ADRENOCORTICOSTEROID RECEPTOR EFFECTS ON HIPPOCAMPAL NEURON VIABILITY

Deanna Lynn McCullers

University of Kentucky

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ABSTRACT OF DISSERTATION

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The Graduate School
University of Kentucky
2001
ADRENOCORTICOSTEROID RECEPTOR EFFECTS ON HIPPOCAMPAL NEURON VIABILITY

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By
Deanna Lynn McCullers
Lexington, Kentucky

Director: Dr. James P. Herman, Associate Professor of Anatomy and Neurobiology
Lexington, Kentucky

2001

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Glucocorticoid activation of two types of adrenocorticosteroid receptors (ACRs), the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR), influences hippocampal neuron vulnerability to injury. Excessive activation of GR may compromise hippocampal neuron survival after several types of challenge including ischemic, metabolic, and excitotoxic insults. In contrast, MR prevents adrenalectomy-induced loss of granule neurons in the dentate gyrus. The present thesis addresses the respective roles of MR and GR in modulating neuronal survival following two forms of neuronal injury, excitotoxicity and traumatic brain injury. Male Sprague-Dawley rats were pretreated with MR antagonist spironolactone or GR antagonist mifepristone (RU486) and subsequently injected with kainic acid, an excitotoxic glutamate analog, or injured with a controlled cortical impact. Twenty-four hours following injury, hippocampal neuron survival was measured to test the hypotheses that MR blockade would endanger and GR blockade would protect hippocampal neurons following injury. Messenger RNA levels of viability-related genes including bcl-2, bax, p53, BDNF, and NT-3 were also measured to test the hypothesis that ACR regulation of these genes would
correlate with neuronal survival. In addition, ACR mRNA levels were measured following receptor blockade and injury to test the hypothesis that glucocorticoid signaling is altered following neuronal injury via regulation of ACR expression.

Mineralocorticoid receptor blockade with spironolactone increased neuronal vulnerability to excitotoxic insult in hippocampal field CA3, and GR blockade with RU486 prevented neuronal loss after traumatic brain injury in field CA1. These results are consistent with the hypotheses that MR protects and GR endangers hippocampal neurons. Adrenocorticosteroid receptor blockade decreased mRNA levels of the anti-apoptotic gene bcl-2 in select regions of uninjured hippocampus, yet ACR regulation of bcl-2 did not consistently correspond with measures of neuronal survival after injury. Kainic acid decreased MR mRNA levels in CA1 and CA3, while both kainic acid and controlled cortical impact dramatically decreased GR mRNA levels in dentate gyrus. These data suggest that injury modulation of glucocorticoid signaling through regulation of ACR expression may influence hippocampal neuron viability following injury.

KEYWORDS: Mineralocorticoid Receptor, Glucocorticoid Receptor, Messenger RNA, Kainic Acid, Traumatic Brain Injury

Deanna L. McCullers

December 6, 2001
ADRENOCORTICOSTEROID RECEPTOR EFFECTS ON HIPPOCAMPAL NEURON VIABILITY

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DISSERTATION

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ACKNOWLEDGMENTS

I would like to acknowledge my advisor and Dissertation Chair, Dr. James Herman, for his instrumental role in my training and in the completion of this work. For many years Dr. Herman has been an excellent role model as a scientist, a teacher, and a person. I also thank the members of my committee, Dr. Brian Davis, Dr. Steve Estus, Dr. Kurt Hauser, Dr. Jim Geddes, and Dr. Joe Springer, and the outside examiner, Dr. Thomas Foster, for their comments, insight, and commitment to my academic development. The former and current Directors of Graduate Studies, Dr. Harold Traurig and Dr. Brian MacPherson, have also been of great assistance throughout the dissertation process. I would further like to acknowledge Dr. Stephen Scheff for providing assistance and resources necessary for performing the studies on traumatic brain injury.

Expert technical assistance was provided by several members of the Herman lab including: Garrett Bowers, Mark Dolgas, Dr. Helmer Figueiredo, Erin Murphy, Megan Paskitti, Dr. Kim Sipe, and Dr. Dana Ziegler. Dr. Patrick Sullivan, Katie Kraft, and other members of the Scheff lab provided technical assistance essential for the completion of the studies on traumatic brain injury, and Cynthia Long assisted with several histological procedures. I would also like to express my appreciation to Kelly Bolte Svec, Susan Herman, Liz Jones, Betty Newsom, Kim Stroth, and Kim Wilkerson for their valuable administrative assistance. Finally, I am indebted to my parents, Lynda J. McCullers and L. Arnold McCullers, for their continuing support.

The contents of Chapter Two have previously been published as an article in the journal Neuroreport titled, “Mineralocorticoid receptors regulate bcl-2 and p53 mRNA expression in hippocampus”, by Deanna L. McCullers and James P. Herman, Copyright © 1998 Lippincott
Williams & Wilkins. This work is reprinted with permission of Lippincott Williams & Wilkins. The contents of Chapter Three have been previously published as an article in the *Journal of Neuroscience Research* titled, “Adrenocorticosteroid receptor blockade and excitotoxic challenge regulate adrenocorticosteroid receptor mRNA levels in hippocampus”, by Deanna L. McCullers and James P. Herman, Copyright © 2001 Wiley-Liss Inc. This work is reprinted with permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc. The contents of Chapter Four are currently in press to be published as an article in the journal *Neuroscience* titled, “Mifepristone protects CA1 hippocampal neurons following traumatic brain injury in rat”, by D.L. McCullers, P.G. Sullivan, S.W. Scheff, and J.P. Herman, Copyright © 2001 IBRO. Work contained in the following dissertation was supported by AG12962 (J.P.H.), AG10836 (J.P.H), AG00242 (D.L.M), NS39828 (S.W.S.), and KSCHIRT (S.W.S.).
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Chapter One: Introduction

Adrenocorticosteroid receptors in brain

Glucocorticoids are steroid hormones that play an essential role in adaptation to stress. Named for their profound effects on glucose metabolism, glucocorticoids stimulate gluconeogenesis, mobilize stored energy, and inhibit subsequent energy storage during the stress response. In addition to regulating metabolism, glucocorticoids also increase cardiovascular tone, inhibit growth and reproductive capabilities, restrain immune and inflammatory responses, and influence learning and memory (Munck et al., 1984; Sapolsky et al., 2000). Glucocorticoids are essential for surviving stress, but excessive exposure can have detrimental effects such as hypertension, steroid diabetes, immunosuppression, inhibition of growth, and infertility (Munck et al., 1984; Sapolsky et al., 1986; McEwen and Stellar, 1993). Elevated levels of the steroid may also contribute to the development of affective disorders such as depression (De Kloet et al., 1998) and neuropathological processes including Alzheimer's disease (Landfield et al., 1992) and age-related neurodegeneration (Sapolsky et al., 1986; Landfield and Eldridge, 1991; McEwen, 1992). Furthermore, many studies have demonstrated that excessive exposure to glucocorticoids increases neuronal vulnerability to many types of challenge including ischemia, excitotoxicity, and oxidative stress (see Sapolsky, 1994).

At the cellular level, glucocorticoid effects are mediated by two types of ligand-activated receptors, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). These adrenocorticosteroid receptors (ACRs) are differentially activated in different glucocorticoid contexts; MR is extensively activated at basal hormone levels, while GR is activated by high glucocorticoid concentrations such as those experienced during stress or circadian peak (Reul and de Kloet, 1985; Reul et al., 1987). This arrangement suggests that the MR mediates tonic influences of glucocorticoids, while GR drives glucocorticoid actions aimed at restoring homeostasis (De Kloet and Reul, 1987). The dichotomous activation of the receptors also implies that MR maintains normal neuronal function while GR mediates the deleterious effects of excessive glucocorticoids. Studies demonstrating rescue of dentate gyrus granule neurons from adrenalectomy-induced death provide some support for the notion that MR has neurotrophic properties (Woolley et al., 1991). In addition, numerous studies have demonstrated
that chronic GR activation compromises neuronal survival in hippocampus (see Sapolsky, 1994), a structure particularly rich in ACR expression (Reul and de Kloet, 1985) and particularly vulnerable to neuronal injury and degeneration (see Sapolsky, 1994).

Improved understanding of the mechanisms underlying pathogenic effects of glucocorticoids is of crucial importance to facilitate treatment of neurodegenerative and psychiatric illness. The negative effects of chronic exposure to supraphysiological glucocorticoid levels have been well established in animal models (Sapolsky, 1985; Sapolsky and Pulsinelli, 1985; Sapolsky, 1986a, b; Elliott et al., 1993; Stein-Behrens et al., 1994a), and in vitro studies provide convincing evidence that deleterious glucocorticoid effects are mediated by GR (Packan and Sapolsky, 1990; Behl et al., 1997b). Some studies demonstrate that GR blockade with antagonist drug mifepristone (RU486) can protect cultured cells against glucocorticoid toxicity (Packan and Sapolsky, 1990; Behl et al., 1997b; Behl et al., 1997a). However, the potential for attenuating physiological glucocorticoid neurotoxicity with GR blockade has not been thoroughly examined in vivo. One previous study has demonstrated transient RU486 protection against ischemia-induced hippocampal cell loss (Antonawich et al., 1999), but RU486 protection against other forms of neuronal injury has not been studied in animal models. Furthermore, the possible neuroprotective role of normal MR activation has not been previously addressed.

