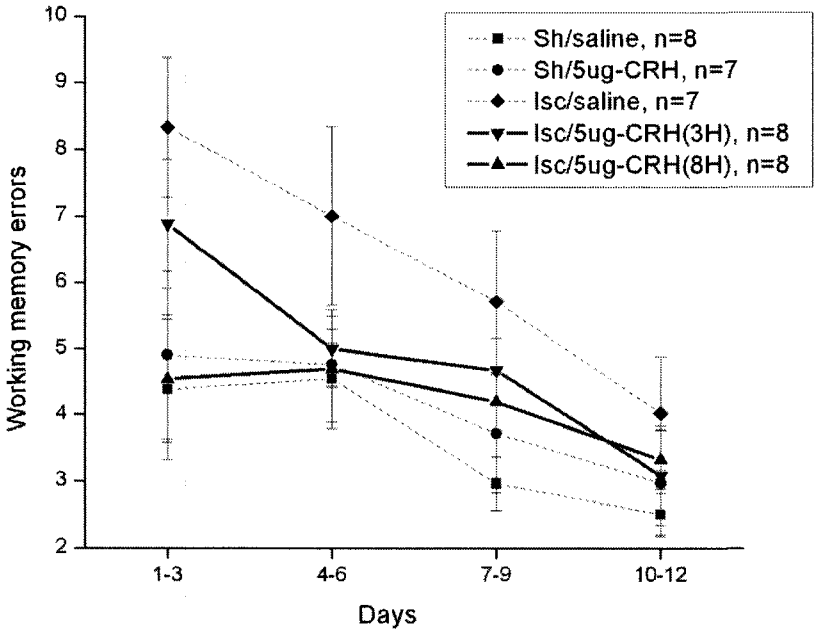


Figure 6: (a) Relationship between CA1 neuronal density and the total number of working memory errors; (b) Number of working memory errors made in the 8-arm radial maze by the various groups. Rats post-treated with CRH 8h following ischemia exhibited a comparable performance as sham-operated animals.

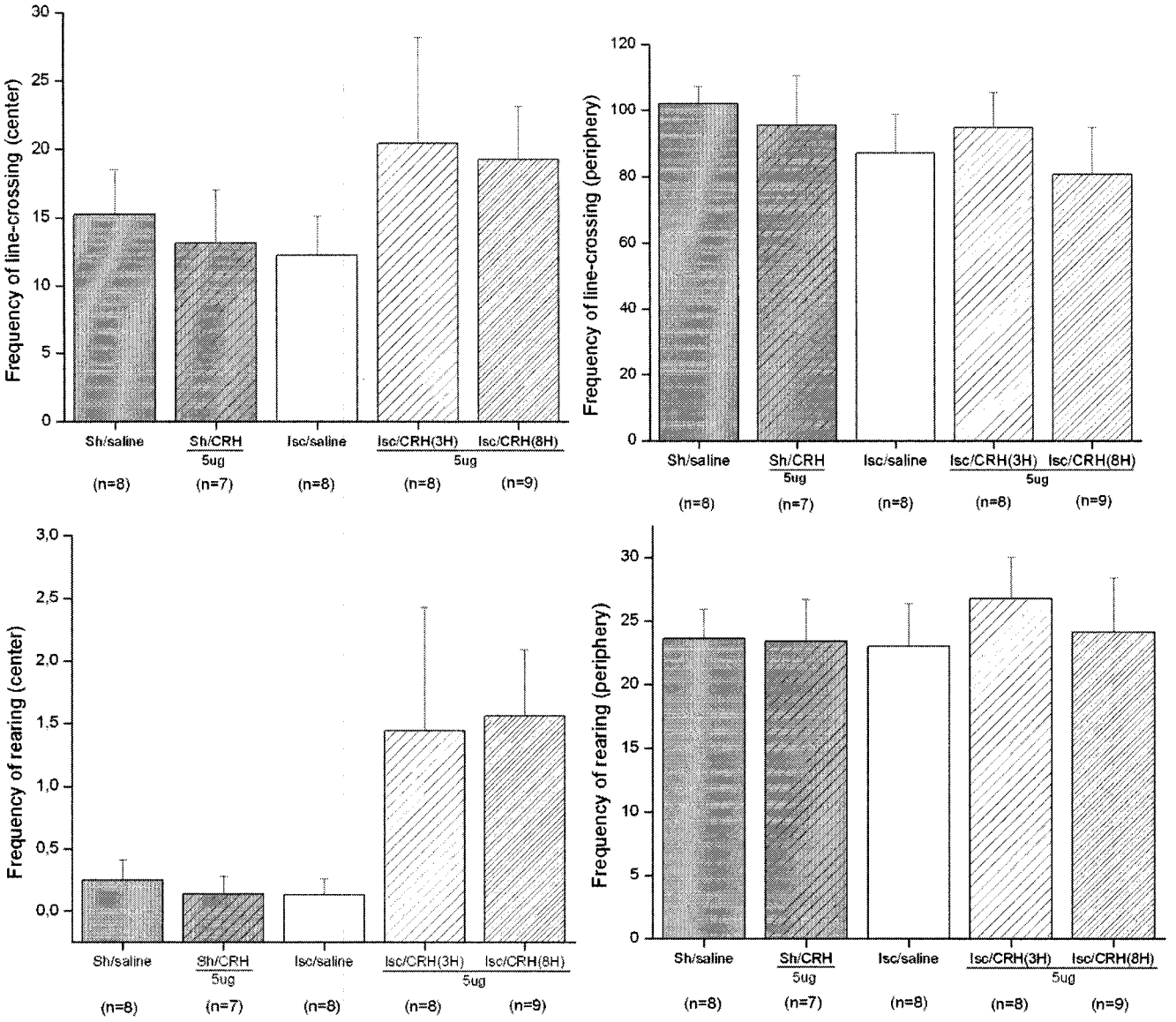


Figure 7: Activity profile in the open field revealed no difference between the various groups of rats over the 5 min monitoring period.

4. Discussion

The present results show that pre-ischemic CRH treatment reduced the ischemia-associated CA1 neuronal death, and that blockade of CRH receptors abolished CRH neuroprotection. The 0.1 and 5μg doses of CRH also reversed the ischemia-induced increase rearing behavior in the center of the open field, while the antagonist α-helical CRH (9-41) reversed this tendency. Ischemic rats pre-treated with NAL or BIN before central administration of CRH showed decreased neuronal density 7 days later compared to CRH-treated ischemic rats suggesting an involvement of opioid receptors in the protective effect of CRH. Finally, CRH administration 8h following ischemia increased cell survival and improved working memory. Together, these results make a clear case for the usefulness of CRH in neuroprotective strategies. The results also raise a number of questions that need to be discussed.

Different *in vitro* studies have supported the contention that CRH plays a role in the modulation of ischemia-induced cell death. In the present study, our findings represent a first *in vivo* demonstration of CRH-induced protection in CA1 pyramidal neurons following global ischemia in rats, with 37% increase in neuron survival in animals pre-treated with 5μg of CRH compared to vehicle-treated ischemic animals. This effect appears to be receptor-dependent, as treatment with the antagonist α-helical CRH (9-41) prior to CRH (5μg) administration significantly reversed the neuroprotection conferred by this peptide. Since this antagonist is non-selective, it is not possible to isolate the role of both receptor subtypes to the observed protection. Nonetheless, results from previous *in vitro* studies indicate that $CRHR_1$ is likely the receptor mediating CRH-induced neuroprotective effects [142,276,394]. In the present study, we did not include a group of

ischemic rats pre-treated with α-helical CRH (9-41) alone to see whether administration of the antagonist had any intrinsic effect on neuronal density. However, previous findings in our lab have shown that this antagonist had no impact on hippocampal neuronal density and produced no change in the open field behaviors [407].

Our findings revealed that only the highest dose of CRH tested (5μg) conferred significant hippocampal protection against a 10-min global ischemia when administered prior to vessel occlusion. Although not significant, the lowest dose (0.1μg) tended to produce similar neuroprotection. If this tendency represents a true effect, it would suggest that central administration of CRH could have an U-shape dose-response, the lowest and highest doses conferring increased protection in the CA1 subfield of the hippocampus compared to the middle dose (1μg). One possible explanation for this putative dose relationship may be that different mechanisms could account for the neuroprotective actions induced by the higher and lower CRH doses. A similar case is the well-documented U-shaped dose-response relationship between the effects of higher and lower doses of corticosterone in the CA1 area of the hippocampus: higher and lower doses acting on two different sets of glucocorticoid receptors with different affinities [239]. In the present case, the specific mechanisms of CRH actions at distinct doses remain to be determined.

The present finding that CRH was effective 8h but not 3h after ischemia is consistent with Elliott-Hunt et al.'s *in vitro* findings in which the maximum neuroprotective effects of CRH were observed when the peptide was administered between 8-12h following glutamate challenge [138]. This remote time window of opportunity suggests that endogenous action of CRH within this time interval may

contribute to secondary mechanisms involved in the rescue of vulnerable neuronal populations. This therapeutic window shows promises for clinical use for CRH, as many stroke patients seek help to the hospital many hours following symptoms onset.

In vitro studies have suggested that the beneficial actions of CRH occur through PKA- [26,276,393,394,426], and a PKC- [394] dependent mechanisms. Other possible mechanisms for CRH-induced neuroprotection at remote post-ischemic intervals include activation of mitogen-activated protein kinase [MAPK] [138,394], suppression of NF-kB [276] and stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) [138], and the stabilization of calcium homeostasis [393]. In addition to its direct pharmacological and physiological effects, evidence also suggests that CRH may confer protection by decreasing the ischemia-induced increase in blood-brain barrier (BBB) permeability following cerebral ischemia [206].

One potential mechanism by which exogenous administration of CRH could promote neuroprotection is if there is a CRH depletion during reperfusion, which would contribute to cellular damage. Recent work in our laboratory however failed to show any fluctuations in post-mortem CRH concentrations in the CA1 region of the hippocampus 4, 24, and 72h following global cerebral ischemia [249]. Similarly, *in vivo* CA1-CRH release remained unaltered during the initial 3h sample period following the ischemia insult [249] again suggesting that CRH remains unchanged during the early reperfusion period and that there is no clear evidence of a CRH deficit. Nonetheless, time-dependent alterations of CRH concentrations and release following ischemia observed have been demonstrated in extrahippocampal regions having direct or indirect connections with hippocampal neurons [249,550].

The present study also examined the contribution of opioid receptors to the neuroprotective effect of CRH. This hypothesis was based on a number of previous results. First, CRH and opioid peptides are co-localized in various brain regions [74,215,414,444,450,517] and interactions between these peptide systems have been demonstrated in both central and peripheral nervous system. In the brain, CRH has been shown to stimulate the release of endorphin (END) and DYN [7,366], as well of ENK [367] in the rat hypothalamus *in vitro*. In contrast, administration of α-helical CRH (9-41) reduces the release of END, ENK and DYN from the hypothalamus *in vivo* and *in vitro* [368]. CRH has also been found to induce the release of Met-ENK and DYN from the neostriatum *in vitro* and from the globus pallidus and the caudate nucleus *in vivo* in a dose-related and receptor-specific manner [481]. Conversely, opioid peptides can stimulate the synthesis and release of CRH. For example, stimulation of KOR induces hypothalamic CRH release *in vitro* and *in vivo*, an effect reversed by the administration of KOR antagonist [70,365]. Thus, it appears possible that the selective blockade of DOR or KOR prior to CRH administration significantly reduces the available CRH leading to a reduction of the protection conferred by exogenous CRH by decreasing the overall endogenous CRH availability.

Our findings suggest that the beneficial effects induced by CRH are opioid-mediated, as blockade of KOR or DOR prior CRH administration significantly reduced the level of neuronal protection conferred by the peptide. These data provide another evidence of an interrelationship between CRH and opioids, and suggest an additional physiological role of these peptides in regulation of ischemic neuronal death. However, the present findings do not indicate which endogenous molecular pathways or brain loci

are directly involved in such control. Several studies have demonstrated the presence of KOR and DOR, as well as DYN and ENK in the rat hippocampus [217,310,326,331,369,563]. Time-dependent decrease in DOR and KOR densities have also been reported following focal ischemia in vulnerable infarct zones [53-55]. Thus, one possible mechanism by which CRH infusion may promote neuronal survival may involve increased opioid peptide release or receptor activation at critical time intervals following ischemia. This is consistent with decreased ischemic injury achieved via administration of DYN and ENK or stimulation of KOR and DOR subtypes.

Another goal of the current study was to investigate whether neuronal protection translated into improved functional recovery. We tested animals in the open field, as many studies reported increased behavioral activity following ischemia [16,76,100,169,406,535,557]. In the present experiment, ischemic animals made more rearing and line-crossing but only in the center of the open field. Pre-treatment with low and high doses of CRH (0.1 and 5μg) significantly reversed the ischemia-induced increased frequency of rearing made in the center of the open field. However, despite significant reduction of neuronal density, blockade of DOR and KOR prior CRH administration had no impact on the frequency of rearing made in the center of the open field, suggesting that this measure does not provide a consistent behavioral index of neuroprotection in the CA1 region.

An additional point is that although global activity level in the open field was comparable between groups, ischemic rats displayed more activity in the center zone of the apparatus compared to sham-operated animals. This specific increase in the center zone of the open field suggests that ischemia leads to decreased anxiety rather than

overall increased behavioral activity. Indeed, increased exploratory activity in the center of the open field has been considered an index of decreased anxiety [417,441,479,493]. Such observation is also consistent with previous studies reporting reduced anxiety level in ischemic animals in the elevated plus maze [358,406].

Ischemic animals reliably demonstrate significant memory impairments in testing paradigms such as the radial arm maze [529], where performance has been positively correlated with the number of intact pyramidal cells in the CA1 section of the hippocampus [255]. Consistent with this, our results demonstrated that ischemic animals made significantly more working memory errors and that treatment with 5μg of CRH 8h but not 3h post-ischemia significantly enhanced memory performance in the radial maze. Statistical analysis also revealed that CA1 neuronal density was correlated to memory performance in the radial maze. The observation that a small number of vehicle-treated ischemic rats showing important CA1 damage managed to show an equivalent number of working memory errors as control sham-operated animals suggests that performance in the maze is also dependent on other extrahippocampal regions.

In conclusion, these findings represent the first demonstration of neuroprotection conferred by CRH in an *in vivo* model of global cerebral ischemia in rats. The beneficial effects induced by CRH appeared receptor-dependent and opioid-mediated, as blockade of CRH receptors, KOR or DOR prior CRH administration significantly reduced the level of neuroprotection conferred by this neuropeptide. These data further support the idea that the CRH and opioid systems are closely related, playing crucial role in the regulation of normal as well as pathological states, both within the CNS and the periphery. CRH conferred protection when administered 8h following vessel occlusion, an effect

accompanied by long-term improvement of ischemia-induced working memory deficits. Together, these findings further support a role for CRH in the modulation of ischemia-induced hippocampal degeneration and functional deficits.

MANUSCRIPT 2

Neuroprotection and functional recovery conferred by the administration of the kappa-opioid receptor agonist U50,488H and the selective delta1-opioid receptor agonist DPDPE in a rat model of global cerebral ischemia.

Abstract

Most studies that have evaluated the beneficial effect of pre-ischemic treatment of kappa-opioid receptor agonists have used short-term reperfusion intervals. We examined the long-term impact of the pre-ischemic peripheral injection of U50,488H (trans-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]-benzeneacetamide), a selective kappa-opioid receptor agonist, on neuronal damage and behavioral deficits following global ischemia in rats. Four groups of ischemic rats were pre-treated with various doses of U50,488H (i.p. 0, 5, 15, 30mg/kg) 15 min prior to vessel occlusion. Two groups of sham-operated animals that received either saline or U50,488H (30mg/kg) acted as controls. The injection of 30mg/kg U50,488H led to a 65% increase in CA1 neuron survival 35 days post-ischemia. CA1 neuronal protection translated into significant improvement of ischemia-induced spatial memory deficits assessed in the 8-arm radial maze. However there was no difference in activity in the open field. We also found that the pre-ischemic intracerebroventricular injection of 5μg of the selective delta1-opioid receptor DPDPE ([D-Pen2,D-Pen5]enkephalin) produced a 59% increase in CA1 neuron survival 7 days post-ischemia. However, DPDPE did not produce any significant impact on locomotor activity. These findings support the possible use of kappa- and delta-opioid receptors to attenuate ischemia-induced hippocampal damage and cognitive impairments.

Keywords: Global cerebral ischemia, delta1-opioid receptor, DPDPE, kappa-opioid receptor, U50,488H, neuroprotection, hippocampus, CA1, spatial memory, behavioral hyperactivity, rats.

1. Introduction

Pre-treatment with kappa-opioid receptor (KOR) agonists have been shown to significantly reduce global and focal ischemic damage in rodents [190] [22,105,264,427,552] and improve ischemia-induced learning and memory deficits [235,236,373]. Significant neuronal protection is found when kappa agonists are administered between 4 and 6h post-ischemia [24,37,87], suggesting a prolonged post-ischemia treatment window with KOR agonists.