The current thesis aims to evaluate the respective roles of MR and GR on hippocampal neuron survival in vivo under conditions of neuronal challenge. Adrenocorticosteroid receptor regulation of neuronal viability was assessed after administration of ACR antagonist drugs and injury with kainic acid, an excitotoxic glutamate analog, or controlled cortical impact, a model of experimental traumatic brain injury. Following antagonist pretreatments and injury, survival of challenged neurons, neuronal expression of viability-related genes, and neuronal expression of ACR mRNAs were measured to test the hypotheses that (1) MR blockade would decrease hippocampal neuron survival of injury and expression of genes associated with neuronal viability, (2) GR blockade would increase hippocampal neuron survival of injury and expression of genes associated with neuronal viability, and (3) glucocorticoid signaling is altered through regulation of ACR expression following injury.
The hypothalamic-pituitary-adrenal axis

Glucocorticoid secretion is regulated by the hypothalamic-pituitary-adrenal (HPA) axis (Figure 1.1). Perception of stress or circadian drive stimulates the paraventricular nucleus of the hypothalamus to release corticotropin-releasing hormone (CRH) and other adrenocorticotropic-releasing hormone (ACTH) secretagogues into the hypophysial portal system in the median eminence. The neuropeptides stimulate anterior pituitary release of ACTH, which travels though systemic circulation and promotes synthesis and release of glucocorticoids from the adrenal cortex [see (Antoni, 1986; Jones and Gillham, 1988)]. Circulating glucocorticoids subsequently inhibit their own release by acting at multiple levels of the HPA system including brain and pituitary (Keller-Wood and Dallman, 1984; Jones and Gillham, 1988). This negative feedback system participates in termination of the stress response and prevents overexposure to catabolic glucocorticoid effects that might threaten homeostasis (Munck et al., 1984). Hypothalamic-pituitary-adrenal dysregulation and elevated basal cortisol levels have been associated with affective disease and cognitive decline in humans (Sapolsky et al., 1986; Martignoni et al., 1992; Axelton et al., 1993).
Figure 1.1. The hypothalamic-pituitary-adrenal axis. Stress or circadian drive stimulate the paraventricular nucleus of the hypothalamus (PVN) to release corticotropin releasing hormone (CRH), which stimulates release of adrenocorticotropin-releasing hormone (ACTH) from the anterior pituitary. ACTH promotes glucocorticoid release from the adrenal cortex. Glucocorticoids act at multiple levels to inhibit further HPA activation.
Properties of adrenocorticosteroid receptors

As members of the nuclear hormone receptor superfamily, MR and GR share a characteristic three-domain structure consisting of an N-terminal transactivation domain, a central DNA-binding domain, and a carboxy-terminal ligand-binding domain (Evans, 1988) (Figure 1.2). The non-conserved N-terminal domain provides unique transactivation properties to each receptor (Evans, 1988). The highly conserved DNA-binding domain suggests that both receptor types bind to identical response elements on target genes (Arriza et al., 1987). In addition to interaction with hormone ligand, the carboxy-terminal domain also mediates binding to chaperone proteins, nuclear translocation, dimerization, and transactivation (Bertorelli et al., 1998). Rat and human ACR sequences are highly homologous; comparisons indicate complete identity in MR and GR DNA-binding domains and high coincidence in steroid-binding domains (Hollenberg et al., 1985; Miesfeld et al., 1986; Patel et al., 1989).

Figure 1.2. Functional domains of the rat glucocorticoid receptor (GR) and mineralocorticoid receptor (MR). Each protein consists of three main functional domains: an N-terminal transactivation domain, a central DNA binding domain, and a carboxy-terminal hormone-binding domain (Miesfeld et al., 1986; Picard and Yamamoto, 1987; Evans, 1988; Patel et al., 1989). Approximate percentages of amino acid identity within each domain are based on human MR and GR homology (Evans, 1988).
Differences in ligand binding affinity underlie the differential activation of MR and GR with increasing plasma glucocorticoid levels. The MR, also termed the “type I” receptor, binds corticosterone with high affinity (Kd ∼ 0.5-1 nM), resulting in extensive occupation even at low circulating glucocorticoid levels (Reul and de Kloet, 1985). The GR, or “type II” receptor, has a lower affinity for corticosterone (Kd ∼ 2.5-5 nM) and is activated (in addition to MR) only at high CORT levels as seen at the circadian peak or during stress (Reul and de Kloet, 1985). In contrast to a simple dose-response relationship, this two-receptor system is capable of generating unique cellular responses based on the strength of glucocorticoid signal.

Central nervous system distribution of adrenocorticosteroid receptors

The GR is widely expressed in the brain, in both neurons and glial cells (Fuxe et al., 1985; van Eekelen et al., 1987; Ahima and Harlan, 1990). Particularly high levels of GR expression are found in the hippocampal formation; other regions expressing moderate to high levels of GR include the paraventricular nucleus of the hypothalamus, the lateral septum, brainstem monoaminergic neurons, thalamic nuclei, the central amygdala, striatum, and layers of cortex (Aronsson et al., 1988; Van Eekelen et al., 1988; Chao et al., 1989). Expression of MR is limited mainly to limbic areas, with highest expression in the hippocampal formation (Reul and de Kloet, 1985; Ahima and Harlan, 1990). Colocalization of both MR and GR has been reported in dorsal subiculum, septum, amygdala, and particularly in hippocampus (Reul and de Kloet, 1985; Van Eekelen et al., 1988). The distribution of ACRs varies by subregion in hippocampus (Herman et al., 1989a; Herman, 1993) (Figure 1.3). The rich expression of ACRs in hippocampal neurons may contribute to their unusual vulnerability to challenge and their extreme sensitivity to the neurotoxic effects of glucocorticoids.
Regulation of adrenocorticosteroid receptor expression

Adrenocorticosteroid receptor expression is subject to short-term and long-term regulation by many factors, including glucocorticoids. Autoregulation of hippocampal ACR expression has been demonstrated in adrenalectomized animals and in subjects treated with ACR antagonists (i.e. receptor expression is increased in the absence of receptor activation). Results from several studies are consistent with regulation of MR expression by MR (Chao et al., 1998a; McCullers and Herman, 2001) and with regulation of GR expression by both MR (O'Donnell and Meaney, 1994; Chao et al., 1998a; McCullers and Herman, 2001) and GR (Herman et al., 1989a; Reul et al., 1989; Chao et al., 1998a; Herman and Spencer, 1998; McCullers and Herman, 2001) (Chapter Three). Furthermore, ACR expression is subject to regulation by glucocorticoid secretion during acute stress (Herman and Watson, 1995; Paskitti et al., 2000), chronic stress (Sapolsky et al., 1984; Gomez et al., 1996; Paskitti et al., 2000), social conflict (Johren et al., 1994), and the circadian rising phase (Herman et al., 1993). It is important to note that ACR expression is differentially regulated by brain region, particularly across subfields of the hippocampus (Herman, 1993).

Adrenocorticosteroid receptor expression is also regulated by numerous factors other than glucocorticoids, emphasizing the importance of ACR regulation as a means for modulating glucocorticoid signaling. Neurotransmitters appear to play a prominent role in regulating ACR
levels; there is evidence for serotonergic (Seckl et al., 1990; Mitchell et al., 1992), noradrenergic (Yau and Seckl, 1992), and dopaminergic (Antakly et al., 1987) regulation of neuronal ACR mRNAs and/or receptor binding. Antidepressant drugs also affect ACR density, but whether antidepressants act directly to alter ACR expression or indirectly by increasing monoamine levels is unclear (Seckl and Fink, 1992). Disruption of hippocampal circuitry also influences ACR mRNA expression; lesions of the medial septum (Yau and Seckl, 1992), dentate gyrus (Brady et al., 1992), entorhinal cortex (O'Donnell et al., 1993), and central 5-HT lesions (Yau et al., 1994) can regulate hippocampal ACR mRNA levels. Excitatory amino acids (Lowy, 1992) and growth factors (Sarrieau et al., 1996) are among numerous other factors shown to regulate ACR expression.

Expression of ACR mRNAs is regulated at multiple levels including transcription, polyadenylation, and/or alternative splicing (see De Kloet et al., 1998). Splicing at the 3'-end of human GR heteronuclear RNA results in at least two GR mRNA variants, GRα and GRβ (Hollenberg et al., 1985). Notably, GRβ is not expressed in rat (Otto et al., 1997). Rat GR and MR mRNAs exhibit heterogeneity in 5'-untranslated exon 1 (McCormick et al., 2000) (Kwak et al., 1993; Zennaro et al., 1995). Interestingly, expression of rat GR mRNA variants can be regulated by environmental manipulations (i.e. postnatal handling) (McCormick et al., 2000), and select MR transcripts are regulated by glucocorticoids (Kwak et al., 1993). Splice variants may exhibit different stabilities and/or translation efficiencies, influencing overall levels of ACR expression based on the differential expression of transcripts (Kwak et al., 1993; Zennaro et al., 1995).

Adrenocorticosteroid receptor activation

Adrenocorticosteroid receptors are activated upon the binding of glucocorticoid ligand, specifically cortisol in human or corticosterone (CORT) in the rat. Unliganded ACRs reside in the cytoplasm as part of a multi-protein complex containing two molecules of heat shock protein (HSP) 90, a molecule each of hsp70, hsp56, p23, and an immunophilin (Pratt, 1993; Bruner et al., 1997; Bertorelli et al., 1998). The chaperone complex facilitates hormone binding to the receptor and participates in receptor trafficking (Pratt, 1993). Glucocorticoid binding induces a conformational change in the ACR molecule that favors dissociation from the chaperone complex (Pratt, 1993). Following dissociation, the liganded receptors become
hyperphosphorylated (Orti et al., 1992) and translocate into the nucleus (Picard and Yamamoto, 1987) where they act as transcription factors (Yamamoto, 1985).