One limit of previous studies was the short reperfusion intervals (less than 8 days) at which the effect of kappa agonists were tested, precluding firm conclusions about the efficacy of such treatment to induce longer-term post-ischemic neuronal protection and functional recovery. As important as shorter-term neuronal survival may be, many therapeutic approaches have been shown to gradually lose their beneficial impact with prolonged reperfusion intervals (for a review, see [108]). The present experiment thus examined the impact of the selective KOR agonist U50,488H on CA1 neuronal degeneration at a longer (35 days) post-ischemic interval, and determined whether the neuroprotection translated into improved behavioral activity and spatial memory. U50,488H is highly selective for kappa sites with poor affinity for mu or delta receptors [97,531]. Among all existing KOR agonists, U50,488H was selected in this study because it has previously been shown to reduce CA1 hippocampal damage [190,512] and to improve ischemia-induced memory deficits [236,373].

In contrast to KOR, the role of delta-opioid receptors (DOR) in the cascade of events following cerebral ischemia remains largely unexplored. The cardioprotective properties of DOR are, however, well established. Thus, administration of DOR agonists have been shown to increase myocardial [48,438], and hypoxic [46] tolerance, a phenomenon

reversed by pre-treatment with DOR antagonist [440]. A number of studies have also demonstrated the participation of DOR in the cellular protection conferred by cardiac ischemic preconditioning [375,457-459,542]. Similarly, morphine-induced cardioprotection has been shown mediated through DOR activation [375,391,459]. DOR stimulation also enhances cardiomyocyte protection in chicks [225,284,332,333] and rabbits [71,508]. Recently, Watson et al. [539] reported significant inhibition of infarct size in rats treated with ARD-353, a non-peptide DOR agonist, either before or after acute myocardial infarction. *In vitro*, activation of DOR has been shown to protect cortical neurons against glutamate-induced injury [572] and reduce hypoxia-induced neuronal injury [571].

Despite an acknowledged role of DOR in cardiac ischemia, the contribution of these opioid receptors to cerebral ischemia remains controversial. Although Borlongan et al. [51] demonstrated significant reduction of ischemia-induced striatal infarction in rats pre-treated with the delta opioid (D-ala 2, D-Leu5) enkephalin, DADLE (4mg/kg, 4 injections at 2h intervals) prior to middle cerebral artery occlusion, Iwata et al. [238] recently failed to observe inhibition of hippocampal damage in rats treated with DADLE (0.25, 1, 4, or 16 mg/kg) prior to a 10 min global ischemic insult. However, its limited blood brain barrier (BBB) permeability [88] and the non-selective binding of DADLE to delta-opioid receptors could explain these findings, as ischemic cardioprotection has been shown selectively mediated through delta1-opioid receptor activation [225,284,457,542]. Recently, Zhao et al. [574] reported that opioid preconditioning with Tan-67 (3mg/kg), a selective delta1-opioid receptor agonist, 24h prior focal cerebral ischemia significantly reduced infarct size and improved neurologic function in rats, whereas no improvement was found when administered 12 or 48h before middle cerebral arteries occlusion.

Thus, another goal of the present study was to determine the effects of central administration of DPDPE, a selective delta1-opioid receptor agonist [4,6] on ischemia-induced CA1 neuronal degeneration and level of behavioral activity in the open field.

2. Materials and methods

2.1 Animals

Male Wistar rats weighing between 225-275g at time of arrival in our facility were obtained from Charles River Laboratories (Rochefort, Québec, Canada). They were individually housed and maintained on a 12 h light/dark cycle (lights on at 7:00 AM) with free access to water and standard rat chow. The room temperature was maintained at 21-23°C with 60% relative humidity. Upon arrival, animals were acclimatized 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 were approved by the University of Ottawa Animal Care Committee.

2.2 Surgical Procedures

Global ischemia

Rats underwent surgery for forebrain ischemia using the four-vessel occlusion procedure (4-VO), as described by Pulsinelli and Brierley [420]. Briefly, rats were deeply anesthetized using 2% halothane. Core temperature was regulated throughout the surgery using a temperature-regulated heating blanket connected to a rectal thermometer (Homeothermic Blanket Control Unit, Harvard Instruments, Natick, MA), and kept warm with a heating pad during vessel occlusion and for 12-16h following surgery and

reperfusion. The vertebral arteries were irreversibly occluded by electrocoagulation, and a small-diameter silk thread was placed 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. 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, the control animals' carotid arteries were exposed but not clamped.

Intracerebral cannula

Rats were implanted with a cannula aimed at the third ventricle for subsequent intracerebroventricular (i.c.v.) administration of DPDPE. Animals were placed in a stereotaxic frame with a horizontal skull position. A guide cannula (22 gauge) was 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 and Watson [390]. The guide cannula was anchored to the skull using 4 stainless steel screws and acrylic dental cement. Following one week of recovery, rats underwent 4-VO, as described above.

2.3 Drug treatments

Peripheral injection

U50,488H (obtained from Sigma-Alrich, Oakville, ON) was diluted in 0.9% saline immediately before administration. Fifteen minutes prior to vessel occlusion, separate groups of ischemic rats received an intraperitoneal (i.p.) injection of either saline (n=12)

or one of three doses of U50,488H (5, 15 or 30mg/kg, n=10 per group). Two groups of sham-operated animals received either vehicle (n=11) or 30mg/kg of U50,488H (n=8) and were taken as controls. The 30mg/kg dose of U50,488H was selected based on prior observations showing that this dose significantly reversed ischemia-induced memory deficits [236] and reduced the development of behavioral activity and preserved hippocampal neurons from ischemic death [512].

Intracerebral injection

DPDPE (American Peptide Company, Sunnyvale, CA) was diluted in 0.9% saline and administered immediately into the 3rd ventricle in a volume of 5μl. Fifteen minutes prior to vessel occlusion, the groups of ischemic rats received an i.c.v. injection of saline (ischemic control group, n=7) or one of four doses of DPDPE (5, 12.5, 25 or 50μg; n=9, 7, 12 and 10, respectively). Two groups of sham-operated animals received an i.c.v. injection of either vehicle (n=9) or DPDPE (50μg; n=9). The range of DPDPE doses (5-50μg, i.c.v.) was selected based on their ability to alter other behavioral paradigms [561]. In the present study, DPDPE induced transient sedation and/or drowsiness following administration that was dose-related and lasted a few minutes at the low doses and reached approx. 60 minutes at the highest 50μg dose in sham and ischemic rats.

2.4. Apparatus and procedures for behavioral testing

Open Field

Five days following reperfusion, rats were tested in an open field and their behavior was monitored for 30 min by the experimenter using a computer and data logging software. The observation arena was made of gray Plexiglas (LWH: 75 X 75 X

30 cm), with a gray opaque floor. The floor was painted with a grid made of 25 10X10 cm squares. The entire arena was kept on a table 90cm above the floor. Rats were brought in the testing room at least 30 min prior testing to minimize the impact of anxiety and/or stress on behavioral performance. Black curtains surrounded the open field, and monitoring of the behavior was possible using an overhead camera. Frequency of line-crossing and rearing in the center and the periphery of the open field was counted, and the time during which animals groomed or were inactive was recorded. Behaviors displayed within the first row of squares adjacent to the walls of the apparatus were recorded as activity in the periphery of the open field.

8-arm radial maze

Two days following the open field test, animals that received peripheral injections were also tested in the radial maze. The maze consisted of eight arms (60 X 12 cm, with a 5 cm high lip around each arm) extending from a central octagonal area (32 cm in diameter with a 30-cm high clear Plexiglas wall). Plexiglas sliding doors permitted entry into each arm. The floor of the arms and central area was 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 sidewalls. At the end of each of the maze's arm, was a food cup (recessed 1 cm into the floor) that held a piece of Fruit Loop cereal. The animals only received 4-5 rat chow pellets/day to motivate them to eat the food reward.

During habituation, rats were placed in the radial arm maze for 10-min exploration periods during 6 days. Baits were initially available throughout the maze to encourage exploration, but was gradually restricted to the food cup. Following this habituation

period, each animal was placed individually in the center of the maze with doors to all arms closed. Upon opening all doors, each rat could enter any of the eight arms. When the rat had consumed the reward at the end of one arm, it eventually 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. Doors were then reopened, and the procedure repeated. A trial ended when all baits were consumed or when 15 min had elapsed. An arm entry was recorded when all four limbs were inside one of the maze arm. Number of working memory errors (entering an arm that was previously entered) and the time taken to consume all baits were recorded. Before the doors were opened for each trial, the experimenter ensured that the orientation of the animal's head was randomly changed from one trial to the other in order to minimize the development of a positional habit. The maze floor was cleaned after each trial in order to minimize olfactory cues. Animals were tested every day for 15 days.

2.5 Analysis of neuronal density on thionin-stained sections

Upon completion of behavioral testing and 35 (peripheral injections) or 7 days (intracerebral injections) following reperfusion, rats were euthanized and their brain removed, frozen on dry ice and stored at -80°C. Serial coronal frozen sections (14 μm) of the hippocampal region were subsequently obtained and stained with thionin. Neuronal density of the hippocampal CA1 subfield was determined using the method of Kirino et al. [252]. Analysis of neuronal density was performed on coronal sections located between 3.14 and 4.16 mm posterior to bregma. Total linear length of the CA1, as defined in the Atlas of Paxinos and Watson [390], was measured using a digitizer. Neurons in the stratum pyramidale within the CA1 subfield were observed with a LEICA DAS microscope

attached to a SONY digital camera and their number was estimated using a computer-assisted cell counting procedure using Norton Eclipse software (v 6.0). Neurons that had shrunken cell bodies with surrounding empty spaces were excluded. Neuronal density of the CA1 region was quantified by taking the number of visually intact pyramidal cells per 1 mm linear length of the CA1 stratum pyramidale. The neuronal density was given by the average of number of neurons counted from six sections on the right and left hippocampus.

3. Results

3.1 Effect of the peripheral injection of the KOR agonist U50,488H on ischemia-induced hippocampal degeneration and behavioral and memory deficits.

3.1.1 CA1 neuronal density

All statistical analyses were conducted using SPSS (V 15.0). The alpha level was at 0.05 for all omnibus tests. Figure 1 presents the CA1 neuronal density of control animals and those that received U50,488H pre-treatment. Results are expressed as means ± SEM. A two-way randomized group ANOVA was performed on all vehicle- and 30mg/kg U50,488H-treated animals to assess the effect of surgery (ischemic vs. sham) and drug (0 vs. 30mg/kg of U50,488H). The analysis revealed significant effects of both factors (surgery, $F_{(1,37)}=58.081$, $p<.001$; drug, $F_{(1,37)}=23.976$, $p<.001$) and their interaction ($F_{(1,37)}=29.086$, $p<.001$) on cell density. Bonferroni-adjusted pair-wise comparisons indicated a significantly lower cell density in ischemic compared to sham animals, as well as in vehicle- compared to drug-treated subjects ($p<.001$). Thus, vehicle-treated ischemic animals had ≈ 70% less neurons than that of sham rats. A higher cellular density in

30mg/kg U50,488H-treated ischemic animals compared to vehicle-treated ischemic rats was also found (≈ 65% increase in CA1 neuron survival; $p < .001$). No other significant difference was found.

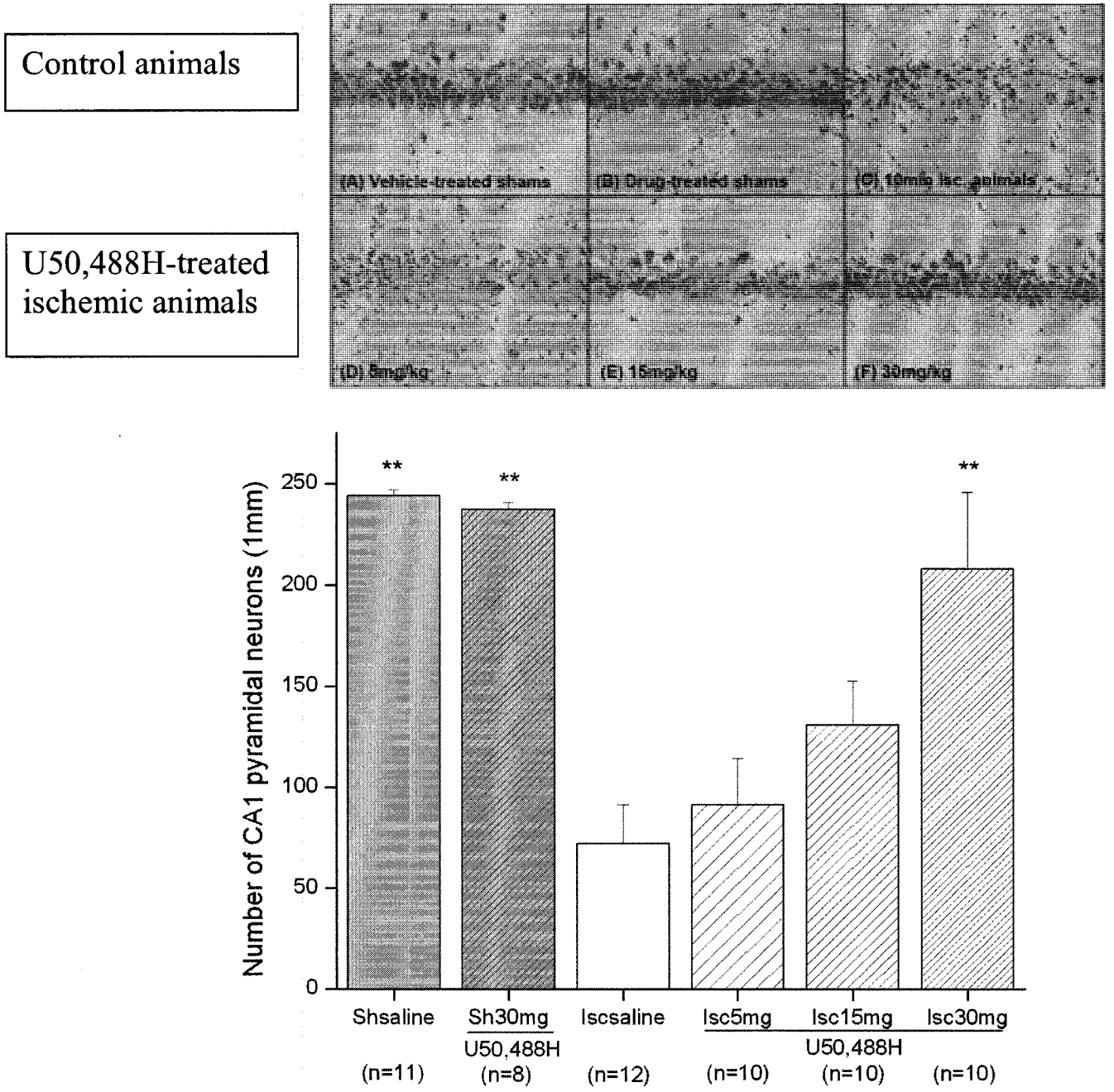


Figure 1: Representative photomicrographs (Thionin staining, Top panel) and histogram (bottom panel) representing neuronal density in the CA1 hippocampal subfield for the different experimental groups. ** Indicates significant different from vehicle-treated ischemic rats ($p < .001$).

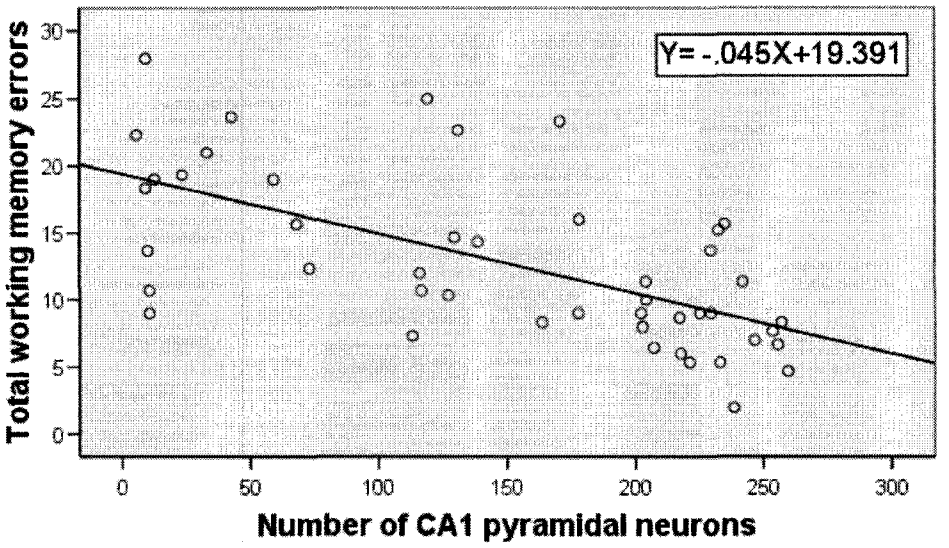
3.1.2 8-arm radial maze

Drug-treated sham-operated animals were excluded from the behavioral analysis as this group of animals was tested under slightly different conditions. Since no difference in neuronal density was found between vehicle- and drug-treated shams, it appears improbable that any behavioral differences would be hippocampal-dependent. Hippocampal neuronal density was negatively correlated to the total number of working memory errors made in the 8-arm radial maze, defined as the sum of errors made over the 15-day experimental tests, (Figure 2a; $F_{(1,44)}=28.921$, $r = -.630$, $p<.001$). Although performance on the last 3-day time interval was influenced by performance on earlier trials, data collected on the final block is best correlated with the histological findings. This relationship was also found to be negative ($F_{(1,44)}=12.242$, $r = -.467$, $p<.001$).

Figure 2b presents the mean number of errors made by shams, vehicle-treated ischemic animals and groups pre-treated with U50,488H in the 8-arm radial maze. Because of poor mobility or performance in the 8-arm radial maze, three vehicle-treated ischemic rats, two vehicle-treated shams, and two U50,488H-treated ischemic animals (15 and 30mg/kg) were not included in the statistical analysis. For this analysis, the 15 test days were grouped into 3-day periods (i.e. days 1-3, 4-6, 7-9, 10-12, 13-15) and mean errors within these 3-day periods were analyzed. The number of working memory errors made in the 8-arm radial maze was evaluated using a 5 X 5 mixed ANOVA design, with group (vehicle-treated, ischemic and sham and 5, 15 and 30mg/kg U50,488H-treated ischemic animals) as the independent factor and time (five blocks of 3-day intervals) as the repeated factor. The Huyndt-Feldt correction for violations to the assumption of sphericity was applied and the degree of freedom adjusted for all repeated variables

having more than two levels [221]. The analysis revealed a significant effect of time ($F_{(3.37,138.09)}$=54.713, p<.001) and group ($F_{(4,41)}$=6.724, p<.001), but no significant interaction ($F_{(13.47,\ 138.09)}$=0.918, p=.538). Bonferroni-adjusted pair-wise comparisons revealed that vehicle-treated ischemic rats made significantly more working memory errors than vehicle-treated shams and ischemic animals pre-treated with 30mg/kg of U50,488H (p<.05). A 5 X 5 mixed ANOVA design performed on the duration (min.) to complete the task in the 8-arm radial maze revealed a significant effect of time ($F_{(3.19,130.62)}$=56.555, p<.0001), but no significant effect of group ($F_{(4,41)}$=2.214, p=.084) or time X group interaction ($F_{(12,74,\ 130,62)}$=1.224, p=.270). An ANCOVA performed on all blocks using CA1 neuronal density as the covariate eliminated the group effect ($F_{(1,40)}$=1.354, p=.267).

a)



b)

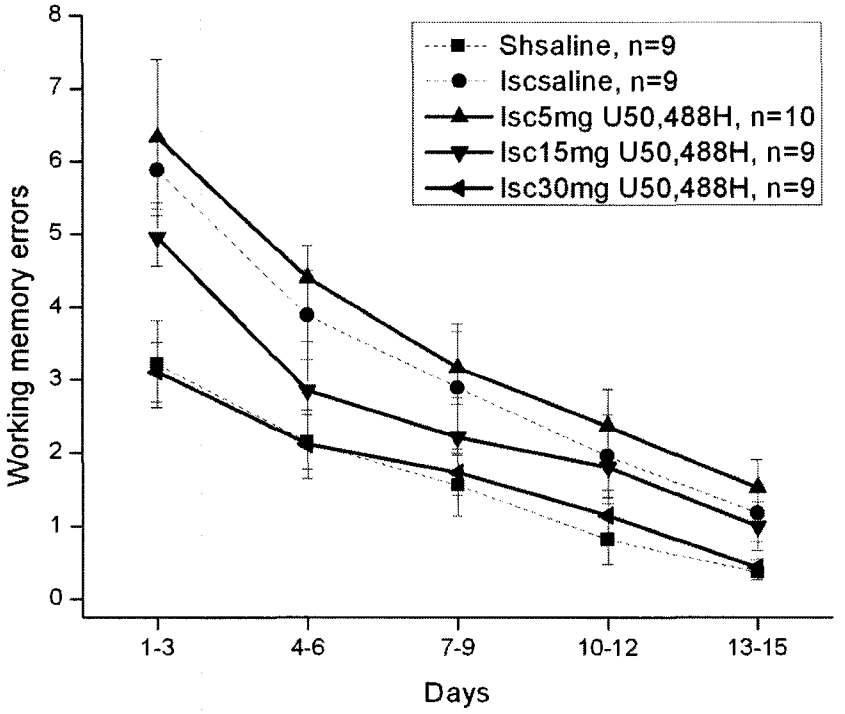


Figure 2: (a) Scatter plot demonstrating a negative correlation between neuronal density and total number of working memory errors made over the 15-day experimental trials, (b) Number of working memory errors in the 8-arm radial maze. U50,488H (30mg/kg)-treated ischemic rats exhibited a performance comparable as sham-operated animals.

3.1.3 Open Field

Figure 3 presents the frequency of total behavioral activity in the open field every 5 min during the 30-min monitoring period. One vehicle- and one 5mg/kgU50,488H-treated ischemic rat were not included in the analysis because they did not produce any behaviors in the open field. Analysis of correlation revealed no relationship between neuronal density and behavioral activity in the open field ($p=.903$). The statistical analysis performed on total behavioral activity was assessed using a 5 X 6 mixed ANOVA design, with group as the independent factor and time (six blocks of 5 min intervals) as the repeated factor. The results revealed a significant effect of time ($F_{(3.91,180.01)}=121.100$, $p<.001$) and time X group interaction ($F_{(15.65,180.01)}=2.127$, $p=.009$), but no group effect ($F_{(4,46)}=.795$, $p=.535$). Simple main effects followed by Bonferroni-adjusted pair-wise comparisons revealed a between-group difference in the first and last 5-min interval, due to significantly more behaviors in the 15mg/kg U50,488H-treated ischemic group than the 5mg/kg U50,488H-treated ischemic animals ($p<.05$). No significant group difference was found for the duration of grooming or inactivity (Tables 1 and 2). Taken together, these results indicate similar behavioral profiles across groups in the open field, suggesting that performance in this test is not related to CA1 neuron survival.

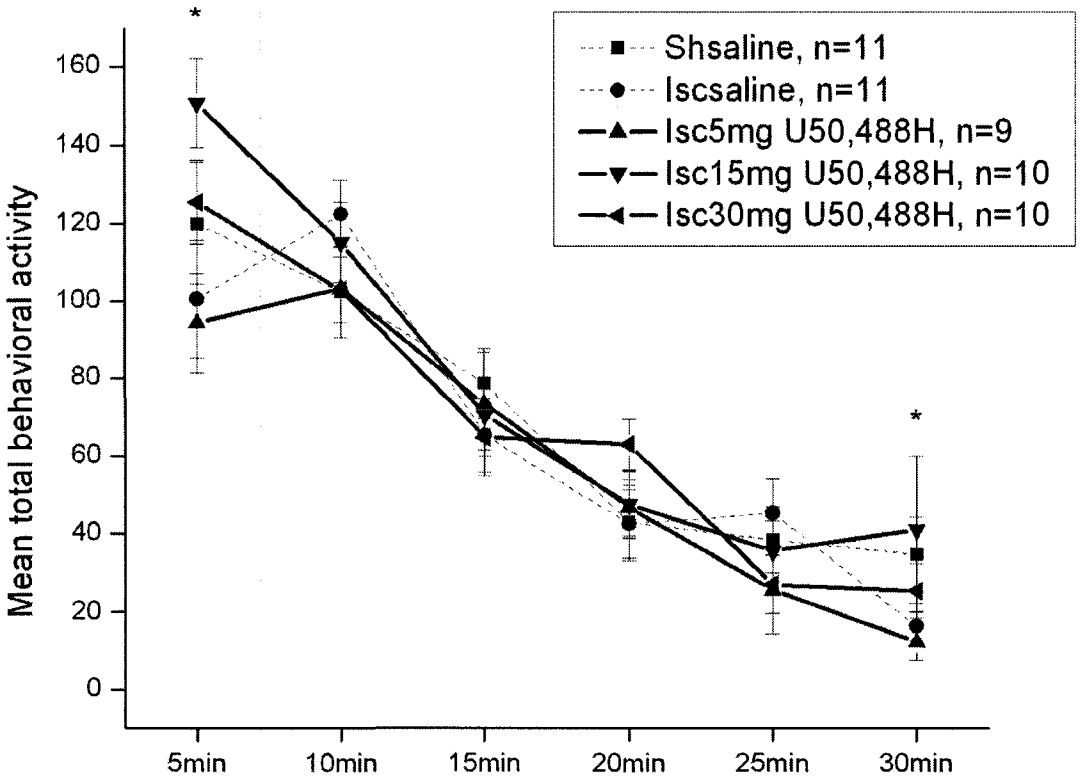


Figure 3: Global activity (frequencies of rearing and line-crossing in the center and periphery) in the open field every 5 min over the 30 min monitoring period for all groups of rats. *, Indicates 15mg/kg U50,488H-treated ischemic rats significantly different from 5mg/kg U50,488H-treated ischemic animals ($p < .05$).

Table 1
Effect of ischemia and drug treatments on duration of grooming.

Groups	5 min	10 min	15 min	20 min	25 min	30 min
U50,488H						
Shsaline	12.79±3.83	30.56±8.43	45.44±12.60	74.38±23.00	78.61±23.47	31.23±12.34
Iscsaline	18.87±4.58	39.96±12.61	62.81±14.14	82.64±18.04	50.19±16.11	70.55±22.73
Isc5mg	45.33±7.71	45.78±9.16	72.44±18.08	78.33±11.42	100.67±24.29	90.22±32.70
Isc15mg	10.8±2.29	23.30±8.49	47.80±14.19	72.20±22.17	75.10±26.14	50.00±16.84
Isc30mg	14.3±3.25	22.40±7.53	50.60±18.07	59.70±11.53	74.40±16.06	84.80±23.48
DPDPE						
Shsaline	14.53±5.36	39.66±7.77	71.28±16.37	81.22±24.3	94.66±32.26	77.72±24.28
Sh50μg	30.68±11.29	41.06±12.99	62.76±12.91	76.68±18.45	95.04±23.07	78.92±17.42
Iscsaline	16.91±8.6	27.00±10.35	66.87±12.71	56.76±14.76	99.37±27.65	110.60±26.03
Isc5μg	17.59±9.02	58.64±17.43	60.76±19.71	83.08±18.11	61.01±15.94	104.48±27.47
Isc12.5μg	12.19±6.34	26.44±9.80	57.11±12.62	89.07±23.99	58.47±22.87	75.59±17.39
Isc25μg	11.98±5.65	31.93±7.54	40.48±15.40	44.78±10.96	42.43±11.86	65.70±21.64
Isc50μg	23.23±14.42	32.09±9.58	52.13±18.34	53.62±12.32	46.22±12.97	87.95±22.08

Each cell represents the mean ± S.E.M. of duration grooming (sec) for every 5 min over the total 30 min monitoring period.

Table 2
Effect of ischemia and drug treatments on duration of inactive episodes.

Groups	5 min	10 min	15 min	20 min	25 min	30 min
U50,488H						
Shsaline	5.65±4.33	9.09±5.40	20.21±9.91	41.82±21.14	52.69±18.66	111.65±30.25
Iscsaline	13.04±7.91	1.20±0.65	19.38±6.46	50.85±11.10	72.71±24.89	155.35±31.15
Isc5mg	7.33±7.21	22.44±10.64	52.78±31.93	50.56±22.01	79.00±32.81	155.22±34.18
Isc15mg	0.20±0.13	14.90±10.22	38.2±17.65	44.60±20.64	66.40±28.83	100.60±31.93
Isc30mg	3.10±2.78	13.90±8.54	37.6±17.00	47.10±15.82	100.40±30.09	94.30±21.51
DPDPE						
Shsaline	6.42±6.42	2.57±1.57	11.01±6.46	19.71±7.56	41.2±13.96	56.13±22.32
Sh50μg	0.81±0.81	1.58±1.34	3.31±1.99	6.64±5.00	18.04±6.84	26.74±9.69
Iscsaline	0.00±0.00	1.07±0.84	15.57±5.98	39.06±16.40	35.33±20.24	31.26±13.20
Isc5μg	0.84±0.84	4.17±2.77	28.08±22.53	54.71±19.35	57.57±30.29	114.04±36.65
Isc12.5μg	7.79±3.83	10.46±5.09	12.56±5.55	37.97±13.17	52.09±21.09	89.91±27.32
Isc25μg	10.70±9.11	12.21±9.96	30.23±14.04	47.78±17.88	56.73±12.17	85.53±26.14
Isc50μg	0.52±0.52	0.00±0.00	2.89±1.64	9.61±4.85	45.83±20.42	41.89±20.18

Each cell represents the mean ± S.E.M. of duration of inactivity (sec) for every 5 min over the total 30 min monitoring period.

3.2 Effect of intraventricular injection of the selective delta1-opioid receptor agonist DPDPE on ischemia-induced behavioral deficits and hippocampal degeneration.

3.2.1 CA1 neuronal density

Figure 4 demonstrates the impact of vehicle and DPDPE treatments in sham-operated and ischemic animals on hippocampal CA1 neuronal density. A two-way ANOVA performed on vehicle- and 50μg-treated sham and ischemic animals revealed a significant effect of surgery ($F_{(3,31)}$=125.304, p<.001), but failed to show an effect of drug ($F_{(1,31)}$=.081, p=.777) or a group X drug interaction ($F_{(1,31)}$=.536, p=.469). Bonferroni pair-wise comparisons indicated that the main effect of group was attributable to significantly a lower cellular density in ischemic animals compared to sham-operated ones (p<.001). In comparison to sham-operated rats, vehicle-treated ischemic rats displayed a 71% loss of CA1 neurons following 10 min global ischemia. A one-way ANOVA including all ischemic animals revealed a significant effect of group ($F_{(4,40)}$=3.274, p<.021). Bonferroni pair-wise comparisons attributed this effect to significantly reduced CA1 damage in the 5μg DPDPE-treated compared to vehicle-treated ischemic rats (p<.05; ≈ 59% increase in CA1 neuron survival). However, the three highest tested doses (12.5, 25 and 50μg) failed to significantly alter ischemic hippocampal damage.

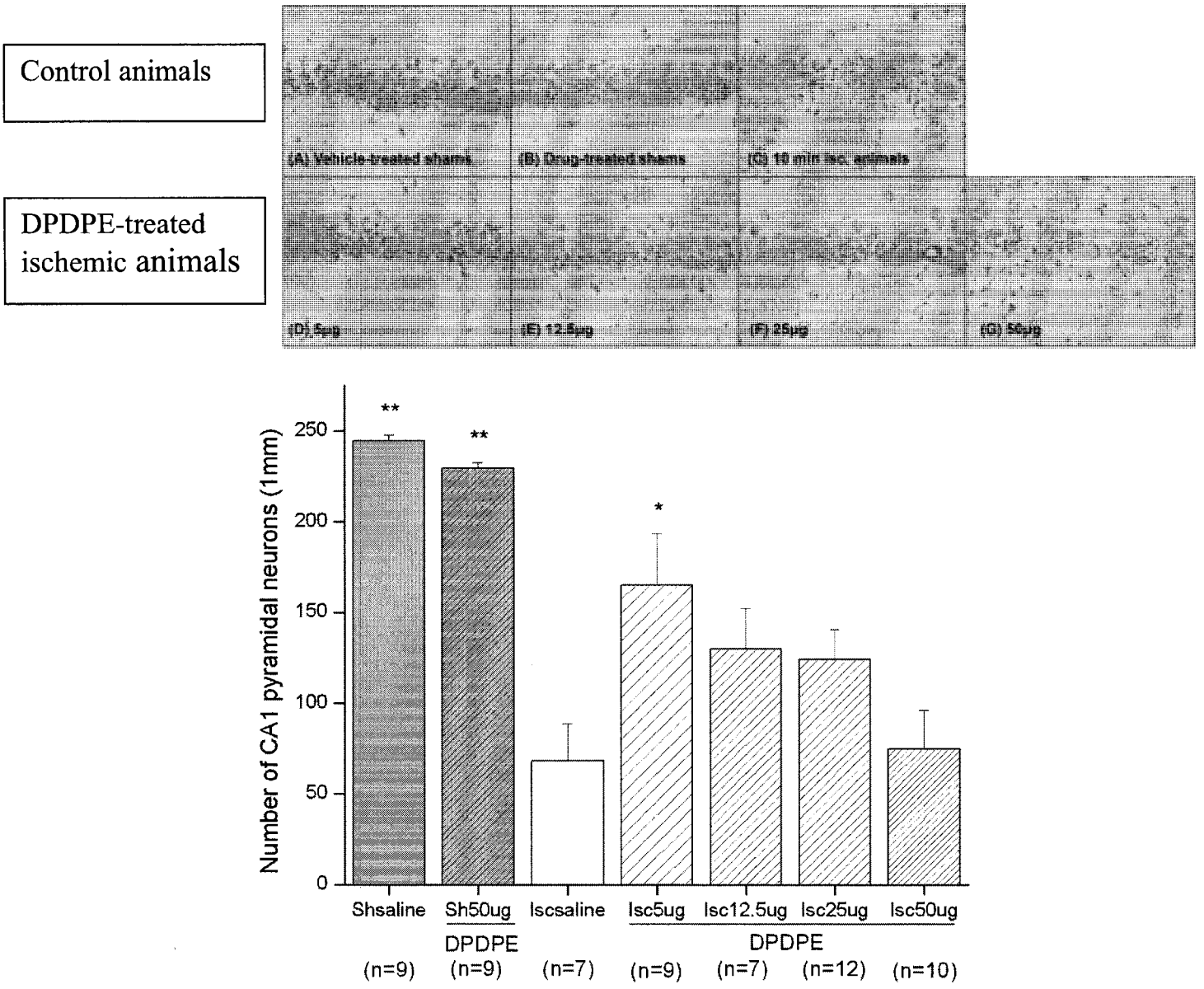


Figure 4: Representative photomicrographs (Thionin staining, Top panel) and histogram (bottom panel) showing neuronal density in the CA1 hippocampal subfield for the different experimental groups. *,** Indicates significant different from vehicle-treated ischemic rats ($p < .05$ and .001, respectively).