**Adrenocorticosteroid receptor regulation of gene transcription**

Models proposed to explain ACR regulation of gene transcription identify chromatin remodeling and stabilization of the transcriptional preinitiation complex as essential mechanisms (Jenster et al., 1997). Adrenocorticosteroid receptor regulation of these processes is accomplished via binding to DNA response elements or through interactions with other transcription factors (Figure 1.4). In the case of the former, the activated receptors can bind as homo- or heterodimers (Luisi et al., 1991; Trapp et al., 1994) to specific DNA sequences called glucocorticoid response elements (GREs). These palindromic sequences are located in or near promoter regions of glucocorticoid-responsive genes and serve as inducible enhancer elements (see Yamamoto, 1985). Response element-bound ACRs recruit coactivator proteins capable of remodeling chromatin structure via histone acetyltransferase activity. By acetylating histone N-termini, coactivators loosen nucleosomal structure and increase accessibility for other transcription factors. The steroid receptors and coactivators may also stabilize assembly of the preinitiation complex, thereby increasing the rate of transcription initiation (Jenster et al., 1997). The MR and GR can repress gene transcription by recruiting corepressor proteins or by acting at negative GREs, which differ in sequence from positive GREs (Drouin et al., 1993). It is important to note that interactions with coactivator proteins will vary depending on dimer configuration, with distinct transactivation properties conferred by each type of homo- or heterodimer (Trapp et al., 1994).

Ligand-activated ACRs may also influence transcription of genes via interactions with other transcription factors (Karin, 1998) (Figure 1.4). For instance, GR modulates activity of transcription factors including activator protein-1 (AP-1), cyclic AMP response element binding protein (CREB), and nuclear factor-κB (NF-κB), in the absence of DNA binding (Herrlich, 2001). Steroid receptor cross-talk with transcription factors most frequently results in negative interference, but synergism is also possible under specific conditions (i.e. when AP-1 is a homodimer of c-Jun) (Diamond et al., 1990). The exact molecular mechanism of steroid receptor cross-talk has yet to be elucidated. Models for direct protein-protein interactions between GR and other transcription factors have been proposed (McEwan et al., 1997).
Adrenocorticosteroid receptors may also repress gene transcription by inducing specific inhibitors of other transcription factors. For example, GR may repress NF-κB activity by inducing IκB, an inhibitor of NF-κB (Auphan et al., 1995; Scheinman et al., 1995). The evidence indicates that multiple mechanisms are likely to account for glucocorticoid-mediated gene repression (see De Bosscher et al., 2000).
Figure 1.4. Models for transcriptional regulation by adrenocorticosteriod receptors. Activated mineralocorticoid receptors (MRs) or glucocorticoid receptors (GRs) bind DNA hormone response elements as dimers or interact with other transcription factors to influence transcription (McEwan et al., 1997; Karin, 1998; De Bosscher et al., 2000). Possible mechanisms for modulating transcription include: recruitment of transcription coactivators or repressors (A, B); binding to negative hormone response elements (nGREs)(C); interference with activity of other transcription factors (D); competitive DNA binding (E, F); and induction of inhibitors of other transcription factors (G).
Adrenocorticosteriod receptor roles in neuronal viability

Differential ACR activation at varying glucocorticoid levels can elicit unique cellular responses due to the distinct regulatory effects of MR and GR. Collaborative binding of MR/GR heterodimers to GREs demonstrates the potential for ACR cooperation in regulating gene expression (Trapp et al., 1994). In some cases, however, MR and GR effects appear to be quite opposite. For instance, predominant MR activation at low glucocorticoid concentrations results in very small depolarization-induced calcium (Ca\(^{2+}\)) currents in CA1 pyramidal neurons (Karst et al., 1994), while the additional occupation of GR at high glucocorticoid levels results in relatively large Ca\(^{2+}\) currents (Kerr et al., 1992). Further, MR activation by low glucocorticoid levels or aldosterone decreases accommodation and afterhyperpolarization amplitude in CA1, enhancing excitatory transmission (Joels and de Kloet, 1990); in contrast, high glucocorticoid levels and GR agonist RU28362 increase accommodation and the duration of afterhyperpolarization, suppressing CA1 excitability (Kerr et al., 1989). Differential activation of GR can also elicit structural effects on hippocampal neurons that do not occur at lower, MR-activating levels. For instance, chronic exposure to GR-saturating glucocorticoid levels achieved by 21-day corticosterone (CORT) administration (Woolley et al., 1990; Magarinos and McEwen, 1995) or repeated stress (Magarinos and McEwen, 1995) reversibly decreases both numbers and length of apical dendritic branches in CA3 pyramidal neurons.

The preferential activation of MR or GR can also influence the survival of hippocampal neurons. The vast majority of literature to date examines negative GR effects on hippocampal neuron viability, as GR activation is associated with the exacerbation of several forms of neuronal insult (Sapolsky, 1985; Sapolsky and Pulsinelli, 1985; Sapolsky, 1986a, b; Sapolsky et al., 1988; Packan and Sapolsky, 1990; Elliott et al., 1993; Stein-Behrens et al., 1994a; Goodman et al., 1996; McIntosh and Sapolsky, 1996a; Behl et al., 1997b). In contrast, MR activation is not associated with reduced neuronal survival (Packan and Sapolsky, 1990), and there is some evidence to indicate that MR may have trophic or neuroprotective effects (Woolley et al., 1991).

Excessive glucocorticoid receptor activation compromises hippocampal neuron viability

Chronic exposure to high concentrations of glucocorticoids can be toxic to neurons, with preferential damage to the hippocampus (see Sapolsky, 1994). Studies describing hippocampal
degeneration in aging male rats with elevated basal glucocorticoid levels provided the first evidence of a physiological role for glucocorticoid neurotoxicity. Landfield and colleagues demonstrated a correlation between the degree of glucocorticoid hypersecretion and the amount of hippocampal degeneration in mid-aged rats (Landfield et al., 1978). Removal of glucocorticoids via adrenalectomy was subsequently shown to prevent the hippocampal cell loss and glial reactivity (Landfield et al., 1981). These studies led to the formation of the “glucocorticoid hypothesis of brain aging and neurodegeneration”, which proposes that glucocorticoids contribute to age-related degeneration of the hippocampus (Landfield and Eldridge, 1991). Sapolsky and colleagues later demonstrated that chronic exposure to high levels of glucocorticoids, achieved by repeated injection of corticosterone, resulted in the death of hippocampal pyramidal neurons in rat (Sapolsky et al., 1985). It remains unclear whether glucocorticoids alone are sufficient to induce neuronal death in hippocampus.

While glucocorticoids may not kill neurons directly, there is ample support for the notion that chronic exposure to high levels of glucocorticoids increases the vulnerability of hippocampal neurons to subsequent challenge (Sapolsky, 1985). In rat, administration of high physiologic concentrations of CORT potentiates ischemic injury to CA3 neurons produced by the four-vessel occlusion method of forebrain ischemia (Sapolsky and Pulsinelli, 1985). High CORT levels also enhance damage induced in CA3 by the excitotoxin kainic acid (Sapolsky, 1985, 1986a, b; Elliott et al., 1993; Stein-Behrens et al., 1994a) and in DG by the antimetabolite toxin 3-acetylpyridine (Sapolsky, 1985, 1986b). Glucocorticoid exacerbation of excitotoxic (Sapolsky et al., 1988; Packan and Sapolsky, 1990; Goodman et al., 1996) and metabolic (Sapolsky et al., 1988) challenge is also seen in primary hippocampal cultures. Furthermore, in vitro experiments have shown that glucocorticoids increase the amount of hippocampal cell death induced by oxidative stress (Sapolsky et al., 1988; Goodman et al., 1996; McIntosh and Sapolsky, 1996a; Behl et al., 1997b) and amyloid β-peptide toxicity (Goodman et al., 1996).

Despite the graded activation of GR with increasing CORT levels, it remains difficult to differentiate between specific MR and GR effects at high CORT concentrations, as both receptor types are activated. Likewise, both MR and GR are inactivated in adrenalectomized animals due to the removal of endogenous CORT. In order to dissect the individual effects of each receptor type, selective MR/GR agonists may be administered in adrenalectomized animals or in culture to study the selective activation of a receptor type. Conversely, MR/GR antagonists may be
administered to intact animals or in culture to observe the effects of loss of function of a specific ACR type. A number of studies using ACR agonists and antagonists in vitro have provided convincing evidence for the hypothesis that the endangering effects of excessive glucocorticoid exposure are mediated by the GR. In primary hippocampal cultures, a specific GR agonist, dexamethasone, exacerbates kainic acid damage, where the MR agonist aldosterone has no effect (Packan and Sapolsky, 1990). In the same culture system, GR antagonist RU486 protects against glucocorticoid/kainic acid synergy, but MR antagonist RU28318 does not prevent the damaging glucocorticoid effects (Packan and Sapolsky, 1990). Other in vitro studies provide strong support for GR enhancement of amyloid β protein, hydrogen peroxide, and glutamate toxicity, as dexamethasone exacerbates (Behl et al., 1997b) and RU486 prevents (Behl et al., 1997b; Behl et al., 1997a) negative glucocorticoid effects with these types of insult. In vivo, RU486 transiently protects gerbil CA1 neurons from ischemia-induced cell loss, indicating that blockade of GR can prevent the detrimental effects of post-ischemia CORT elevations (Antonawich et al., 1999).