3.2.2 Open Field

Figure 5 presents the frequency of total behavioral activity in the open field every 5 min during the 30-min monitoring period. A correlation revealed no relationship between hippocampal neuronal density and behavioral activity in the open field (p=.424). A 2 X 2 X 6 mixed ANOVA design was performed on vehicle- and 50μg-treated sham and ischemic animals to assess the effect of surgery (ischemic vs. sham), drug (0 vs. 50μg of DPDPE) and time (six blocks of 5 min intervals). Results revealed a significant effect of time ($F_{(4.68,145.01)}$=55.978, p<.001), but no significant effect of surgery ($F_{(1,31)}$=.353, p=.557), drug ($F_{(1,31)}$=1.021, p=.320) or any interaction. Similarly, a 5 X 6 mixed ANOVA design performed on all ischemic animals revealed a significant effect of time ($F_{(3.77,150.73)}$=88.784, p<.001), but no significant effect of group ($F_{(4,40)}$=1.251, p=.305) or time X group interaction ($F_{(15.07,150.73)}$=1.165, p=.305). No difference was found between groups for the duration of grooming or inactivity (see Tables 1 and 2). Overall, these results indicate that neither the 10-min global ischemic insult nor the DPDPE pre-treatments exerted a significant impact on the overall activity profile of rats in the open field, suggesting that performance using this measure is not dependent on CA1 neuron survival.

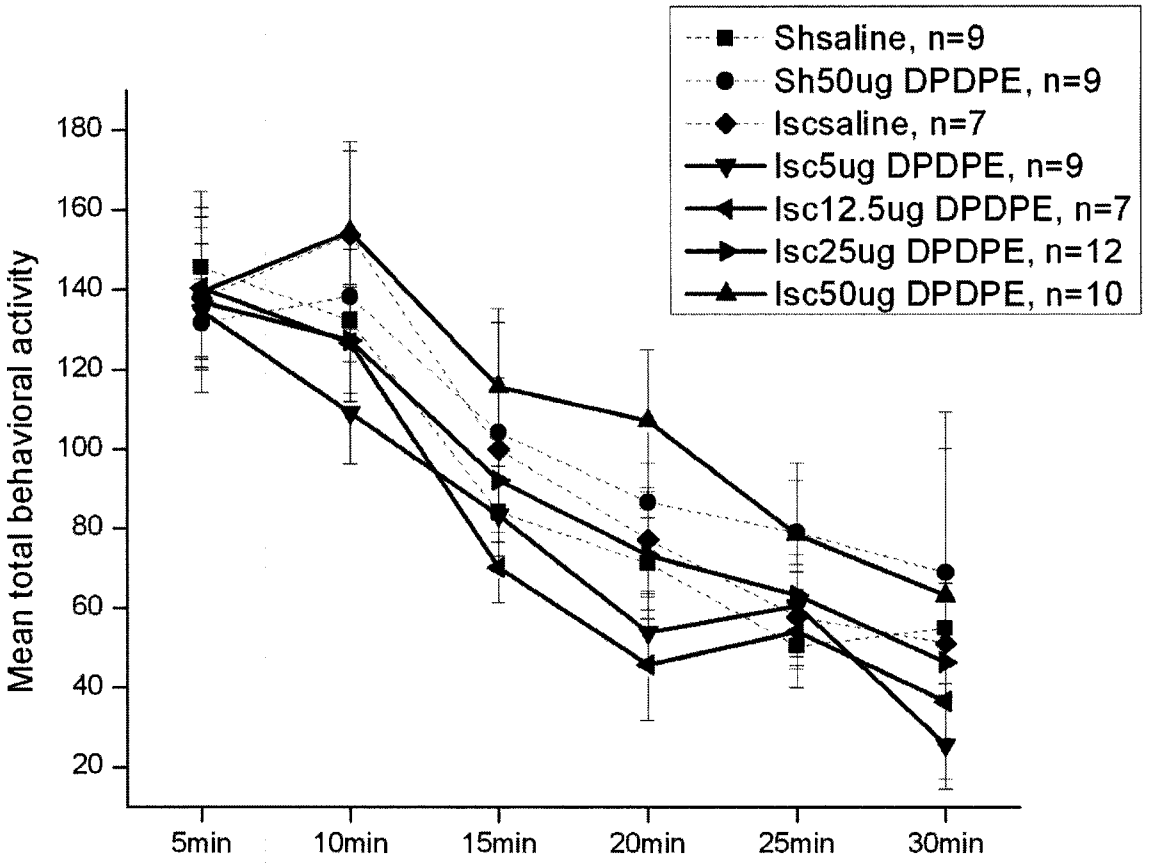


Figure 5: Global activity (frequencies of rearing and line-crossing in the center and periphery) in the open field every 5 min over the 30 min monitoring period for all groups of rats.

4. Discussion

The present study demonstrates that pre-treatment with 30mg/kg of U50,488H increased the survival rate of CA1 pyramidal neurons and improved radial maze performance in ischemic rats, 35 days after ischemia. This long-lasting protection extends findings from previous studies that have examined the effects of kappa agonists on ischemia at shorter post-ischemic delays. This is a significant finding given that many therapeutic approaches gradually lose their beneficial impact with prolonged reperfusion intervals. Secondly, we demonstrated for the first time, that pre-treatment with 5μg of the selective delta1-opioid receptor agonist DPDPE led to a 59% increase in CA1 neuron survival following a 10 min global ischemia. These results raise a number of questions that need to be addressed.

One limit of this study is the absence of the use of the selective KOR and DOR antagonists to determine whether the effects induced by U50,488H and DPDPE were receptor-dependent. However, findings from previous studies suggest that the neuronal and memory improvements associated to KOR agonists utilization are reversed by administration of the selective KOR antagonist nor-binaltorphimine [235,236,573]. Similarly, the selective DOR antagonist naltrindole has been shown to reverse the beneficial effects of ischemic preconditioning (IPC)- as well as morphine-induced myocardial protection in rat and rabbit hearts [375,459].

The mechanisms by which kappa agonists would exert their protective effects in ischemia are suggested by a number of previous studies. For example, *in vitro*, kappa agonists have been shown to inhibit the release of glutamate [56,163,212,268,309] by blocking calcium entry into presynaptic terminals [163]. Consistent with such findings, significant

inhibition of glutamate release following focal ischemia in animals pre-treated with the kappa agonist CI-977 was observed [301]. The neuroprotective effects of KOR agonists have also been associated with inhibition of ischemia-evoked nitric oxide production in rats [178,570]. Recently, KOR agonists have been shown to exert neuroprotective actions through modulation of the mitogen-activated protein kinase and extracellular signal-regulated kinase (MAPK/ERK) signalling systems [29,30]. Finally, different studies have also suggested that the neuroprotective effects of KOR agonists are independent of an impact on brain and/or body temperature in ischemic animals [37,235,236,573].

Research investigating the potential role of delta opioid receptors has been equivocal. For example, DADLE was ineffective to reduce the impact of focal [51] and global ischemia [238]. This could be due to DADLE's non-selective binding to DOR or to poor BBB crossing of the peptide following systemic administration [88]. However, there is a large set of studies showing the beneficial effects of delta opioid agonists in cardiac ischemia. Huh et al. [225] reported a dose-dependent cardioprotection against ischemic damage using the selective delta1 agonist, TAN-67 (0.01-10μM), the 1μM dose conferring higher protection as compared to a 10μM dose. Similarly, treatment with SNC-121 (0.1-1μM), a selective non-peptide DOR agonist, has been shown to induce a concentration-dependent cell death reduction in isolated cardiac myocytes. Again, consistent with the present results, the lowest dose conferred the highest protection [387].

Possible mechanisms underlying the neuroprotective action of delta opioid agonists are suggested by research in cardiac ischemia. Consistent with the present experiment, cardioprotection appears mediated through delta1- and not delta2-opioid receptors [323,324,391,457,542]. Some studies have suggested that the delayed protection

following delta1-opioid receptor stimulation in cardiac ischemia was partly mediated through ATP-sensitive potassium channels (K^+(ATP)), p38 MAPK and ERK [160] or by a burst of reactive oxygen species (ROS) [388], as well as through activation of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) [389,471]. Although the involvement of similar mechanisms in the present study remains unknown, activation of K^+(ATP) channels in these effects appears plausible in light of the participation of these modulators to CA1 neuron survival following global ischemia [209] and cerebral IPC [211,405].

The behavioral observations in the present experiment also raise a number of issues. In the current study, we selected the 8-arm radial maze to determine the impact of U50,488H on spatial memory function, as working memory performance of rats in this test has been shown to strongly correlate with CA1 neuronal damage [255]. This is consistent with our observation that sham and ischemic rats pre-treated with 30mg/kg of U50,488H displayed similar profiles in the 8-arm radial maze. Both groups made significantly less working memory errors compared to vehicle-treated ischemic animals and spatial memory performance in the radial maze varied as a function of CA1 neuronal density. Our observation that ischemic animals do eventually learn this spatial task after a few days is consistent with other studies showing recovery of ischemia-induced working memory impairments following extensive testing using other paradigm [229]. Together, this data suggest that other hippocampal subfields and/or extrahippocampal structures can eventually allow learning after a long post-ischemic period despite CA1 extensive neuronal loss [101].

The lack of a large increase in locomotor activity in our ischemic animals tested 5 days following reperfusion, despite significant CA1 neuronal damage, contrast with previously reported ischemia-induced hyperactivity [16,76,100,169,535]. However, consistent with the present results, Gulinello et al. [183] recently reported that global ischemia did not affect locomotion in the open field in rats tested 6 days post-occlusion, while other studies reported reduced locomotor activity following bilateral occlusion of the carotid arteries in rodents [164,558,559]. Differences between sham and ischemic animals have also been shown considerably reduced or absent with repetitive testing [16,134,145,242]. Furthermore, some evidence suggests that cerebral ischemia fails to alter open field activity in animals tested several weeks following the insult [294,424]. Thus, marked differences in behavioral activity profile of ischemic animals appears to be limited to when animals are initially tested in the open field, within hours to a few days following the ischemic insults.

While some studies have reported a positive correlation between hippocampal damage and locomotor activity [9,107,134,169,247], others have not. Thus, in spite of significant hippocampal CA1 protection, preconditioned gerbils still displayed habituation deficits in the open field [107,134]. One possible explanation for these discordant results is suggested by the absence of changes in open field activity in rats with ischemic damage restricted to hippocampal CA1 neurons [176] . However, when more extensive damage was produced in the cerebral cortex and caudate nucleus by two 7-min occlusions, persistent locomotor hyperactivity was observed [232].

In conclusion, the present study found that pre-ischemic peripheral injection of a kappa opioid agonist produced long-lasting significant sparing of CA1 neurons and improved radial maze performance. Secondly, we report that pre-ischemic

intracerebroventricular injection of a selective delta1-opioid receptor agonist significantly reduced global ischemia-induced CA1 neuronal damage. Additional studies examining the effects of post-ischemic injection of DPDPE will help validate this treatment as a potential therapeutic tool.

MANUSCRIPT 3

Protection conferred by Corticotropin-releasing hormone in rat primary cortical neurons against chemical ischemia is partially mediated via opioid receptor activation.

Abstract

Many *in vitro* studies have supported neuroprotective properties of Corticotropin-releasing hormone (CRH) against various excitotoxic and oxidative insults. However, to date none have demonstrated whether CRH can protect against a KCN-induced form of chemical ischemia. In the current study, we determined the impact of administration of CRH pre- and post-potassium cyanide (KCN)-induced insult in rat primary cortical neurons. We also assessed whether CRH effects were receptor-dependent, using the CRH receptor antagonist α-helical CRH (9-41) (1μM). Our findings revealed that both pre- (200fmol to 2nmol) and post- (20pmol and 2nmol, 3 and 8h post-KCN insult) treatments with CRH conferred significant protection against KCN-induced cortical injury, an effect blocked in cultures treated with α-helical CRH (9-41) prior to KCN administration.

Interactions between CRH and opioid systems have been demonstrated in the regulation of different physiological processes in the peripheral and central nervous system. The current study examined whether kappa- and delta-opioid receptors (KOR and DOR) blockade, using nor-binaltorphimine and naltrindole respectively (10μM), reversed the protection induced by CRH. Blockade of KOR and DOR prior to CRH administration significantly reduced the protection at the 3h time interval, but failed to significantly alter neuronal death 8h post-KCN insult. Using reverse transcription polymerase-chain reaction (RT-PCR) we demonstrated increased enkephalin, DOR and KOR mRNA expression in CRH-treated primary cortical neurons. Together, these findings demonstrate neuroprotective effects of CRH against KCN- induced injury in cortical neurons, and suggest a participation of the opioid system in modulating CRH actions.

Keywords : Primary cortical neurons, Corticotropin-releasing hormone, chemical ischemia, kappa-opioid receptors, nor-binaltorphimine, delta-opioid receptors, naltrindole, α-helical CRH (9-41), reverse transcription polymerase-chain reaction.

1. Introduction

The 41 amino acid polypeptide Corticotropin-releasing hormone (CRH) plays a crucial role in the regulation of various stress responses principally via activation of the hypothalamic-pituitary-adrenal (HPA) axis and regulation of autonomic, endocrine and behavioral responses (for a review, see [381]). Recently, a participation of CRH in modulation of neuronal degeneration has been proposed, although the exact role of the peptide remains elusive. For example, blockade of CRH receptors has been shown to reduce neuronal damage following traumatic brain injury [437], epileptic seizures [305], and cerebral ischemia [295,497]. In contrast, Fox et al. [152] reported dose-related protection against hypoxic insult in hippocampal neurons using either CRH or the antagonist α-helical CRH (9-41). More recently, Craighead et al. [110] showed no alterations of excitotoxic cellular damage following CRH administration both *in vitro* and *in vivo*. In a recent study, we also failed to observe changes in CA1 neuronal damage following 10 min global ischemia in rats pre-treated with the non-selective CRH receptor antagonist α-helical CRH (9-41) or CP154,526, a selective non-peptide $CRHR_1$ antagonist [407].

In recent years, numerous *in vitro* studies have suggested neuroprotective actions of CRH. CRH has been shown to prevent glutamate-induced neurotoxicity in rat organotypic hippocampal slices [138], protect neurons against amyloid-beta (Aβ) toxicity, and inhibit apoptotic cell death in hippocampal, cerebellar and cortical cultures following various oxidative insults [26,142,276,393,426]. To date, none of the existing studies have assessed the participation of CRH in modulation of neuronal damage following potassium cyanide (KCN) exposure, considered a chemical form of ischemia [3], which compromises cellular respiration and ATP production. One goal of the current *in vitro* study aimed to determine

the impact of CRH pre- and post-treatments against KCN-induced neuronal injury in rat primary cortical neurons.

Different physiological effects of CRH in the central and peripheral nervous systems are mediated via activation of the opioid system [68,69,454,455], and CRH stimulates the release of opioid peptides [7,366,367]. Conversely, other studies have demonstrated alterations of CRH synthesis and release in the brain by opioid peptides and receptors [52,70,365,505]. We recently have observed that CRH-induced protection against global cerebral ischemia in rats *in vivo* was receptor-dependent and opioid-mediated (unpublished observations). Thus, a second goal of the current thesis aimed to determine whether CRH neuroprotective effects on KCN-induced injury are altered by prior blockade of either CRH, delta- or kappa-opioid (DOR and KOR) receptors using α-helical CRH (9-41), naltrindole and nor-binaltorphimine, respectively. Finally, we assessed mRNA expression of enkephalin (ENK), dynorphin (DYN) and DOR and KOR in CRH-treated primary cortical neurons using reverse transcription polymerase-chain reaction (RT-PCR).

2. Methods

2.1. Primary cortical neuron culturing

Primary cortical neurons were cultured as described previously [208]. Briefly, pregnant Sprague-Dawley rats (Charles River Canada, St. Constant, QC, Canada) at E-15/16 were anesthetized with halothane and euthanized by cervical dislocation. Fetuses were decapitated and the cortical region dissected out and collected under sterile conditions. The tissue was then placed into 5 ml of Hank's Basic Salt Solution (HBSS)

(Gibco-BRL). Upon completion of tissue collection, 100 µl of Trypsin-EDTA solution was added to dissociate tissue connection for 25 min at 37 °C. Subsequently, Trypsin inhibitor 125 µl (Sigma) and DNase I (10 µg/ml) (Sigma) were added and incubated for 5 min at 25 °C. The cells were centrifuged at 1000 rpm for 5 min, and the supernatant removed. The cells were resuspended by triturating 10 times using a 5 ml pipette with the same mixture as above. Tissue and cells were allowed to settle for 5 min and the supernatant containing the neurons was transferred to a new tube. These were then centrifuged at 1000 rpm for 5 min at room temperature. After removal of the supernatant, the cells were resuspended in serum-free Neurobasal Medium (Gibco-BRL) supplemented with B-27, N-2, L-glutamine (0.5 mM) and a combination of penicillin (100 IU/ml) and streptomycin (100 µg/ml). The cells were plated on poly-D-lysine (100 µg/ml, Sigma) dishes and kept for 7 days *in vitro* (DIV) without replacing the culture medium in a humidified atmosphere of 95% air and 5% CO^2 at 37 °C. Cultures were grown for another 6 to 7 days, with one half of the medium being changed every 2 to 3 days, before being used in an experiment. For RNA isolation, cells were grown in 60 mm diameter culture dishes (2.5 × 106 cells/dish). At 12–14 DIV, all of the cells appeared healthy with no sign of crenated nuclei or axonal blebbing.

2.2. Treatments

Impact of CRH pre- and post-treatments on KCN-induced injury.

Pre-treatment

In order to determine the impact of pre-treatment with CRH (American Peptide Company) on KCN-induced chemical ischemia, cultures of rat primary cortical neurons

were treated with different doses of CRH for 24h (i.e., control, 2fmol, 200fmol, 2pmol, 20pmol, 200pmol, 2nmol). They were then exposed to potassium cyanide (KCN, 1 mM, 4h) and returned to normal medium for 24h prior to cell survival assay. The selected doses of CRH were chosen based on dose-related hippocampal protection against glutamate-induced neurotoxicity *in vitro* [138].

Post-treatment

Since higher cell survival level was obtained using the 20pmol and 2nmol doses of CRH in the pre-treatment conditions, these two doses were retained for the subsequent post-treatment analysis. Thus, cortical neurons were treated for 24h with CRH (20pmol and 2nmol) 3h or 8h following the KCN insult, and then returned to normal medium.

Impact of pre-treatments with α-helical CRH (9-41), nor-binaltorphimine and naltrindole on KCN-induced cell death and CRH-mediated protection.

To determine whether the protection conferred by CRH was receptor-dependent and mediated via activation of distinct opioid receptors, cortical neurons were treated with either the CRH receptor antagonist α-helical CRH (9-41) (1μM; Phoenix pharmaceuticals), the KOR antagonist nor-binaltorphimine (BIN; 10μM; Sigma) or the DOR antagonist naltrindole (NAL; 10μM; Sigma) for 1h prior to CRH (20pmol for 24h) administration 3h or 8h post-KCN insult. The doses of BIN and NAL were selected based on efficacy to reverse kappa opioid agonist-induced release of serotonin in hippocampal slices [113,114] and block delta-opioid induced protection against glutamate excitotoxicity in neocortical neurons [572], respectively. The α-helical CRH (9-41) dose was selected based on its ability to prevent CRH-induced hyperpolarization-activated cation current [422].

2.3 Cell survival determination

Twenty-four hours following the end of treatment, cell survival was determined by MTT assay (Cell Proliferation Kit I (MTT), Roche Applied Science, Mannheim, Germany). The assay was performed using manufacturer's instructions. Briefly, 10μl of MTT solution per 100μl of media was added to the cells and incubated for 4h at 37°C with 5% $C0_2$. Following this incubation, 100μl of solubilization solution was added to each well. The plate was allowed to stand overnight in the incubator at 37°C with 5% $C0_2$. The following day, plates were checked for complete solubilization of the purple formazan crystals and absorbance was measured using a spectraMax 340 microplate ELISA reader (Molecular Devices). The wavelength used to measure the absorbance of the product was 550 nm and the reference wavelength used was 690 nm.

2.4. mRNA expression of opioid peptides and their receptors in CRH-treated primary cortical neurons using RT-PCR

Opioid peptide (ENK and DYN) and receptor (DOR and KOR) mRNA expression was determined in cortical neurons cultures treated for 24h with different doses of CRH (i.e., control, 2fmol, 200fmol, 2pmol, 20pmol, 200pmol, and 2nmol). Total RNA was then isolated and subjected to reverse transcription followed by polymerase-chain reaction (RT-PCR) analysis.

2.5. Total RNA isolation

Primary cortical neurons were lysed in Trizol reagent (Invitrogen) and total RNA was extracted following the manufacturer's instructions. The concentration of the RNA was determined by measuring the absorbance at 260 nm.

2.6. Reverse transcription and PCR

Total RNA (2 μg) from the primary cortical neurons was subjected to reverse transcription for 60 min at 42 °C using SuperScriptTM II (200 units) and First Strand Buffer (Invitrogen), in the presence of 0.5 μg/μl oligo dT, 10 mM DTT and 1 mM dNTP mix. Amplification of cDNA (1 μl) obtained from RT was then performed in ThermoPol Buffer (NEB), containing 1 mM dNTP mix, in the presence of 5 μM of each specific primer and 2.5 units of Taq DNA polymerase (NEB). Control samples lacking reverse transcriptase were processed in parallel with the same experimental protocol. Amplification was performed in an Eppendorf thermocycler (Brinkmann) and PCR products were obtained after 33 cycles (typically 95 °C for 1 min, 1 min at an annealing temperature at 60 °C, 72 °C for 1 min), after an initial denaturation at 95 °C for 5 min. The PCR primers were designed according to the published sequences from GenBank and synthesized by Applied Biosystems. The sense and antisense primers used to detect rat DOR, KOR, DYN and ENK mRNA in CRH-treated primary cortical neuronal cultures are reported in Table 1. Each sample was run in duplicate. cDNA was also amplified with GAPDH primers (sense 5′-CAT GGC CTT CCG TGT TCC TAC CC-3′; antisense 5′-CCT CGG CCG CCT GCT TAC-3′) as a loading control. Five microliter aliquots of the PCR products were size-separated by electrophoresis on a 2% agarose gel.

Table 1 : The sense and antisense sequences, annealing temperatures, source and product lengths for primers used to detect KOR, DOR, DYN and ENK in CRH-treated cortical neurons.

Primers	Tm (°C)	Source	cDNA (bp)
KOR			
(S) 5'- CCAGCCTGTACATTTCTCAAGGA -3'	60	Rattus norvegicus opioid receptor, kappa 1, mRNA	71
(A) 5'- AATATCCCAGCATGAACTTTCCA -3'		NM_017167	
DOR			
(S) 5'- AGTGGTATGCACGCTCCAGTT -3'	60	Rattus norvegicus opioid receptor, delta 1, mRNA	74
(A) 5'- AGAGGAACACGCAGATCTTGGT -3'		NM_012617	
DYN			
(S) 5'- CATCAGCCCAACCGCTCTAT -3'	60	Rattus norvegicus prodynorphin, mRNA	70
(A) 5'- GGTACAAAGACAAGGTTCTCTGGATT -3'		NM_019374	
ENK			
(S) 5'- CCTCCGACCTGCTGAAAGAG-3'	60	Rat enkephalin mRNA, complete cds	72
(A) 5'- TGCTTTCCTGTTGGTGGCTAT-3'		M28263	

3. Results

3.1. Pre- and post-treatments of cultured primary cortical neurons with CRH significantly reduces KCN-induced neuronal death.

Figure 1 shows the effect of CRH pre-treatment on the survival of cultured cortical neurons subjected to a KCN insult. Results are expressed as mean percentage of living neurons ± SEM. A one-way ANOVA (SPSS, 15.0) performed on all KCN-treated cultures demonstrated a significant effect of treatment ($F_{(6,21)}$=187.160, p<.001). A planned comparison revealed a significant decrease in the percentage of living cells in KCN-treated cultures as compared to that of untreated cultures ($t_{(6)}$=39.421, p<.001). Bonferroni-adjusted pair-wise comparisons showed that pre-treatment with 200fmol, 2pmol, 20pmol, 200pmol or 2nmol of CRH significantly reversed the KCN-induced neuronal death (p<.001). In contrast, treatment with the lowest dose of CRH (2fmol) failed to reduce KCN-induced cell death.

The effect of CRH (20pmol or 2nmol) administered following the KCN insult on cultured cortical neurons is shown in Figure 2. A one-way ANOVA performed on all KCN-treated cultures demonstrated a significant effect of treatment ($F_{(4,15)}$=32.0350, p<.001). There was a significant decrease in the percentage of living cells in KCN-treated cultures compared to that of untreated cultures ($t_{(6)}$=10.081, p<.001). Bonferroni-adjusted post-hoc tests revealed that treatment with both doses of CRH either 3 or 8h following the KCN insult significantly reduced cortical neuronal death (p < .05).

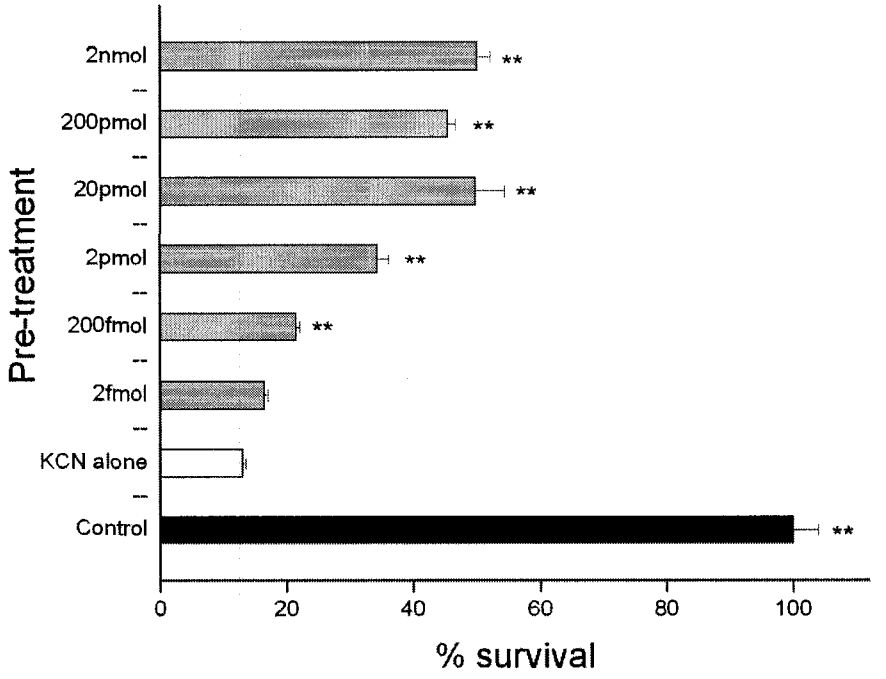


Figure 1: Pre-treatment with CRH dose-dependently significantly increased neuronal survival of KCN-treated cultured cortical neurons. ** Indicates significant different from KCN-treated cultures ($p < .001$).

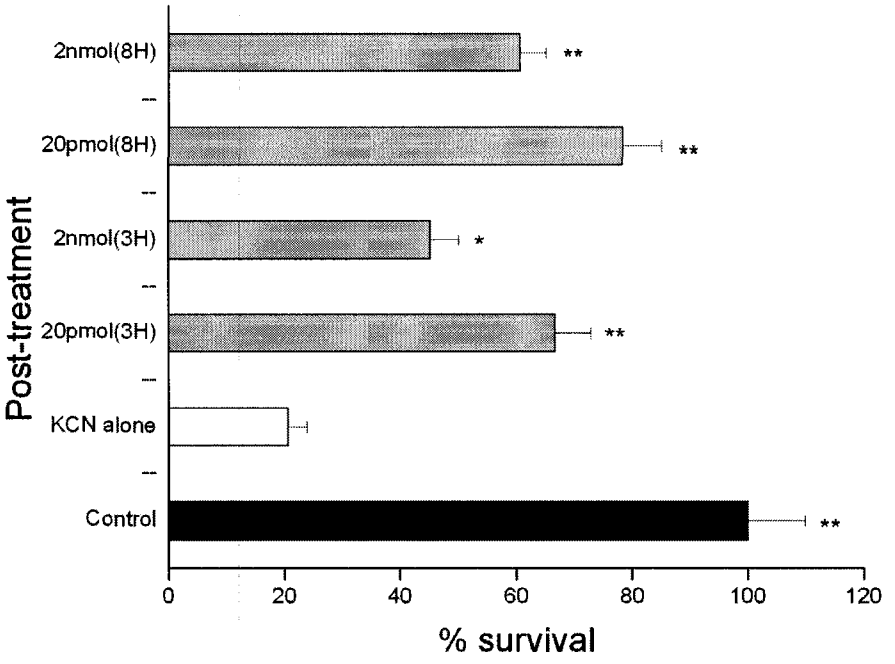


Figure 2: Treatment with CRH (20pmol or 2nmol) either 3 or 8h following the KCN insult significantly reduced cortical neuronal death. *'** Indicates significant different from KCN-treated cultures ($p < .05$ and .001, respectively).

3.2 Blockade of CRH, kappa- and delta-opioid receptors prior to CRH (20pmol) administration significantly reduces the protection conferred by this neuropeptide on cultured cortical neurons against chemical ischemia.

The effect of CRH receptor blockade prior to CRH administration is presented in Figure 3. Statistical analysis involved planned comparisons in the form of pair-wise t tests with Bonferroni correction. As the omnibus results were of no interest, one-way ANOVAs were not conducted [221]. Statistical analysis revealed that treatment with CRH 3h and 8h following the KCN insult significantly increased the percentage of living cells ($t_{(6)}$=5.716, p=.001 and $t_{(6)}$=3.520, p=.013, respectively). In addition, treatment with α-helical CRH (9-41) prior to CRH administration significantly decreased the protection conferred by the neuropeptide administered 3 or 8h post-KCN insult ($t_{(6)}$=4.798, p=.003 and $t_{(6)}$=4.973, p=.003, respectively). To determine whether α-helical CRH antagonist (9-41) had any intrinsic effects on neuronal density, we tested the antagonist alone and in combination with KCN and compared neuronal survival with that observed in cortical neurons treated with KCN (positive control) or not (negative control). Our findings demonstrated no intrinsic activity of α-helical CRH and suggest that the beneficial effects of CRH are dependent on interaction of the peptide with its receptors.

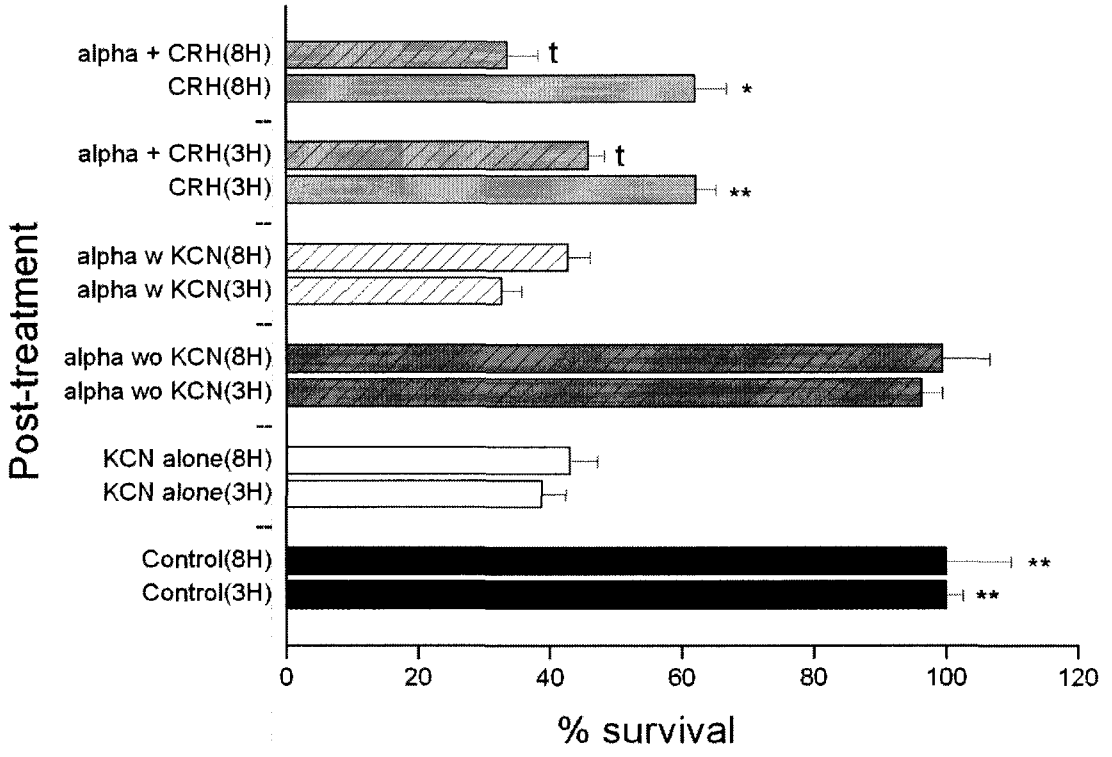


Figure 3: Treatment with the non-selective antagonist α-helical CRH (9-41) prior to CRH administration significantly decreased the protection conferred by this neuropeptide administered 3 or 8h post-KCN insult. *'** Indicates significant different from KCN-treated cultures ($p < .05$ and .001, respectively); ^{t} Indicates significant different from CRH-treated cultures ($p < .05$).