Mechanisms for glucocorticoid receptor endangerment of hippocampal neurons

Glucocorticoids can increase neuronal susceptibility to injury by inducing a state of metabolic vulnerability (Sapolsky, 1985). In the absence of additional challenge, temporary energy crisis induced by glucocorticoids is not likely to threaten neuronal survival. However, by compromising cellular energy supplies, glucocorticoids can greatly intensify the toxicity of a wide variety of insults that impair generation of energy (i.e. ischemia) or place pathogenic demands on the neuron for energy (i.e. excitotoxicity) (Sapolsky, 1990). Glucocorticoids impair glucose metabolism in regions including hippocampus, locus coeruleus, hypothalamic paraventricular nucleus, median eminence, and pituitary anterior lobe (Kadekararo et al., 1988). Activation of GR may compromise neuronal energy stores by impairing glucose transport, as demonstrated in cultured hippocampal neurons and glia in vitro (Horner et al., 1990). Chronic glucocorticoid exposure also markedly induces expression of ATP-requiring enzymes such as glutamine synthetase and glycerol-phosphate dehydrogenase, which may result in the further depletion of cellular ATP stores (Nichols and Finch, 1994). Energy supplementation with glucose or mannose has been shown to attenuate glucocorticoid neurotoxicity in vivo (Sapolsky, 1986b) and in vitro (Sapolsky et al., 1988).
Neuronal energy depletion following excessive glucocorticoid exposure has been proposed to exacerbate hippocampal insult by contributing to excitotoxic cascades (Sapolsky, 1990). Under normal conditions, glutamate interaction with postsynaptic receptors mobilizes free cytosolic Ca\(^{2+}\) in the postsynaptic neuron in a highly regulated fashion. Glutamate is removed from the synapse by energy-dependent glutamate transporters (Kanai et al., 1993). Calcium in the postsynaptic neuron is effectively buffered by Ca\(^{2+}\) binding proteins including calbindin and parvalbumin, sequestered by cellular organelles such as smooth endoplasmic reticulum and mitochondria, or extruded via ATP-dependent transporters including a Na\(^{+}/Ca\(^{2+}\) exchanger and the plasma membrane calcium pump (PMCA) (Blaustein, 1988). However, energy depletion and necrotic insult can lead to the pathologic accumulation of synaptic excitatory amino acids, leading to excessive influx of Ca\(^{2+}\) into the postsynaptic neuron. Energy crisis compromises glutamate removal from the synapse and may even result in reversal of glutamate transporters (Takahashi et al., 1997), resulting in the further depolarization-induced opening of voltage-gated Ca\(^{2+}\) channels and NMDA-gated Ca\(^{2+}\) channels on the postsynaptic neuron. Energy deficits can also lead to the failure of ATP-driven Ca\(^{2+}\) sequestration and extrusion systems, exacerbating increases in intracellular Ca\(^{2+}\) levels (see Tymianski and Tator, 1996). Excessive Ca\(^{2+}\) influx can set in motion many destructive processes including mitochondrial failure, the degradation of structural proteins by Ca\(^{2+}\)-activated proteases (i.e. calpain) (Siman et al., 1989), and the generation of free radicals [see (Choi, 1992; Tymianski and Tator, 1996)].

Many experimental studies support the theory that glucocorticoids contribute to excitotoxic glutamate/Ca\(^{2+}\) cascades. Chronic exposure to high levels of glucocorticoids has been shown to increase extracellular levels of excitatory amino acids following kainic acid administration (Stein-Behrens et al., 1992; Stein-Behrens et al., 1994b). Glucocorticoids also prolong intracellular Ca\(^{2+}\) elevation after kainic acid treatment (Elliott and Sapolsky, 1992) and increase basal intracellular Ca\(^{2+}\) levels (Elliott and Sapolsky, 1993) in culture. Furthermore, glucocorticoids exacerbate Ca\(^{2+}\)-mediated degenerative events in the hippocampus following intrahippocampal kainic acid infusion, as measured by spectrin proteolysis and altered tau immunoreactivity (Elliott et al., 1993; Stein-Behrens et al., 1994a). Glucocorticoid exacerbation of 3-acetylpyridine toxicity is eliminated with administration of an NMDA receptor antagonist, aminophosphonovaleric acid, providing evidence that negative glucocorticoid effects are dependent on activation of the NMDA receptor (Armanini et al., 1990).
Glucocorticoids can also influence neuronal survival via genomic mechanisms. High glucocorticoid concentrations may modulate Ca\(^{2+}\) homeostasis by increasing P/Q- and L-type Ca\(^{2+}\) channel subunit mRNA levels, as demonstrated in isolated CA1 neurons (Nair et al., 1998). Glucocorticoids also decrease hippocampal mRNA levels of plasma membrane calcium pump isoform 1 (PMCA1) (Bhargava et al., 2000), which regulates intracellular Ca\(^{2+}\) levels by coupling ATP hydrolysis with Ca\(^{2+}\) extrusion. These changes in gene expression, if indicative of changes in protein expression, are consistent with increased intracellular Ca\(^{2+}\) accumulation with GR activation. Furthermore, glucocorticoid regulation of neurotrophin expression may lead to decreased neurotrophic support and the exacerbation of neuronal insults. Immobilization stress (Smith et al., 1995a) and CORT administration (Chao and McEwen, 1994; Smith et al., 1995a) decrease hippocampal brain-derived neurotrophic factor (BDNF) mRNA levels and increase hippocampal neurotrophin-3 (NT-3) levels in hippocampus.

Neurotrophic effects of mineralocorticoid receptor activation

The potentially damaging effects of GR activation at high glucocorticoid concentrations are well documented. In contrast, the MR, which is persistently activated even at basal glucocorticoid levels (Reul and de Kloet, 1985), is not associated with neuronal energy depletion or exacerbation of neuronal insult (Packan and Sapolsky, 1990). On the contrary, MR may function in a neurotrophic manner, as activation of MR is required for the survival of dentate gyrus granule neurons. Removal of endogenous glucocorticoids by adrenalectomy causes granule cell loss within as little as three days (Gould et al., 1990), and nearly all granule cells are lost within three to four months after surgery (Sloviter et al., 1989). Administration of the selective MR agonist aldosterone prevents adrenalectomy-induced granule neuron loss, but treatment with GR agonist RU28362 provides only partial protection for DG granule neurons (Woolley et al., 1991). Thus, MR occupation is necessary for maintenance of DG neuron survival, but the role for GR is less clear (Woolley et al., 1991). MR activation also regulates granule cell neurogenesis in DG, maintaining a balance between granule cell birth and cell death (Gould et al., 1991). MR may regulate neurogenesis by enhancing excitatory input to the region (Gould, 1994) or indirectly through regulation of neurotrophin expression (Lindholm et al., 1994; Qiao et al., 1996).
Kainic acid as a model of excitotoxicity

Kainic acid (KA) is a glutamate analog notable for its excitotoxic and epileptogenic effects. Systemic or intracerebral injection of KA induces epileptiform seizures and cell loss in hippocampus in a pattern similar to human temporal lobe epilepsy (Nadler et al., 1978; Ben-Ari and Cossart, 2000). The extensive literature demonstrating glucocorticoid modulation of KA excitotoxicity, the selective toxicity of KA in hippocampus, and the substantial but incomplete neuronal loss following seizure indicate KA as an ideal model for use in studying ACR effects on hippocampal neuron viability.

Glutamate receptors

Glutamate receptors are divided into two major categories - metabotropic and ionotropic receptors. Metabotropic glutamate receptors are coupled to G-protein-mediated second messenger pathways; ionotropic glutamate receptors allow the direct influx of cations upon activation. Ionotropic glutamate receptors are responsible for the majority of fast excitatory synaptic transmission in the central nervous system. Three types of ionotropic glutamate receptors have been identified based on their preferential activation by each compound – the AMPA, kainate, and NMDA receptors (Dingledine and McBain, 1999).

Non-NMDA glutamate receptor subunits are approximately 900 amino acid residues in length, have a calculated molecular weight of approximately 100 kDa, and are predicted to have four transmembrane domains (Keinanen et al., 1990). Non-NMDA receptors are formed by homomeric or heteromeric associations of AMPA (GluR-1/-2/-3/-4) or kainate (GluR-5/-6/-7 and KA-1/-2) receptor subunits. Subunit composition may confer distinct functional and pharmacological properties (Hollmann et al., 1991; Bettler and Mulle, 1995). Kainate receptors exhibit significant structural homology to AMPA receptors and can be expressed in the same cells as AMPA receptors, yet KA receptor subunits and AMPA receptor subunits do not seem to coassemble (Frerking and Nicoll, 2000). Kainate receptors generate excitatory postsynaptic potentials (EPSPs) in response to glutamate release (Frerking and Nicoll, 2000), and kainate responses undergo rapid and total desensitization from which recovery is slow (Lerma et al., 1997). As compared to AMPA receptor-mediated EPSPs, kainate-mediated EPSPs have smaller
peak amplitude and slower decay kinetics. A controversial presynaptic role for kainate receptors has also been proposed (see Malva et al., 1998).

NMDA receptors are believed to play a key role in glutamate neurotoxicity due to their high Ca\(^{2+}\) permeability as compared to non-NMDA ionotropic glutamate receptors (Rothman, 1987). NMDA receptors are unique in that they require bound ligand and the voltage-dependent removal of a Mg\(^{2+}\)-dependent block to initiate activation, and the presence of glycine is required for full activation (Malva et al., 1998). NMDA receptors are insensitive to AMPA and kainate (Hollmann and Heinemann, 1994) but may be activated by kainate-induced seizures or excitotoxic cascades (Ben-Ari and Gho, 1988).

**Kainic acid excitotoxicity**

The distribution of KA binding sites roughly corresponds to regional susceptibility to KA excitotoxicity. Regions displaying KA binding and sensitivity to KA toxicity include the hippocampus, amygdala, entorhinal cortex, piriform cortex and insular cortex (Kohler and Schwarcz, 1983). High-affinity kainate receptors are expressed at very high density in rat brain in the mossy fiber synaptic region, or stratum lucidum of CA3 (Foster et al., 1981; Represa et al., 1987). The epileptogenic effects of KA are attributed to excitation of CA3 neurons by postsynaptic kainate receptors at mossy fiber synapses (Ben-Ari and Gho, 1988). It is important to note that degeneration of CA3 neurons is probably due to the sustained release of glutamate subsequent to recurrent seizures, not to the activation of KA receptors per se (Coyle et al., 1981; Ben-Ari, 1985; Ben-Ari and Cossart, 2000). Seizures generated in CA3 propagate to CA1 via a network of recurrent collateral glutamatergic axons that interconnect pyramidal neurons. Secondary activation of CA1, the major source of hippocampal output, leads to the spread of seizures to other limbic structures including entorhinal cortex (Figure 1.5) (Ben-Ari and Cossart, 2000).
Figure 1.5. Schematic diagram of the intrinsic circuitry of the hippocampal formation (adapted from Amaral and Witter, 1989). The hippocampal formation is composed of four basic regions: the entorhinal cortex (EC), the dentate gyrus (DG), the hippocampus proper (which may be divided into subfields CA1, CA2, and CA3), and the subicular complex (S). The major connections linking hippocampal subfields are glutamatergic and largely unidirectional. Input from EC reaches DG via the perforant path. Granule cells of the DG project to the CA3 field via mossy fibers. CA3 pyramidal neurons give rise to Schaffer collaterals, which terminate on CA1 pyramidal neurons. CA1 pyramidal neurons project to S, which projects to regions including EC.

The behavioral effects of systemic KA administration have been described in detail (Sperk et al., 1983). Approximately five minutes after injection, most animals assume a catatonic posture with staring behavior. Within approximately one hour, subjects develop generalized clonic-tonic convulsions accompanied by frequent rearing, falling over to one side, “wet dog shakes”, and hemorrhagic foam from the mouth. Convulsions often subside two hours after injection and are replaced by a phase of motor hyperactivity. In more severely affected animals, the convulsive phase may last up to 4-5 hours after KA injection. After twenty-four hours the animals appear physically exhausted. Rare convulsions may still occur spontaneously or in response to noise or handling (Sperk et al., 1983).

Kainic acid lesion preferentially destroys hippocampal pyramidal cells, particularly those of region CA3 (Nadler et al., 1978). Neurons of CA1 are vulnerable to a lesser degree, and DG
granule cells are relatively insensitive to KA excitotoxicity (Nadler et al., 1978; Kohler and Schwarcz, 1983). As early as one to three hours after exposure, damaged neurons appear shrunken, with darkly stained cytoplasm and pyknotic nuclei (Sperk et al., 1983) (Figure 1.6). Some neurons appear swollen, containing small vacuoles in mitochondria and in cytoplasm due to swelling of endoplasmic reticulum and Golgi apparatus (Sperk et al., 1983). Cell death induced by KA administration has been classified as necrotic (Sperk et al., 1983; Fujikawa et al., 2000) yet exhibits some characteristics consistent with apoptosis such as TUNEL positivity and DNA laddering (Fujikawa et al., 2000). Damaged neurons appear to be partially removed by three days after injection but may be seen for at least two weeks (Nadler et al., 1978). Neuronal degeneration is accompanied by edema, perivenous hemorrhage, gliosis, and vacuolization of the neuropil (Nadler et al., 1978; Sperk et al., 1983). The entorhinal cortex, piriform cortex, frontal cortex, and medial thalamic nuclei are also subject to degrees of KA-induced degeneration (Nadler et al., 1978; Kohler and Schwarcz, 1983; Sperk et al., 1983).

**Figure 1.6.** Photomicrograph illustrating the effects of (A) saline versus (B) kainic acid on CA1 hippocampal pyramidal neurons (40X magnification). Twenty-four hours following systemic administration, saline-treated neurons are healthy and densely packed with rounded borders and prominent nucleoli. Neurons damaged by kainic acid appear pyknotic and shrunkeled with dark-staining somata and shrunken processes.
Kainate-induced death of hippocampal neurons is likely due to the sustained release of glutamate during recurrent seizures (Coyle et al., 1981; Ben-Ari, 1985; Ben-Ari and Cossart, 2000) and subsequent increases in intracellular Ca\(^{2+}\) levels (Hori et al., 1985; Sun et al., 1992). Excessive accumulation of glutamate in the synapse will activate post-synaptic glutamate receptors, resulting in Ca\(^{2+}\) influx. Calcium accumulation is mediated predominantly by the NMDA receptor, but AMPA or kainate receptors may also contribute to rises in intracellular Ca\(^{2+}\) (Choi, 1992). The pathologic energy demands of repeated seizures lead to impairment of Ca\(^{2+}\) sequestration and extrusion systems, contributing to Ca\(^{2+}\) elevations (see Tymianski and Tator, 1996). Calcium homeostasis may be further disrupted by the release of Ca\(^{2+}\) from internal stores, activation of certain enzymes (such as calpains and phospholipases), and changes in immediate early gene expression. Glutamate efflux from injured neurons may further exacerbate excitotoxic cascades (Choi, 1992).

**Glucocorticoid modulation of kainic acid excitotoxicity**

Glucocorticoids exacerbate KA toxicity (Sapolsky, 1985, 1986a, b; Sapolsky et al., 1988; Packan and Sapolsky, 1990; Stein-Behrens et al., 1994a), making KA administration an excellent model for studying ACR effects on neuronal viability. Glucocorticoids intensify KA-induced accumulation of extracellular glutamate (Stein-Behrens et al., 1992; Stein-Behrens et al., 1994b) and KA-induced calcium elevations (Elliott and Sapolsky, 1992, 1993). Chronic exposure to glucocorticoids also impairs antioxidant defenses in response to KA (McIntosh et al., 1998a) and increases the electrophysiological sensitivity to KA in hippocampal neurons (Talmi et al., 1995). In addition, glucocorticoids influence immediate early gene responses to KA (Unlap and Jope, 1995a) and exacerbate KA-induced spectrin proteolysis (Elliott et al., 1993; Lowy et al., 1994).

Glucocorticoids may also influence neuronal viability to KA via genomic mechanisms. Kainate administration results in the altered expression of a multitude of genes, including: cell survival/death genes (bcl-2, bax, p53, KROX-20, COX-2, cyclin D1, and GADD45) (Sakhi et al., 1994; Gillardon et al., 1995; Vreugdenhil et al., 1996; Zhu et al., 1997; Hashimoto et al., 1998; Timsit et al., 1999), transcription factors (AP-1 and NF\(\kappa\)B) (Kaminska et al., 1994; Pennypacker and Hong, 1995; Won et al., 1999), neurotrophic factors [BDNF and nerve growth factor (NGF)] (Ballarin et al., 1991; Gall et al., 1991; Hashimoto et al., 1998), neuropeptides (Pennypacker and Hong, 1995; Friedman, 1997), heat shock proteins (Zhang et al., 1997;
Hashimoto et al., 1998), immediate early genes (Honkaniemi and Sharp, 1999), GABA-A receptor subunits (Tsunashima et al., 1997), and countless others. Altered gene expression is likely to influence cellular response to KA toxicity; accordingly, glucocorticoid modulation of gene expression may modulate cell survival. Experiments in the current thesis focus on glucocorticoid regulation of members of the Bcl-2 family, p53, and neurotrophins BDNF and NT-3, all of which have been implicated in KA-induced neuronal death (see below).

Regulators of cell survival and death

Members of the bcl-2 family of genes are critical regulators of cell viability. Interactions between pro-survival genes, including bcl-2, bcl-xL, and bcl-w, and pro-death genes, such as bax and bad, are thought to influence cellular susceptibility to necrotic and apoptotic death (Oltvai et al., 1993; Korsmeyer, 1995; Merry and Korsmeyer, 1997). Bcl-2 family members influence cell survival via multiple mechanisms including stabilization of mitochondria and interference with cell death pathways (see below).

A key event associated with cell death is the opening of a large conductance channel called the mitochondrial permeability transition pore. Permeability transition can be induced by a number of stimuli, including sustained increases in intracellular Ca^{2+} levels, oxidative stress, caspase activation (see below), uncoupling of the respiratory chain, and modification of the Bcl-2 complex (Green and Reed, 1998). Opening of the nonselective permeability transition pore allows molecules up to approximately 1.5 kD to pass through the mitochondrial inner membrane into the matrix. The loss of inner membrane selectivity causes collapse of the mitochondrial transmembrane potential and dissipation of the H^{+} gradient, resulting in the uncoupling of the electron transport chain, a decrease in ATP production, and an increase in production of reactive oxygen species. In addition, permeability transition pore opening causes release of caspase-activating proteins such as cytochrome c and apoptosis-inducing factor (AIF) into the cytoplasm, promoting apoptosis [see (Kroemer et al., 1997; Green and Reed, 1998)].

Mitochondrial release of cytochrome c promotes apoptosis by initiating protease cascades programmed to dismantle the cell (Eldadah and Faden, 2000). Cytochrome c facilitates interaction between apoptotic protease activating factor-1 (Apaf-1) and procaspase-9 (Li et al., 1997; Zou et al., 1997). Subsequent processing yields caspase-9, which activates “executioner” caspases such as caspase-3 (Li et al., 1997; Pan et al., 1998b). Caspase-3 cleaves ICAD, the
inhibitor of CAD, a caspase-activated deoxyribonuclease (Enari et al., 1998; Sakahira et al., 1998). Once released from ICAD inhibition, CAD is responsible for internucleosomal DNA degradation characteristic of apoptosis (Enari et al., 1998; Sakahira et al., 1998).

Conditions favoring apoptosis trigger bax translocation from the cytoplasm to mitochondrial membranes (Green and Reed, 1998), promoting the release of cytochrome c from the intramembranous compartment (Jurgensmeier et al., 1998). Anti-apoptotic proteins such as Bcl-2 and Bcl-xL may inhibit apoptosis by preventing molecules such as Apaf-1 from activating caspases (Pan et al., 1998a). Bcl-2 can also prevent mitochondrial permeability transition (Green and Reed, 1998), the release of cytochrome c (Kluck et al., 1997; Yang et al., 1997), and the release of AIF from mitochondria (Green and Reed, 1998), each of which could prevent caspase activation. Although the functional significance is not fully understood, Bcl-2 family members can also form cation-selective pores, which could speculatively influence ion transport, regulate volume of the matrix space, control pH in the intermembrane space, or induce permeability transition in mitochondria (Green and Reed, 1998). In addition, Bcl-2 acts at cell nuclei (Marin et al., 1996), endoplasmic reticulum (He et al., 1997), and mitochondria (Green and Reed, 1998) to influence subcellular Ca^{2+} distribution. Whether the many effects of Bcl-2 are separate functions or consequences of Bcl-2 regulation of a single cell death process remains uncertain.