The effects of blockade of DOR on CRH-induced protection are presented in Figure 4. Administration of NAL had no intrinsic effect on neuronal density. The analysis demonstrated that treatment with CRH 3h and 8h following the KCN insult increased the percentage of living cells ($t_{(6)}$=6.307, p=.001 and $t_{(6)}$=2.716, p=.035, respectively), although the level of protection obtained at the 8h time interval did not reach significance with Bonferroni correction. The analysis also revealed that treatment with NAL prior to CRH administration reduced neuronal survival induced by the 3 or 8h post-ischemic CRH administration ($t_{(6)}$=5.214, p=.002 and $t_{(6)}$=2.576, p=.042, respectively), although NAL inhibition of CRH-induced neuronal survival at the 8h time interval did not reach significance when corrected using Bonferroni test.

Figure 5 illustrates the effect of KOR blockade on neuronal density and CRH-induced protection. The analysis demonstrated that treatment with CRH 3h and 8h following the KCN insult increased the percentage of living cells ($t_{(6)}$=5.731, p=.001 and $t_{(6)}$=3.738, p=.010, respectively). In addition, treatment with BIN prior to CRH administration significantly reduced the percentage of neuronal survival induced by CRH when administered 3h following the KCN insult ($F_{(5)}$=23.779, p=.005). In contrast, KOR blockade failed to reverse the CRH-induced protection at the 8h time interval ($F_{(6)}$=2.055, p=.202). Similar to NAL, BIN showed no intrinsic effects on neuronal density. These results suggest that the protection produced by CRH appears mediated in part via activation of DOR and KOR and suggest that opioid receptors preferentially contribute to neuroprotective mechanisms activated when CRH is administered 3h following KCN exposure.

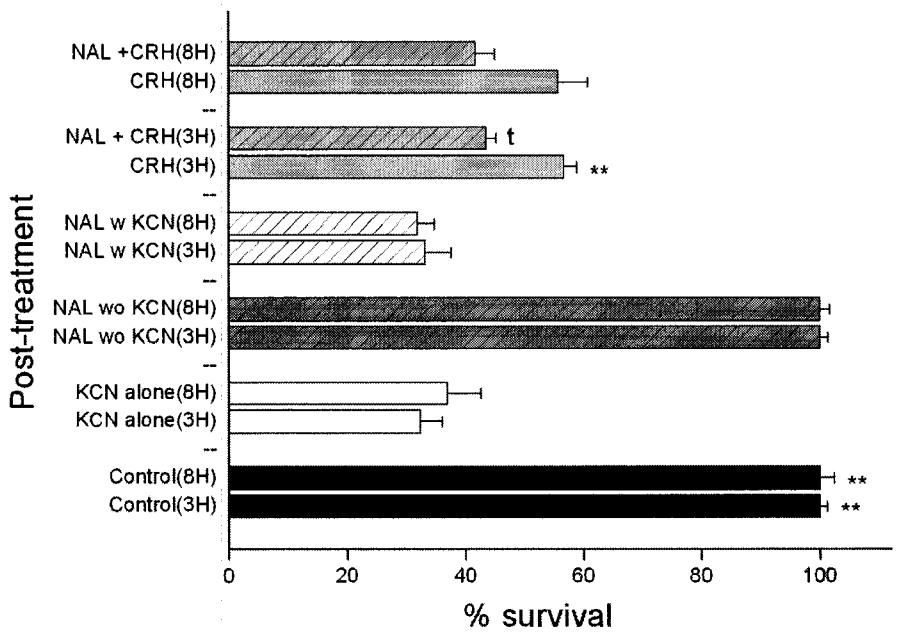


Figure 4: Treatment with the DOR antagonist NAL prior to CRH administration significantly decreased the protection conferred by this neuropeptide administered 3h post-KCN insult. ** Indicates significant different from KCN-treated cultures ($p < .001$); ^{t} Indicates significant different from CRH-treated cultures ($p < .05$).

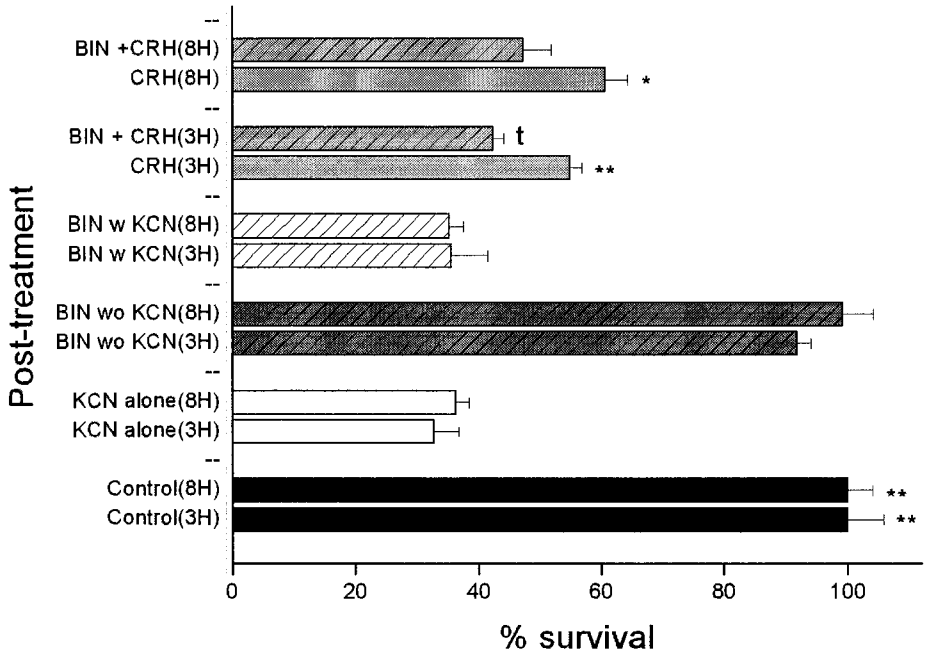


Figure 5: Treatment with the KOR antagonist BIN prior to CRH administration significantly decreased the protection conferred by this neuropeptide administered 3h post-KCN insult. *'** Indicates significant different from KCN-treated cultures ($p < .05$ and .001, respectively); ^{t} Indicates significant different from CRH-treated cultures ($p < .05$).

3.3 Treatment of cortical neurons with CRH increases the mRNA expression of opioid peptides and receptors.

Figure 6 presents the changes in mRNA expression of opioid peptides and their receptors in CRH-treated primary cortical neurons using RT-PCR. Expression of ENK responded to CRH treatment in an inverted-U shape manner, the lowest and highest dose tested (2 fmol and 2 nmol, respectively) having no impact, while the middle doses (200 pmol, 20 pmol, 2 pmol and 200 fmol) increased its expression. The mRNA levels of DOR increased with addition of CRH to the cultures at all concentrations with the exception of the lowest dose (2 fmol). In contrast, mRNA levels of DYN increased solely after treatment with 200 fmol CRH. Finally, all doses of CRH slightly increased expression of KOR.

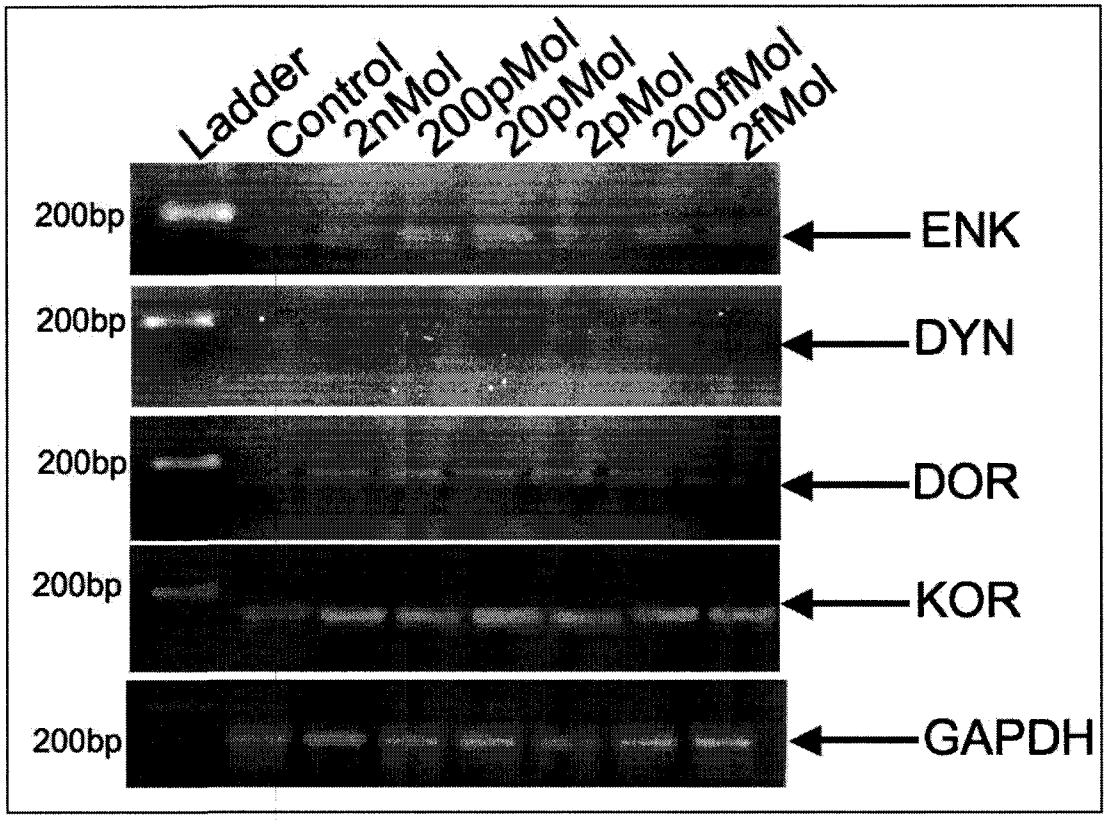


Figure 6: Alteration of opioid peptide and receptor mRNA expression following treatment with CRH in primary cortical neurons.

4. Discussion

In the present study, both pre- and post-treatments with CRH significantly enhanced survival of primary cortical neurons *in vitro* in a model of neuronal injury using KCN. Our findings demonstrated protective CRH effects initiated at low physiological CRH dose (200fmol), with maximal neuronal protection achieved with the 20pmol CRH dose. These findings are consistent with CRH dose response profiles (10^{-9}-10^{-12} mol/L) reported by *in vitro* studies using oxidative stress or glutamate-induced neurotoxicity in primary cerebellar neurons or organotypic hippocampal slices, respectively [138,276], and suggest that submaximal receptor activation appears sufficient to promote survival of injured neurons. Findings from the present study also suggest that CRH maintains comparable efficacy when administered at remote time intervals following KCN administration. Thus, the 20 pmol CRH dose administered 8h after the 4h KCN insult led to ~ 30-50% enhanced survival in primary cortical neurons as compared to neuronal damage in KCN treated cortical cultures. Similarly, Elliott-Hunt et al. [138] reported maximum neuroprotective effects when CRH was added 8-12 h after a 3h excitotoxic glutamate insult in hippocampal slices. This suggests that a cascade of physiological processes is initiated before neuronal death is irreversible.

Blockade of CRH receptors using 1μM of α-helical CRH (9-41) prior to CRH (20pmol) administration significantly reduced neuronal protection, suggesting that this effect is mediated via CRH receptor activation. Since this antagonist is non-selective, it is not possible to isolate the role of the $CRHR_1$ and $CRHR_2$ subtypes to the observed protection. Recently, type 1 CRH receptors have been shown widely expressed in cortical neurons and capable of activating multiple G-proteins, suggesting that CRH can regulate

multiple signalling pathways in the rat cerebral cortex [180]. Interestingly, recent *in vitro* studies suggested that $CRHR_1$ is the principal receptor subtype mediating CRH-induced neuroprotective effects [142,276,394]. CRH protection was also shown dependent of PKA- [26,276,393,394,426], and/or PKC- [394] activation. *In vitro*, activation of mitogen-activated protein kinase (MAPK) [138,394], suppression of NF-kB [276] and stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) [138], as well as stabilization of calcium homeostasis [393] have also been implicated in CRH action. The contribution of these endogenous mechanisms to CRH-induced neuronal protection against KCN injury remains to be established.

The present study assessed the possibility that CRH protective actions be mediated via interaction with the opioid system. This hypothesis was based on findings showing that opioid peptides and receptors enhanced neuronal survival following a variety of insults. Thus, DYN was shown to significantly inhibit lipopolysaccharide (LPS)-induced neurotoxicity in mixed cortical neuron/glia cultures [258], and reduce neuronal damage [24] and memory deficits [235] *in vivo* following cerebral ischemia. Similarly, DOR activation has been shown to protect cortical neuronal cultures against hypoxic- [571] and glutamate-induced injury [572], and naltrindole increased hypoxia-induced neuronal injury, an effect reversed by DOR agonist administration [571]. Numerous *in vitro* and *in vivo* studies also demonstrated neuroprotective actions of KOR against various excitotoxic insults as well as focal and global ischemic damage [22,105,190,264,427,552].

In addition to these effects, CRH and opioids interactions have been shown in various physiological systems. In the brain, CRH has been shown to stimulate the release of endorphin (END) and DYN [7,366], as well of ENK [367] in the rat hypothalamus *in vitro,* a

phenomenon blocked by pre-treatment with α-helical CRH (9-41) [368]. CRH has also been found to induce the release of Met-ENK and DYN from the neostriatum *in vitro* and *in vivo* in a dose-related and receptor-specific manner [481]. In the present study, blockade of DOR and KOR, using NAL or BIN (10μM), reduced the neuronal protection conferred by CRH administration 3 and 8h post-KCN insult, although this effect was significant only at the 3h post-injury interval. These results suggest that CRH-induced protection is partly mediated through activation of DOR and KOR.

In support of the previously described findings, results from RT-PCR revealed that treatment of primary cortical neurons with several doses of CRH influenced the mRNA expression levels of ENK, DOR and KOR. This appeared less evident in the case of DYN. These observations suggest that CRH may attenuate cell death in rat primary cortical neurons in part through upregulation of discrete opioid peptide and receptor gene expression. The addition of Western blotting showing time-dependent alterations of protein expression for DYN, ENK, DOR and KOR following CRH administration would help confirm the participation of these endogenous signals and receptors as modulators of CRH-induced cortical protection.

In conclusion, results from this study represent the first demonstration of protection produced by CRH pre- and post-treatments following a KCN insult. Our findings further suggest that the CRH-induced beneficial effects involve the release of endogenous opioid ligands and activation of opioid receptors. Additional studies examining interactions between CRH and opioid peptides will help clarify the discrete contribution of each peptidergic system in the modulation of neuronal protection.

General discussion

The main objective of this thesis was to examine the potential role of CRH in global ischemia. The current thesis examined the impact of CRH on neuronal survival following global ischemia and determined dose- and time-dependent CRH actions *in vivo*. Findings from the current thesis demonstrated a clear neuroprotection produced by CRH against ischemic damage.

The second goal of this thesis was derived from the co-localization of CRH with opioid peptides and the neuroprotective actions of opioid peptides. We first examined the effects of manipulating the opioid systems and found that kappa and delta receptor activation protected neurons following global ischemia and led to functional sparing. We also found that CRH neuroprotection could be mediated by the activation of opioid peptides and their receptors. In the next sections, we will summarize and discuss the specific results obtained.

CRH exerts neuroprotective actions against ischemic damage in vivo.

Our findings demonstrate that pre-treatment with 5μg of CRH 15 min prior vessel occlusion conferred significant protection against damage to the hippocampal CA1 pyramidal neurons (≈ 37% increase in CA1 neuron survival compared to vehicle-treated ischemic rats). This effect appeared receptor-dependent, as non-selective blockade of both CRH receptors using the antagonist α-helical CRH (9-41) (25μg) abolished the protection conferred by CRH.