Accumulating evidence supports a role for bcl-2 family members in modulating neuronal viability after KA-induced seizure. Systemic KA administration decreases bcl-2 mRNA levels in rat hippocampus (McCullers and Herman, 1998) (Chapter Two) and Bcl-2 immunoreactivity in mouse hippocampus and cortex (Gillardon et al., 1995). In contrast, systemic KA increases bax mRNA levels in rat hippocampus (McCullers and Herman, 1998; Lopez et al., 1999) (Chapter Two) and mouse brain (Gillardon et al., 1995). Increased Bcl-w expression has also been observed in rat hippocampus and piriform cortex following KA microinjection into the amygdala (Henshall et al., 2001). Bcl-2 promotes neuronal survival of KA insult, as Bcl-2 overexpression with viral vectors protects hippocampal neurons against KA toxicity in culture and in vivo (Phillips et al., 2000). Furthermore, KA-induced degeneration is attenuated in Bax-deficient cultured motor neurons (Bar-Peled et al., 1999), demonstrating Bax participation in KA-induced cell death.

The p53 tumor-suppressor gene has been described as a guardian of the genome. In response to DNA damage, the transcription factor induces either growth arrest or apoptosis depending
upon the cell type, environment, and expression of other regulatory factors. In either scenario, the end result is the genetic death of the damaged cell, preventing potential neoplastic risk (Evan and Littlewood, 1998). The mechanisms by which p53 promote apoptosis are not fully delineated, but many studies indicate the involvement of repression or induction of specific target genes such as bcl-2 and bax (Miyashita et al., 1994; Miyashita and Reed, 1995; Evan and Littlewood, 1998). Transrepression of anti-apoptotic genes and non-transcriptional mechanisms may also be involved (Evan and Littlewood, 1998). The signaling pathways by which insults activate p53 are mostly unknown (Evan and Littlewood, 1998).

Experimental evidence suggests that p53 influences hippocampal vulnerability to KA-induced neuronal degeneration. P53 mRNA is induced in KA-sensitive brain regions including hippocampus, amygdala, piriform cortex, and thalamus following systemic KA injection (Sakhi et al., 1994) (Chapter Two), and nuclear accumulation of p53 protein has been observed in degenerating neurons after KA administration (Sakhi et al., 1996). Furthermore, p53-deficient mice ("knock-out" mice) exhibit little or no hippocampal cell loss after KA treatment (Morrison et al., 1996; Xiang et al., 1996).

Neurotrophic factors (i.e. nerve growth factor, brain-derived neurotrophic factor, neurotrophins-3 and 4/5, basic fibroblast growth factor, ciliary neurotrophic factor, and insulin-like growth factors) support neuronal differential and survival during development (Barde, 1989) and may protect against select forms of neuronal injury (Lindsay et al., 1994; Mattson and Scheff, 1994; Mocchetti and Wrathall, 1995). A variety of neurotrophic factors have been reported to protect against injury resulting from physical, ischemic, excitotoxic, metabolic, and oxidative insults (Mattson and Scheff, 1994; Mocchetti and Wrathall, 1995). The neuroprotective function of these factors is proposed to involve enhanced maintenance of Ca\textsuperscript{2+} homeostasis and free radical metabolism (Mattson and Scheff, 1994).

Several studies have examined the involvement of two members of the neurotrophin family, brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), in relation to KA excitotoxicity in hippocampus. Intrahippocampal (Ballarin et al., 1991) or systemic (Dugich-Djordjevic et al., 1992; Lee et al., 1997) KA administration dramatically increases hippocampal BDNF mRNA levels. Levels of BDNF mRNA remain elevated for as long as two weeks following intracranial ventricular injection of KA (Garcia et al., 1997a). Increased BDNF protein levels have also been demonstrated following systemic KA administration (Rudge et al.,
In contrast, NT-3 mRNA levels are decreased following systemic (Lee et al., 1997; Katoh-Semba et al., 1999) or intracranial ventricular (Garcia et al., 1997a) KA administration, with decreases lasting up to two weeks post-injection in the latter model (Garcia et al., 1997a). NT-3 protein levels correspond with altered mRNA levels, decreasing following systemic KA injection (Katoh-Semba et al., 1999). Experiments indicate that BDNF and NT-3 protect cultured rat hippocampal, septal, and cortical neurons against damage induced by glucose deprivation or glutamate neurotoxicity by attenuating harmful rises in intracellular Ca\(^{2+}\) levels (Cheng and Mattson, 1994). BDNF also protects cultured rat cerebellar granule neurons against glutamate neurotoxicity (Lindholm et al., 1993). In contrast, a study examining the effects of in vivo intrahippocampal neurotrophin administration reports no neuronal protection against KA toxicity with NT-3 infusion and exacerbation of KA-induced injury in CA3 with BDNF administration (Rudge et al., 1998).

**Controlled cortical impact as a model of traumatic brain injury**

Hypothalamic-pituitary-adrenal abnormalities are prevalent in human cases of head injury (King et al., 1970; Pentelenyi and Kammerer, 1984; Chiolero and Berger, 1994; Lieberman et al., 2001), indicating the urgent need for research examining the physiological effects of glucocorticoid secretion after traumatic brain injury (TBI). Synthetic glucocorticoid steroids such as methylprednisolone have been extensively used in the clinical treatment of central nervous system trauma. In 1990, the American National Acute Spinal Cord Injury Study demonstrated significant benefits of high-dose methylprednisolone in the treatment of human spinal cord injury (Bracken et al., 1990). However, the utility of these compounds with respect to brain injury requires further evaluation (see McIntosh et al., 1998b). Not surprisingly, neuroprotection conferred by methylprednisolone is thought to be independent of hormonal (receptor-mediated) glucocorticoid activities; rather, the primary neuroprotective mechanism of methylprednisolone is hypothesized to be the inhibition of oxygen free-radical induced lipid peroxidation via direct membrane effects (Hall et al., 1992). While the nonhormonal effects of exogenous synthetic steroids have been researched extensively, the physiological role for endogenous glucocorticoids after TBI remains largely unexamined. Increased cortisol levels reported with human TBI (King et al., 1970; Pentelenyi and Kammerer, 1984) emphasize the
potential importance of receptor-mediated glucocorticoid effects on neuronal viability after trauma.

Many animal models of mechanical brain injury have been developed in an attempt to reproduce aspects of human closed-head injury. Of these, several commonly used techniques are: fluid percussion, which produces brain injury by the rapid injection of fluid into the closed cranial cavity (Dixon et al., 1987); closed head injury, in which a weight-drop device delivers a standard blow to the skull (Shapira et al., 1988); and the cortical impact model, which uses a pneumatic impactor to deliver a controlled impact to the exposed dura, with measurable velocity and cortical deformation (Dixon et al., 1991). Experiments in the present thesis employ the controlled cortical impact (CCI) model of TBI in conjunction with ACR antagonist treatments in order to study ACR effects on hippocampal neuron viability following TBI.

**Controlled cortical impact pathology**

Traumatic injury of the human brain causes neuronal loss in regions including cortex, hippocampus, thalamus, and cerebellum (Adams et al., 1985; Kotapka et al., 1992; Ross et al., 1993). Neuronal degeneration caused by TBI is the product of direct mechanical insult (primary injury) and delayed pathological events including ion disequilibration, free radical formation, membrane damage, protease activation, and cytoskeletal disruption (secondary injury) (Faden, 1993). The delayed component of TBI pathology is of great significance as it provides opportunity for therapeutic intervention after injury. The pattern of neuronal loss in experimentally injured rat brain mimics that of human TBI, with neuronal degeneration observed in cortical areas overlying the evolving contusion, in hippocampus, and in lateral thalamus (Sutton et al., 1993; Dietrich et al., 1994; Hicks et al., 1996). Dark, shrunken neurons and swollen astrocytes can be detected in the acute phase, as early as ten minutes following injury (Hicks et al., 1996). With the controlled cortical impact model at moderate severity, neuronal loss in CA3 is evident by one hour after injury (Baldwin et al., 1997). Furthermore, there is no significant difference in the percentage of surviving CA3 neurons estimated 24 hours or 30 days post-injury, indicating that neuronal loss is rapid and complete by 24 hours (Baldwin et al., 1997). Moderate levels of experimental TBI result in neuronal death by both necrosis and apoptosis, with the greatest loss caused by necrosis (Raghupathi et al., 2000; Zipfel et al., 2000).
Secondary neuronal degeneration following traumatic insult is driven by an array of physiological and biochemical changes. Excessive glutamate release (Faden et al., 1989; Bullock and Fujisawa, 1992), increased intracellular Ca\(^{2+}\) levels (Young, 1992; Nilsson et al., 1993; McIntosh et al., 1998c), mitochondrial dysfunction (Sullivan et al., 1998; Sullivan et al., 1999), metabolic changes (Hovda et al., 1992), tissue edema (Wahl et al., 1988), lipid peroxidation (Braughler and Hall, 1992), free radical formation (Lewen et al., 2000), vascular disruption (Cortez et al., 1989), neurochemical alterations (Hayes et al., 1992), inflammation (Feuerstein et al., 1997), astroglial responses (Cortez et al., 1989), axonal injury (Povlishock and Christman, 1995), proteolysis (Kampfl et al., 1997), activation of cell death pathways (Beer et al., 2000; Raghupathi et al., 2000), and altered gene expression (Mattson and Scheff, 1994; Hayes et al., 1995; Raghupathi et al., 2000) are among the many factors identified as contributors to trauma-induced degeneration (see Faden, 1993).