These findings represent the first *in vivo* demonstration of CRH-induced protection against global cerebral ischemia in rats and challenge the CRH neurotoxic role

in ischemia initially proposed [295,497]. Our results are consistent with various *in vitro* studies that have demonstrated CRH neuroprotection against neurotoxic insults [26,138,276,393,394].

CRH is neuroprotective when infused 8h after ischemia.

We show for the first time significant reduction of CA1 neuronal injury in ischemic rats injected i.c.v. with 5μg of CRH 8h but not 3h following global ischemia (≈ 61% increase in CA1 neuron survival). These CRH-injected ischemic animals also showed enhanced spatial memory performance in the 8-arm radial maze. The observation of reduced neuronal death together with improved spatial memory performance provides strong support for a neuroprotective role of CRH. Our results are consistent with the time window of opportunity reported for CRH neuroprotective actions in organotypic hippocampal cultures [138]. The *in vivo* CRH beneficial effects is also consistent with the modulation by CRH of delayed signalling reactions associated with apoptosis [142,426].

The observation of neuroprotective effects upon delayed post-ischemic CRH infusion is clinically important because the known effective pharmacological therapies must all be administered within 3h of symptom onset, while CRH appears effective up to 8h providing a potentially much improved window of opportunity for treatment. Since CRH infused 3h post-ischemia did not alter ischemia-induced CA1 neuronal death, this early 3h post-ischemic time could be used for treatments that aim to restore blood flow (clot busters) while CRH could be used at 8h post-ischemia to protect neurons.

CRH effects are modulated by opioid systems.

The initial step in the analysis of potential interactions of CRH with the opioid system was to replicate, using our global ischemic model, the neuroprotective effects previously reported with KOR agonists in various models of focal and global ischemia [22,105,190,264,427,552]. All these reports have focussed on neuronal protection observed within 8 days following ischemia. Only one study reported significant CA1 neuronal protection at 47 days following global cerebral ischemia in rats using a different kappa agonist PD117302 [168], and the impact of such treatment on spatial memory function was not determined.

We found that pre-ischemic administration of 30mg/kg of U50,488H produced a significant CA1 neuronal protection (≈ 65% increase in CA1 neuron survival compared to vehicle-treated ischemic rats) up to 35 days following global ischemia. This protective effect was accompanied by a significant improvement of spatial memory in the 8-arm radial maze. In the past, various therapeutic approaches have been shown to gradually lose their beneficial impact with prolonged reperfusion intervals (for a review, see [108]). Thus, KOR-induced long-lasting hippocampal protection and the significant improvement of memory impairments following global ischemia together suggest a promising therapeutic avenue.

In vitro and *in vivo* studies have supported the protective effects of DOR stimulation in cardiac ischemia which appeared mediated through the activation of the opioid delta1 receptor subtype [323,324,391,457,542]. Our findings indicate that the pre-ischemia injection of the selective delta1-opioid receptor agonist, DPDPE, conferred a dose-dependent neuroprotection of CA1 neurons (5μg: ≈ 59% increase in CA1 neuron survival

compared to vehicle-treated ischemic rats). These results support the involvement of delta1-opioid receptors in the cascade of events regulating CA1 neuronal damage following global ischemia in rats.

Blockade of specific opioid receptors alters CRH-induced hippocampal damage and functional recovery in ischemic rats.

Our results revealed that prior blockade of DOR or KOR (i.e., using naltrindole (20μg) or nor-binaltorphimine (30μg), respectively) significantly reduced CRH (5μg)-induced CA1 neuroprotection in ischemic animals. These findings represent the first *in vivo* demonstration of opioid modulation of CRH-induced CA1 neuronal protection following cerebral ischemia in rats. These observations are consistent with the observation that CRH actions involve the activation of the opioid system in the peripheral nervous system [297,298,350]. Together, these results further suggest close relationships between the two peptidergic systems in the regulation of normal as well as pathological states, both within the CNS and the periphery.

CRH confers protection against KCN-induced injury in primary cortical neurons in vitro.

We demonstrated *in vitro* that both CRH pre- (200fmol to 2nmol) and post-(20pmol and 2nmol, 3 and 8h post-KCN insult) treatments significantly reduced KCN-induced cortical injury. The blockade of CRH receptors using 1μM of α-helical CRH (9-41) prior to CRH (20pmol) administration significantly attenuated the CRH neuroprotection, suggesting again a receptor-dependent effect. Blockade of KOR and DOR (i.e., using nor-binaltorphimine (10μM) and naltrindole (10μM), respectively) prior

to CRH (20pmol) treatment reduced the level of protection obtained with CRH at the 3h time interval, supporting the idea that CRH-induced protection is partially mediated through the release of opioid peptides and/or the stimulation of discrete opioid receptor subtypes. The use of hippocampal neuronal cultures and organotypic hippocampal slices in future studies involving KCN administration could confirm the existence of similar interactions in hippocampal neurons. These findings expand on previous *in vitro* observations, and suggest that CRH promotes cellular survival in different injury models known to induce necrotic and apoptotic cell death via similar excitotoxic pathways. They also suggest that similar to what was observed in hippocampal neurons *in vivo,* opioid peptides act as a neuromodulator of CRH-induced protection of cortical neurons *in vitro.*

Potential mechanisms mediating CRH-induced protection

Both *in vivo* and *in vitro* findings in the present thesis demonstrate that the neuroprotective effects of CRH depend on specific interactions of the peptide with its receptors. The antagonist α-helical CRH (9-41) being non-selective, it is not possible to isolate the role of both receptor subtypes to the observed protection. *In vitro*, several studies suggest that $CRHR_1$ is the main receptor mediating the CRH-induced neuroprotective effects [142,276,394]. Recently, we have shown that pre-treatment with the selective non-peptide $CRHR_1$ antagonist CP154,526 did not alter ischemia-induced damage in CA1 pyramidal neurons [407]. However, in that study, the 10-min ischemia had left very few surviving CA1 neurons in both saline- and CP154,526-treated ischemic animals, which made it impossible to determine if blockade of $CRHR_1$ increased ischemic damage.

In the heart, urocortin administration before and after the ischemic insult has been shown to enhance myocyte survival against hypoxic/ischemic injury [58,59,374] and reduce infarct size in isolated rat hearts [58]. *In vitro*, administration of urocortin, which binds $CRHR_2$ with 40-fold greater affinity than $CRHR_1$ [527], significantly inhibited oxidative- and excitotoxic-induced cell death [142,394]. Although these results suggest that urocortin confers neuroprotection only via $CRHR_2$, recent evidence suggests otherwise since treatment with the highly selective $CRHR_1$ antagonist CP154,526 reversed the protective effects induced by urocortin [142].

Future experiments will have to test the effect of $CRHR_1$ antagonists in ischemic animals with less neuronal damage. Once commercial agonists selective to $CRHR_1$ are available, the contribution of $CRHR_1$ to ischemia-induced neuronal damage or survival can be fully tested.

Molecular processes in CRH-induced neuroprotective effects

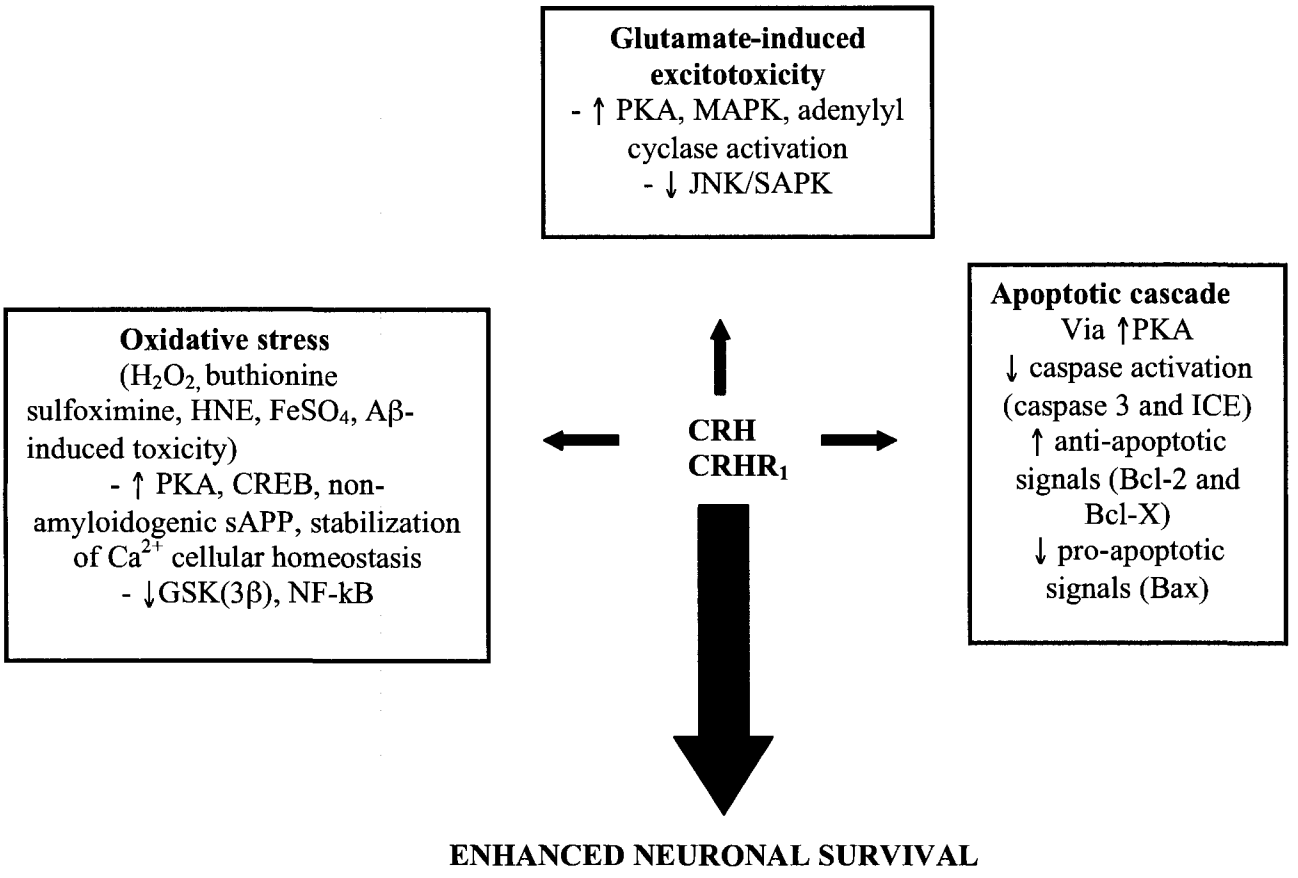
Over the last decade, *in vitro* studies have addressed the participation of distinct molecular processes to CRH neuroprotective actions. In a recent study, Bayatti et al. [26] have demonstrated protection against amyloid β (Aβ)-induced toxicity in hippocampal and cerebellar cultures using low doses of CRH (10^{-8}M), while higher CRH concentrations (10^{-7}M) led to protection of cortical neurons. This protection appeared dependent on protein kinase A (PKA) activation, induction of cAMP response element-binding protein (CREB) and inhibition of glycogen synthase kinase (GSK)3β expression. Consistent with these findings, Facci et al. [142] observed a CRH-induced inhibition of Aβ-induced apoptotic death in cortical, hippocampal and cerebellar granule neurons. This

inhibition was also observed with related peptides such as urotensin I, urocortin and sauvagine (0.3-1.2nM) and involved cAMP-dependent phosphorylation of (GSK)3β. Using lower picomolar CRH concentrations (10pM), Pedersen et al. [393] also reported hippocampal and cortical neuronal protection against Aβ, lipid peroxidation product 4-hydroxynonenal (HNE), ferrous sulfate ($FeSO_4$) and glutamate-induced cell death. CRH-induced protection was mediated through PKA activation. The authors also found that CRH stabilizes calcium homeostasis by preventing the Aβ-induced increase in intracellular calcium levels. Urocortin (0.5-5.0 pM) protection against excitotoxic hippocampal damage involves PKA activation in conjunction with other enzymatic processes involving protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) activation [394]. Lezoualc'h et al. [276] demonstrated that CRH-induced (10^{-8}M) neuronal protection against various oxidative insults (Aβ, H_2O_2, buthionine sulfoximine) is dependent on PKA activation, inhibition of NF-kB factor transcription and enhanced release of non-amyloidogenic soluble amyloid β precursor protein (sAPP). Recently, Elliott-Hunt et al. [138] demonstrated inhibition of glutamate-induced neurotoxicity in organotypic hippocampal slices using physiological CRH and urocortin concentrations (2pM). The observations that low picomolar concentrations of CRH and related peptides protect against a wide range of oxidative and excitotoxic further support the physiological involvement of these molecules in regulation of cell injury.

Interestingly, Elliott-Hunt et al. [138] have demonstrated that CRH-induced protection in organotypic hippocampal slices was mediated via adenylyl cyclase and MAPK activation, and the inhibition of glutamate-induced stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) stimulation. These pro- or anti-apoptotic

protein kinase pathways are known to play a critical role in neuronal degeneration, in part through the modulation of the expression of different caspases (for a review, see [222]). Thus, caspase inhibition has been demonstrated to be protective against focal [91,140,150,277,546] and global [214] ischemia. Recent evidence also suggests that CRH suppress apoptosis in Y79 human retinoblastoma cells at a site upstream of activation of procaspase-3, an effect mediated through PKA activation [426] as well as via inhibition of phosphatidylinositol kinase-3 pathway [142]. Induction of similar anti-apoptotic mechanisms modulating CRH actions in cerebral ischemic models *in vivo* remains to be determined. As summarized in Figure 1, the proposed physiological mechanisms mediating CRH-induced neuronal protection are as follow. CRH could confer protection against glutamate-induced toxicity through activation of PKA, MAPK, adenylyl cyclase and inhibition of JNK/SAPK. In addition, CRH could protect neurons from oxidative stress via activation of PKA, induction of CREB, enhanced release of sAPP, stabilization of Ca^{2+} cellular homeostasis, and inhibition of NF-kB factor transcription and of (GSK)3β expression. Finally, CRH could prevent apoptotic death by inhibiting caspases activation and pro-apoptotic signals, as well as by enhancing anti-apoptotic signals.

Figure 1 Summary of the proposed physiological mechanisms mediating CRH-induced neuronal protection.



Adapted from: Bayatti, N. & Behl, C. (2005). The neuroprotective actions of corticotropin releasing hormone. *Ageing Research Reviews, 4,* 258-270, p. 266.

Neuromodulatory actions of CRH and opioid peptides in the hippocampus

The most severely damaged area of the brain in response to global ischemia is the pyramidal CA1 region of the hippocampus. CRH receptors, particularly $CRHR_1$, are found in CA1 and CA3 pyramidal neurons [75,90,526]. Recently, Chen et al. [89] demonstrated that hippocampal CRH was exclusively located in a subset of GABAergic somata, axons and boutons, whereas $CRHR_1$ resided mainly on dendritic spines of pyramidal cells. Although CRH is localized in inhibitory interneurons, the documented functions of CRH in the hippocampus include facilitation of excitatory activity through inhibition of slow afterhyperpolarizations (sAHP's) [5,486]. Thus, CRH has been shown in a number of instances to significantly enhance the firing rate of hippocampal CA1 pyramidal neurons [477]. In addition, CRH plays a role in long-term potentiation (LTP), by producing a long-lasting enhancement of hippocampal synaptic efficacy [39,536,537]. $CRHR_1$ also appears to mediate hippocampal activation of excitatory neurotransmitters, including serotonin (5-HT) and norepinephrine (NE) [234,398]. These findings would first lead us to conclude that CRH could possibly induce excitatory and potentially neurotoxic effects in the CA1 region of the hippocampus. However, neuronal excitation does not necessarily imply neuronal death and/or detrimental effects. For instance, LTP (for a review, see [353]), 5-HT (for a review see [66]) and NE [147] all play a significant role in learning and memory. Being localized on inhibitory interneurons, CRH could also induce its protective effects by stimulating GABAergic transmission and inhibiting ischemia-induced glutamate release and neuronal degeneration [17,267]. Inhibitory CRH effects have also been reported at the hippocampus. Thus, administration of CRH or CRH agonists has been shown to diminish the amplitude of hippocampal population spike and prevent the onset of LTP *in vitro* [428].