Glucocorticoid modulation of traumatic brain injury

Many factors contributing to secondary neuronal degeneration following TBI are likely influenced by glucocorticoids. Glucocorticoids reduce edema (Xu et al., 1992), influence neurotransmitter systems (Meijer and de Kloet, 1998), reduce inflammation (De Bosscher et al., 2000), and interact with cell death pathways (Reagan and McEwen, 1997). Steroid hormones also prevent lipid hydrolysis and the subsequent formation of oxygen free radicals, as noted above (Hall et al., 1992). Furthermore, glucocorticoids exacerbate cognitive deficits associated with experimental TBI (Hicks et al., 1993; White-Gbadebo and Hamm, 1993; Smith et al., 1994; Scheff et al., 1997). Experiments in the current thesis focus on glucocorticoid and trauma-induced regulation of viability-related genes, specifically members of the bcl-2 family and tumor-suppressor p53.

Regulators of cell survival and death

Bcl-2 family members are critical regulators of cell viability, and an increasing body of evidence suggests that bcl-2 related proteins control neuronal survival following TBI. Bcl-2 expression increases in injured human brain (Clark et al., 1999) and in rat hippocampal and cortical neurons surviving cortical impact with secondary hypoxia (Clark et al., 1997). In contrast, bcl-2 expression is reported to decrease in rat cortical neurons following closed head
injury (Lu et al., 2000). A neuroprotective role for Bcl-2 after TBI has been supported by studies demonstrating reduced neuronal loss in injured cortex and hippocampus of Bcl-2 overexpressing mice (Raghupathi et al., 1998; Nakamura et al., 1999). Bax may also participate in the regulation of TBI-induced neuronal death, as bax translocation to nuclei of injured cortical and hippocampal neurons has been reported forty-eight hours after cortical contusion (Kaya et al., 1999).

P53, a regulator of bcl-2 and bax expression (Miyashita et al., 1994), is also associated with experimental TBI in the rat. Induction of p53 mRNA has been observed in cortex, hippocampus, and thalamus following lateral fluid-percussion injury (Napieralski et al., 1999), and translocation of p53 proteins to nuclei of injured neurons has been reported using the controlled cortical impact model (Kaya et al., 1999). Neuronal apoptosis in thalamus is attenuated in p53 -/- mice (Martin et al., 2001), but a study measuring cortical lesion volume after controlled cortical impact in p53 -/- mice reported no significant differences compared to wild-type (Tomasevic et al., 1999).

Several studies suggest that neurotrophic factors may also influence hippocampal neuron survival following TBI. Nerve growth factor mRNA levels are increased in dentate gyrus after injury by lateral fluid percussion (Truettner et al., 1999), while NT-3 mRNA levels are decreased (Hicks et al., 1997). In addition, increased hippocampal BDNF mRNA levels are observed following injury with CCI (Yang et al., 1996) and lateral fluid percussion (Hicks et al., 1997; Truettner et al., 1999) models of TBI, suggesting the potential for enhanced neuronal survival due to altered neurotrophin expression. In support of a neuroprotective role for BDNF expression after fluid percussion injury, BDNF mRNA levels are decreased in cortical regions containing degenerating neurons, generally unchanged in adjacent areas, and increased in more remote regions such as piriform cortex (Hicks et al., 1999). However, in a recent study intrahippocampal infusion of BDNF did not improve measures of neuronal loss, neuromotor function, learning, or memory following lateral fluid percussion injury (Blaha et al., 2000).
Chapter Two: Mineralocorticoid receptors regulate bcl-2 and p53 mRNA expression in hippocampus

Introduction

Glucocorticoids regulate gene expression in hippocampal neurons through mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) proteins. MR binds glucocorticoids with high affinity but low capacity, whereas GR binds with low affinity and high capacity (Reul and de Kloet, 1985). These differences in binding efficacy allow MR and GR to regulate gene expression in a concentration-dependent manner; MR is predominantly active at low concentrations of glucocorticoids, while GR becomes increasingly bound and dominant at higher glucocorticoid concentrations (Reul and de Kloet, 1985). MR activity at low glucocorticoid levels is associated with increased cell excitability (Joels and de Kloet, 1990), long-term potentiation (Pavlides et al., 1995), and dentate gyrus (DG) granule cell survival (Woolley et al., 1991). In contrast, high levels of glucocorticoids acting mainly through GR have been shown to reduce long-term potentiation (Pavlides et al., 1993), shrink hippocampal pyramidal cell dendrites (Woolley et al., 1990), and exacerbate damaging effects of toxic insults (Sapolsky et al., 1988; Packan and Sapolsky, 1990). Thus, MR and GR seem to promote differential cellular effects; MR appears to enhance and GR appears to compromise neuronal viability.

The influence of MR and GR on survival of hippocampal neurons is likely exerted at the level of gene regulation. To address the role of the MR in this regulation, we tested the effect of spironolactone (SPIRO), a specific, competitive MR antagonist (Sutanto and de Kloet, 1991), on basal and kainic acid (KA) stimulated expression of mRNAs associated with apoptosis or neuronal survival. Messenger RNAs include those that encode: 1) bcl-2, an inhibitor of apoptosis which is down-regulated by KA (Gillardon et al., 1995); 2) bax, a death-inducing member of the bcl-2 gene family which is up-regulated by KA (Gillardon et al., 1995); 3) p53, a tumor-suppressor which is induced by KA-related DNA damage (Sakhi et al., 1994); 4) brain-derived neurotrophic factor (BDNF), a neurotrophin which is induced by KA excitotoxicity (Ballarin et al., 1991); and 5) neurotrophin-3 (NT-3). To investigate the hypothesis that MR promotes neuroprotective gene activity, we evaluated the effects of MR blockade and KA
treatment by in situ hybridization analysis of mRNA expression in CA1, CA3, and DG of the rat hippocampal formation.

**Materials and Methods**

**Animal treatment and tissue preparation**

All animal handling and protocols were approved by the University of Kentucky IACUC. Young adult male Sprague-Dawley rats were subcutaneously injected twice daily, in morning and afternoon, with MR antagonist spironolactone (50 mg/kg) or sesame oil vehicle. Treatment continued for three days until a total of five injections were administered. On day three, two hours after the final morning injection, half of each group received intraperitoneal injection of kainic acid (12 mg/kg), while the remaining half received 0.9% saline vehicle. Rats were monitored for 8 hours following KA injections; only animals exhibiting behavioral changes indicative of KA seizures as described previously (Sperk et al., 1983) were included in the study. Animals were decapitated on day four, twenty-four hours after onset of kainic acid-induced seizure activity. Brains were rapidly removed and frozen in isopentane (Sigma) cooled to -30°C to -40°C on dry ice. Tissue was sectioned (16 μm) with a Bright-Hacker cryostat, thaw-mounted onto Ultrastick Slides (Becton Dickinson), and stored at -20°C.

**In situ hybridization**

Assessment of mRNA expression was accomplished using probes specific for 1) bcl-2, 2) bax, 3) p53, 4) BDNF, and 5) NT-3. Probes were labeled by standard in vitro transcription, using 33P-UTP, according to previously published methods (Seroogy and Herman, 1996). Frozen tissue sections removed from a -20°C freezer were placed in 4% paraformaldehyde for 10 min. Slides were rinsed with 5mM KPBS, 5mM KPBS with 0.2% glycine, and again with 5mM KPBS. Following 10 min in 0.1 M TEA with 0.25% acetic anhydride and rinses in 0.2X SSC, sections were dehydrated. Labeled probes were added to a hybridization buffer containing 50% formamide. 50 μL (1 x 10^6 cpm) of diluted probe were applied to each slide. Slides were coverslipped, placed in moistened chambers, and incubated overnight at 55°C. Following hybridization, coverslips were removed in 2X SSC, rinsed once in fresh 2X SSC, rinsed 3X in
0.2X SSC for 10 min. per wash, followed by a 1 hour wash in 0.2X SSC at 60 °C. After dehydration, slides were exposed for 14 - 21 days to Kodak BioMAX film.

Data analysis

Densitometric measurements were taken from subfields CA1, CA3, and dentate gyrus using NIH Image 1.55 software for Macintosh. Data were analyzed by two-way ANOVA, with planned comparisons used to distinguish significance of KA and SPIRO effects. The relationship between bax and p53 mRNAs was assessed by regression analysis.

Results

Figure 2.1 illustrates expression of bcl-2, bax, and p53 in hippocampus of saline- and KA-treated rats. Bcl-2 mRNA levels were significantly affected by SPIRO (F(1, 25) = 4.305; p < 0.05) and KA (F(1, 25) = 5.845; p < 0.05) in CA1 and by KA in CA3 (F(1,25) = 6.468; p < 0.05). Planned comparisons indicate that SPIRO decreased basal bcl-2 mRNA levels in CA1 and CA3 of saline-treated subjects (p < 0.05). Kainic acid significantly decreased bcl-2 mRNA levels in CA1 (p < 0.05) and CA3 (p < 0.01) of oil-treated subjects (Figure 2.2).

Kainic acid treatment significantly influenced bax mRNA expression in CA1 (F(1, 12) = 5.555; p < 0.05) and CA3 (F(1, 24) = 8.049; p < 0.01) (Figure 2.3). Planned comparisons revealed that following KA injection, bax mRNA expression significantly increased in CA1 (p = 0.05) of SPIRO-treated animals and in CA3 (p < 0.05) of oil-treated animals. MR blockade had no significant effect on bax mRNA expression.