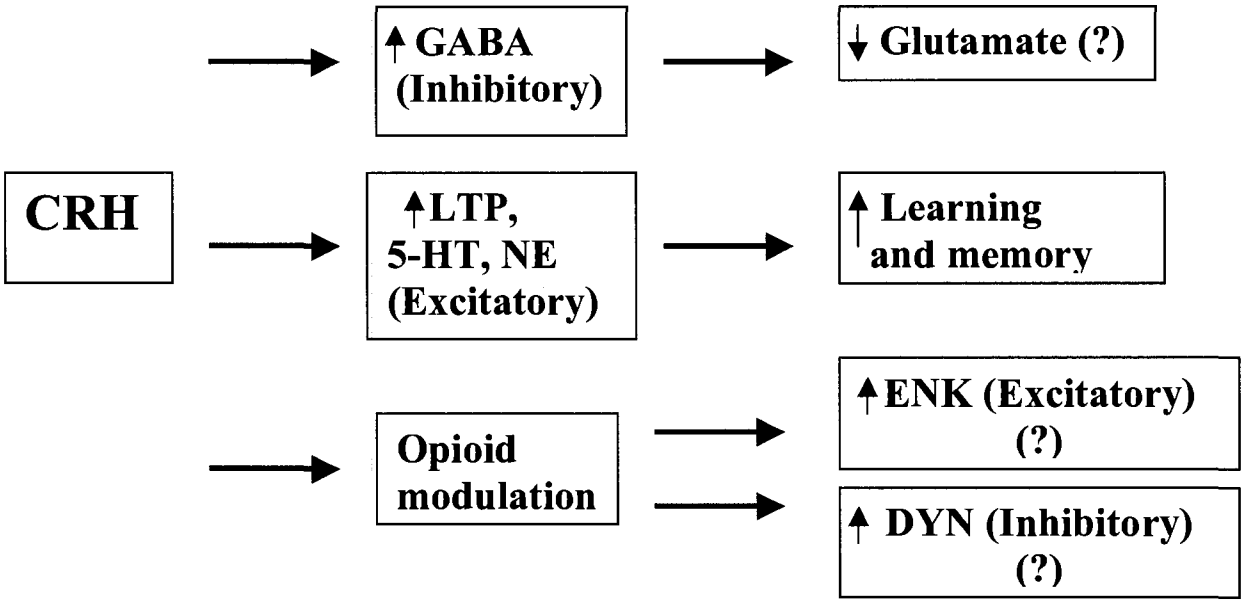
In addition, CRH produces a rise in hippocampal mineralocorticoid receptors (MR), which inhibit HPA activity [170]. Thus, CRH exerts a wide-range of excitatory and inhibitory action at the hippocampus. The physiological significance of these effects on hippocampal pyramidal neurons remains to be elucidated.

CRH being present in numerous loci of the brain, extrahippocampal CRH could well play an indirect role to modulate hippocampal neuronal excitability following ischemia. High densities of CRH-containing cell bodies are present in the central nucleus of the amygdala (CeA) [501], and significant *in vivo* increase in CRH release has been reported at the CeA nuclei combined to time-dependent alterations of CeA-CRH levels in post-mortem tissue in response to global ischemia [249]. The amygdala also plays a significant role in HPA function and could influence other CRH-mediated activities including glucocorticoid release [146].

The opioidergic system also exerts a wide-range of neuromodulatory actions in the hippocampus. In contrast to most brain areas where endogenous opioids decrease the excitability of neurons, ENK elicit potent excitatory responses within the hippocampal CA1 pyramidal cells [131,137,185,213,360,364,577]. This effect does not result from a direct action on pyramidal neurons, but rather occurs through a disinhibitory mechanism produced by a reduction in GABA-mediated inhibitory transmission from interneurons [99,131,293,304,360,363,382,430,478,577]. ENK's inhibition of inhibitory post-synaptic potentials has also been reported in the CA3 subfield of the hippocampus [73]. Similar to what has been observed with hippocampal CRH signals, ENK have been implicated in the modulation of long-term potentiation at mossy fiber/CA3 pyramidal cell synapse [126,318].

In contrast, DYN exerts produces inhibition in the dentate gyrus and hippocampal pyramidal CA1 and CA3 cell layers [73,303,346,533,543], and reduces long-term potentiation in dentate gyrus mossy fibers [543]. In addition, DYN promotes the induction of inhibitory afterpotentials following electrical stimulation of synaptic afferent in the dentate gyrus and CA1 cells in rats [359]. Considering that both DYN [235] and ENK [71,508] confer ischemic protection, but that these endogenous ligands have opposite modulatory effects at the hippocampus, it is possible that they confer protection through different extra-hippocampal mechanisms. It is also possible that these inhibitory or excitatory effects do not directly play a role in the modulation of hippocampal neuronal death. A summary of the potential neuromodulatory actions of CRH at the hippocampus is presented in Figure 2.

Figure 2 Summary of possible CRH neuromodulatory actions at the hippocampus.



Global ischemia and behavior: Is neuroprotection associated with improvement of ischemia-induced behavior and memory impairments?

Despite the importance of the reduction of ischemia-induced neuronal damage, recovery of function remains the primary goal for any ischemic patient. For these reasons, the reduction of memory impairments in the radial maze following treatment with CRH (5μg) administered 8h after the ischemia is clinically very relevant. The attenuation of memory impairments was related to hippocampal protection rather than to acute memory enhancement induced by CRH itself because of the fact that the drug was administered 8h after ischemia and the beneficial effects of CRH were observed many days thereafter.

Consistent with this assumption, ischemic rats treated with CRH (5μg) 3h following vessel occlusion showed decreased neuronal density in the CA1 subfield of the hippocampus and made a comparable number of working memory errors as vehicle-treated ischemic rats. Similar results were observed with KOR, where only rats treated with the highest dose of U50,488H (30 mg/kg) demonstrated preservation of neuronal density and displayed a concomitant enhancement of memory performance in the 8-arm radial maze. It is unlikely that a single dose of CRH and U50,488H administered several days before testing could have influenced memory performance. Rather, correlation analyses suggested that working memory performance was negatively related to the number of intact CA1 pyramidal neurons. These results suggest that CRH is capable of protecting hippocampal neurons and concomitantly improving spatial memory deficits in ischemic rats.

Usefulness of open field measures to evaluate behavioral impact of ischemia

Many studies have reported ischemia-induced hyperactivity in the open field and used this occurrence as a behavioral manifestation of ischemic damage [16,76,100,169,406,535,557]. However, in the present experiments, ischemic animals failed to demonstrate hyperactivity despite significant CA1 neuronal damage. This is not an unusual finding since Gulinello et al. [183] recently reported that global ischemia failed to change locomotor activity in the open field. Other studies have even reported reduced open field locomotor activity following bilateral carotid occlusion [164,558,559]. A number of factors may explain these divergent results.

One of them is related to the severity of CA1 neuronal damage. Ishibashi et al. [232] recently reported contrasting behavioral profiles in gerbils following single versus repeated instances of bilateral common carotid occlusion. Persistent locomotor hyperactivity was observed after two 7-min occlusions leading to selective bilateral neuronal death in the cerebral cortex, caudate nucleus and CA1 hippocampal fields. This persistent locomotor hyperactivity, reduced anxiety and severe cognitive deficits 4 weeks post-ischemia contrasted with the increased anxiety, transient locomotor hyperactivity and mild cognitive impairments observed after a single 5-min ischemic episode. Similarly, Gionet et al. [176] found no change of open field activity in rats with ischemic damage restricted to hippocampal CA1 neurons. Together, these results suggest that performance in the open field may be dependent on CA1 neuronal damage together with neuronal death in other extrahippocampal regions. This conclusion is also supported by the lack of correlation between behavioral activity level in the open field and CA1 neuronal density. Mumby et al. [354] also suggest that lesions of parahippocampal structures better explain

ischemia-induced object recognition deficits, long believed to depend only on disrupted hippocampal activity following ischemia [98,551,580]. Mumby [355] demonstrated that recognition performance was not altered in rats with complete hippocampus ablation, indicating that other brain loci played a critical role in this function. Other factors could also have accounted for the divergent open field results; however, they will require investigation in future studies. For example, these could include time elapsed between vessel occlusion and behavioral testing in the open field and possible interactions of anxiety and locomotion in the open field.

Strengths and limitations of the current studies

The experiments in the present thesis have a number of strengths and limitations. First, the inclusion of vehicle- and drug-treated sham animals in all *in vivo* experiments controlled for possible intrinsic effects of the specific drugs on hippocampal neuronal density and/or behavioral performance. Second, shams received the same surgical incisions as the ischemic group with the exception of electrocoagulation of the vertebral arteries. Twenty-four hours later, their carotid arteries were also exposed but not clamped. Thus, shams were under anesthesia for a time comparable to ischemic animals and received similar surgical procedures with the exception of vessel occlusion. This controlled for the stress associated to the surgical procedures that could conceivably have an impact on behavior and neuronal density. In experiments with i.c.v. administration of drugs, shams were also implanted with a cannula. All sham animals received either a vehicle or a drug treatment and were treated exactly like ischemic animals over the entire course of the studies with the exception of ischemia.

Despite these control procedures, some methodological limitations need to be addressed. All available models of ischemia present difficulties, one of them being the ability of getting reproducible degree of focal or global ischemic injury across animals [484]. Like others, we observed variability in the severity of CA1 damage between animals undergoing ischemia for 10 min. The permanent cauterization of the vertebral arteries and transient clamping of the carotids theoretically should result in a global ischemic episode, characterized by a cessation of cerebral blood flow throughout the entire brain. Behaviorally, this results in a loss of the righting reflex and bilateral pupil dilation during the ischemic episode. However, this is not always the case. No problems are associated with the exclusion of animals that wake up during the procedure: this indicates less than optimal ischemia due to incomplete carotid occlusion or incomplete vertebral arteries cauterization. However, incomplete ischemia can be observed even in animals that show a 10-min loss of righting reflex and bilateral pupil dilation as indicated by CA1 neuron sparing in ischemic animals. One possible explanation is the presence of vascular collateral branches that can supply some blood to the brain, a phenomenon that cannot be controlled.

A final possibility is that the pre-ischemic administration of CRH and drugs could have potentiated the behavioral signs of ischemia, for example by producing sedation, and thus masked an incomplete ischemia. However, this could not be the case in experiments where the CRH or drug injections were performed after ischemia. So, despite these limitations, the beneficial effects of CRH and its interaction with opioid systems appear genuine.

In general, animals that wake up before the 10 min is elapsed have less CA1 damage, while animals that undergo a period of coma following vessel occlusion display more severe damage. However, there are exceptions. Thus, it is extremely difficult to determine whether rats have had a complete ischemia before histology. In the current thesis, all ischemic animals that showed an absence of CA1 neuronal death in post-mortem neuronal assessment were eliminated from statistical analysis, independently of group. On the other hand, a number of vehicle-treated ischemic animals that showed higher CA1 neuronal density than the group averages were not discarded. This controlled for the possibility that the observed protection following any drug treatment could be attributable to an effect in animals with less severe ischemic insults.

Recording of electroencephalogram (EEG) activity can be done to monitor the level of neuronal activity during the ischemic procedure [562]. However, few researchers use this technique since the EEG procedure can interfere with the ischemic procedure: placement of electrodes on the dura using stereotaxic procedure may be uncomfortable for the animals and lead to faster awakening.

In closing, variable ischemia is actually more representative of the clinical reality since stroke patients show a variable pattern of neuronal damage. Moreover, some treatments may work better when neuronal damage is not too extensive but not when the ischemic insult results in almost complete neuronal destruction.

Another limitation that should be addressed in future studies concerns a better monitoring and regulation of brain and body temperature during ischemia and in the hours following reperfusion. Regulation of core and brain temperature is an important factor because hypothermia during ischemia (a few Celsius below normal temperature) has been

associated with less behavioral changes and an increased neuronal protection [103]. Since hypothermia is induced by various pharmacological treatments, these may produce their beneficial effects through a non-specific reduction of temperature rather than by pharmacological action on neurons.

The following data is available for some of the drugs used in the present thesis. Itoh et al. [236] have shown that U50,488H (30mg/kg) does not influence body temperature in sham animals and completely blocked the hypothermia produce by ischemia itself, suggesting that the beneficial effect obtained was due to the activation of the kappa agonist rather than being mediated through hypothermia. Evidence also suggests that DPDPE (4.65 nmol i.c.v.) does not alter body temperature [193,194]. On the other hand, CRH (250 pmol i.c.v.) reduces hypothermia [207], again suggesting that any beneficial effects are not mediated by a non-specific effect on body or brain temperature. Thus, although it is possible that some drug treatments may have produce hypothermia in ischemic animals, few evidence support this hypothesis for the present results.

Different techniques can be used to maintain temperature during ischemia [124]. Most commonly, core body temperature is maintained using a rectal probe and heating lamp and/or electric blanket. However, the use of a lamp involves a risk of burning the animal if it's not kept at a sufficient distance from the animal. On the other hand, telemetric devices connected to a brain probe glued directly on the skull or inserted through a cannula is an efficient technique to regulate brain temperature. Control of brain temperature is usually restricted to the period during vessel occlusion, as post-ischemic regulation is much more difficult in a freely moving animal.

Although core and brain temperatures were not closely monitored during vessel occlusion in our studies, all rats were kept warm using a heating pad placed under the cage during ischemia or sham procedure and continued for 12-16h following reperfusion. When animals regain consciousness, they can choose to stay over the heating pad or go to the part of the cage that is unheated allowing them some control over their body temperature.

Future directions

Results from the current thesis suggest that CRH confers hippocampal protection in part via the release of opioid peptides and/or the activation of opioid receptors. Additional evidence is now required in order to draw more definite conclusions. Few *in vivo* and *in vitro* studies have so far been performed using these modulators so the specific interactions between the two peptide systems in the central nervous system have to be examined. It would also be interesting to see whether such an interaction could be found using other *in vivo* excitotoxic models or pathological states sharing similar injury mechanisms (epilepsy being one example). In addition, studies could be performed to further document co-localization of opioid peptides and CRH and determine whether their expression co-varies following ischemia both in the CA1 and in extrahippocampal areas. More time intervals could be investigated, including 8h post-ischemia, as CRH has been shown to confer maximal hippocampal protection when administered at this time window. Furthermore, *in vivo* microdialysis could be used to measure delayed CRH release from the hippocampal CA1 region following stroke.

The current thesis has focused on the characterization of the role of kappa- and delta-opioid receptors as modulators of CRH-induced protection. Additional studies could examine the role of other receptors, such as mu receptors and the opioid receptor like 1 (ORL_1). In future experiments, it would also be interesting to examine bi-directional actions of CRH and opioid peptides since opioids could exert their beneficial actions on ischemic damage by modulating CRH release.

In an attempt to better characterize potential interactions between CRH and opioid peptides, we have performed another experiment that unfortunately could not be completed due to various methodological problems. The study used laser-capture microdissection (LCM), a technique developed at the National Cancer Institute [139], which allows for the precise isolation of individual cells of interest and subsequent RNA extraction [448]. Once the desired cells are isolated, quantitative polymerase-chain reaction (Q-PCR) provides an accurate method for determination of levels of specific DNA and RNA sequences in tissue samples. Using Q-PCR on LCM-obtained tissue, the main goal of this study was to quantify the expression of delta- and kappa-opioid receptors, as well as their respective endogenous ligands, enkephalin and dynorphin, at distinct time intervals (1, 6 and 24h) in the CA1 subfield of the hippocampus in vehicle- and CRH(5μg)-treated ischemic and sham animals. The selected time intervals were chosen based on results obtained in Boutin et al.'s studies [53-55], which found decreased receptor density at these specific post-ischemic intervals, as well as from the results reported by Haqqani et al. [195], showing an increase in blood-brain barrier permeability 1, 6, and 24h after transient global cerebral ischemia. This technique being novel, it had only been tried at the NRC using blood vessel micropuncture with some success but was tested for the

first time on neuronal material. Various problems were encountered throughout the experiment. For example, since LCM allows capturing single cells, we exclusively sampled CA1 pyramidal neurons but in doing so, we did not have enough material from one animal to get sufficient amount of RNA to do RT-PCR (the main objective in using this particular technique being to get high cellular resolution, we did not want to pool samples from different animals or collect the whole hippocampus). Thus, we had to amplify the extracted RNA using RiboAmp kit (Molecular Devices, Sunnyvale, CA), a 3-day procedure before obtaining the desired concentration. Initially, although closely following provided instructions from RiboAmp kit, we could not succeed with the amplification process. It took many months to solve this problem using a PicoPure extraction kit from Molecular Devices, which was optimized for subsequent RNA amplification. Once this problem was solved, we then performed RT-PCR and Q-PCR, using primers that we designed for opioid receptors and ligands. However, following the Q-PCR analysis, we observed an important DNA genomic contamination, preventing any conclusion as to the expression of the selected genes. The reasons of this contamination are still unknown. Having done many months of work and not knowing the strength and validity of the results obtained with this novel technique, we decided to end the experiment. However, it would be interesting to replicate the study using more established techniques, such as immunohistochemistry.

In conclusion, the compendium of findings reported in the present thesis significantly contributed to further characterize the role of CRH and opioid peptides and receptors in the modulation of endogenous processes associated with an ischemic insult. In addition to the demonstration that CRH confers protection against ischemia-induced CA1 neuronal degeneration when administered both prior and following vessel occlusion, this thesis elucidated possible interactions between discrete peptide systems shown to share common functions in the CNS and for which interactions are present in both the brain and periphery. Importantly, behavioral assessment was performed to determine whether neuronal survival was associated with concomitant changes of ischemia-induced behavioral and memory deficits. It is hoped that research along these lines will clarify the role of discrete peptide signals as mediator of ischemia-induced cellular and behavioral alterations, and provide evidence supporting further research on these neuropeptides as novel therapeutic agents against stroke damage.

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