Spironolactone (F(1,20) = 4.481; p < 0.05) and KA treatment (F(1, 20) = 15.819; p < 0.001) elicited significant main effects on p53 mRNA expression in CA3, the only hippocampal region in which p53 was observed in consistently quantifiable amounts (Figure 2.4). Kainic acid significantly increased p53 mRNA levels in CA3 of oil-treated animals (p < 0.001), while SPIRO pre-treatment significantly attenuated this increase (p < 0.05).
Figure 2.1. Photomicrographs illustrating bcl-2 (A, B), bax (C, D), and p53 (E, F) mRNA distribution in hippocampus of saline- (A, C, E) and KA-treated (B, D, F) rats. KA decreased bcl-2 mRNA expression in CA1 and CA3, increased bax mRNA expression in CA3, and increased p53 mRNA expression in CA3.
Figure 2.2. Effects of MR blockade and kainic acid on bcl-2 mRNA expression in hippocampus. MR blockade with SPIRO significantly decreased basal bcl-2 mRNA expression in CA1 and CA3 of saline-treated animals (*p < 0.05). KA decreased bcl-2 mRNA expression in CA1 and CA3 of oil-treated animals (‡p < 0.05).
Figure 2.3. Effects of MR blockade and kainic acid on bax mRNA expression in hippocampus. Kainic acid (KA) significantly increased bax mRNA expression in CA1 of SPIRO-treated animals and in CA3 of oil-treated animals (†p < 0.05).
Figure 2.4. Effects of MR blockade and kainic acid on p53 mRNA expression in hippocampus. Kainic acid significantly increased p53 mRNA levels in CA3 (p < 0.05). MR blockade with SPIRO significantly attenuated the KA-induced increase (p < 0.05).

Kainic acid treatment significantly altered BDNF mRNA levels in CA1 (F(1, 23) = 26.952; p < 0.0001) and CA3 (F(1, 23) = 13.511; p < 0.01); expression increased significantly (p < 0.05) in CA1 and CA3 of both oil- and SPIRO-treated animals (Figure 2.5). NT-3 mRNA levels were affected by KA in DG (F(1, 24) = 107.00; p < 0.0001), showing significant decreases in expression in both oil- and SPIRO-treated animals (p < 0.0001) (Figure 2.6). MR blockade had no significant effect on BDNF or NT-3 mRNAs.
Figure 2.5. Effects of MR blockade and kainic acid on brain derived neurotrophic factor (BDNF) expression in hippocampus. Kainic acid significantly increased BDNF mRNA expression in CA1 and CA3 ($^{+}p < 0.05$.}
Figure 2.6. Effects of MR blockade and kainic acid on neurotrophin-3 (NT-3) expression in hippocampus. KA decreased NT-3 mRNA expression in DG (\(^{\ddagger} p < 0.05\)).
Discussion

Within the bcl-2 gene family, dimerization among death-repressing members, such as Bcl-2, and death-inducing members, such as Bax, is thought to determine cellular response to apoptotic signals. The ratio of life-promoting to death-promoting proteins determines if dimerization favoring cell survival will occur (Oltvai et al., 1993). We report that MR blockade by SPIRO decreases basal bcl-2 mRNA expression in CA1 and CA3, demonstrating that MR activity is essential in maintaining normal levels of bcl-2 mRNA in these regions. This finding supports the hypothesis that MR functions as a neuroprotective factor, as decreases in bcl-2 mRNA could lead to the alteration of cellular Bcl-2/Bax ratios and predispose cells to apoptotic cell death. We also report a decrease in bcl-2 mRNA expression in CA1 and CA3 of oil-treated animals with KA administration, an effect previously demonstrated in mouse hippocampus (Gillardon et al., 1995). Bcl-2 mRNA levels were similarly decreased in SPIRO and oil-treated animals after KA-treatment. Thus, whereas SPIRO pretreatment decreased bcl-2 mRNA levels in control rats, it did not exacerbate decreases in bcl-2 mRNA expression induced by KA treatment. Overall, the data suggest that MR modulates bcl-2 expression in hippocampus and may therefore play a role in pyramidal cell survival.

The observed induction of bax mRNA expression in CA1 and CA3 following KA treatment is consistent with previous studies (Gillardon et al., 1995). Notably, levels of bax mRNA did not differ significantly between SPIRO or oil-treated groups prior to or following KA administration. In combination with decreased bcl-2 mRNA expression, these results suggest a possible reduced Bcl-2/Bax ratio, a scenario believed to favor the neuroendangering actions of Bax (Oltvai et al., 1993).

In agreement with previous studies (Sakhi et al., 1994), we demonstrated that KA treatment increases p53 mRNA levels in rat hippocampus. We additionally report that SPIRO attenuated this KA-induced increase in CA3, indicating MR involvement in p53 mRNA expression under these conditions. As accumulation of p53 is associated with induction of apoptosis (Yonish-Rouach et al., 1991; Sakhi et al., 1996), these data suggest that, contrary in part to our principal hypothesis, the MR may play a role in regulating both protective and endangering gene products.

Bax mRNA responses parallel those of p53 mRNA in this study. SPIRO appeared to attenuate the KA-induced increase of bax mRNA in CA3 and also significantly reduced the KA
induction of p53 mRNA in the same region. These changes in bax and p53 mRNA levels after KA treatment are significantly correlated ($r = 0.74; p < 0.05$), indicating that the expression of bax and p53 mRNAs are regulated in parallel. P53 is capable of inducing the human bax gene (Miyashita and Reed, 1995) and is thought to participate in a signaling pathway involving bax and bcl-2 that predisposes cells to apoptosis (Miyashita et al., 1994). For these reasons, the similar responses observed in this study invite speculation that increased bax mRNA levels may result from upregulation by p53. However, p53 overexposure has been shown to cause apoptosis in hippocampal pyramidal neurons without bax induction (Jordan et al., 1997), so the possibility remains that the increases in bax and p53 mRNA are not causally linked.

Consistent with previous reports (Ballarin et al., 1991), this study demonstrated that BDNF mRNA levels increase in rat hippocampus following KA treatment. As elevated glucocorticoid levels have been shown to decrease the expression of BDNF mRNA (Schaaf et al., 1997), this induction is most likely a consequence of another aspect of KA excitotoxicity, although possibly influenced by glucocorticoid input. In addition, NT-3 mRNA expression was decreased in DG of animals receiving KA. While neurotrophins are clearly susceptible to glucocorticoid regulation, as demonstrated by previous studies (Chao and McEwen, 1994; Smith et al., 1995a), there does not appear to be any differential regulation of BDNF or NT-3 mRNA with MR blockade prior to or following excitotoxic insult with KA.

It should be noted that blockade of the MR may alter mRNA expression via activity of an unopposed GR. Without the MR available to form MR-GR heterodimers and otherwise compete with GR homodimers at the GC response element, an increase in GR-mediated transactivation may underlie changes in transcriptional regulation. Overall, it is clear that MR blockade affects bcl-2 and p53 mRNA levels, but whether this change is due to a loss of MR or increase in GR activity remains to be determined. Another important consideration is the substantial hippocampal pyramidal cell loss that accompanies KA treatment after 24 hours. It is possible that changes observed in bcl-2, bax, and p53 mRNAs could represent non-specific actions of dying cells, rather than specific regulatory events, or possibly reflect changes in surviving cell numbers.

Our current understanding of the function of GC receptors and cell viability is far from complete. The MR likely participates in apoptosis regulation, but the present evidence does not convincingly support an exclusively neuroprotective role. One problematic issue is the
complexity of receptor interaction involved. It is unclear at this time whether changes in mRNA expression following MR blockade are attributable to an absence of MR activity or an exaggerated presence of GR. Based on the available data, it appears that MR promotes protective gene induction under normal conditions, which may have important implications for survival of pyramidal cells following injury.
Chapter Three: Adrenocorticosteroid receptor blockade and excitotoxic challenge regulate adrenocorticosteroid receptor mRNA levels in hippocampus

Introduction

Glucocorticoids are steroid hormones that convey hypothalamic-pituitary-adrenal (HPA) axis information throughout the body. Two types of adrenocorticosteroid receptors (ACRs) mediate glucocorticoid action in brain: the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). Once the receptors bind glucocorticoid ligand, cortisol in humans and corticosterone (CORT) in rodents, MR and GR act as transcription factors (Yamamoto, 1985) to modulate genes controlling many physiological processes including development, metabolism, memory, inflammation, immune function, and cell death (Munck et al., 1984; McEwen et al., 1986).

Because the MR has greater affinity for binding CORT and the GR greater capacity, the MR is mainly active at basal glucocorticoid concentrations while the GR is extensively bound during periods of elevated glucocorticoid levels (Reul and de Kloet, 1985; Reul et al., 1987). Predominant activation of the MR or GR at different CORT concentrations appears to have opposing effects on neuronal viability in hippocampus, an area of MR and GR colocalization (Reul and de Kloet, 1985; Herman et al., 1989a) that is particularly susceptible to glucocorticoid-mediated effects. For example, activation of the MR protects hippocampal dentate gyrus (DG) granule cells from adrenalectomy (ADX) induced death (Woolley et al., 1991). In contrast, prolonged elevation of glucocorticoid levels, acting mainly through the GR, shrinks pyramidal cell dendrites (Woolley et al., 1990), impairs calcium regulation (Elliott and Sapolsky, 1993), and exacerbates neuronal insults (Sapolsky, 1985; Sapolsky and Pulsinelli, 1985; Sapolsky, 1986a; Sapolsky et al., 1988; Goodman et al., 1996; McIntosh and Sapolsky, 1996a). Thus, evidence exists that the MR protects and the GR endangers hippocampal neurons.

Considering the differential effects of MR and GR on hippocampal cell survival, ACR activation may be of critical importance in the cellular response to injury. To address this issue, the present study is designed to investigate the roles of the MR and the GR in regulating ACR expression and neuron viability under basal conditions and following excitotoxic insult. Subjects
were pretreated with vehicle, the MR antagonist spironolactone (SPIRO), or the GR antagonist mifepristone (RU486) and subsequently challenged with systemic injection of kainic acid (KA). *In situ* hybridization was performed to assess the effects of ACR blockade and subsequent challenge with KA on relative MR and GR mRNA levels. In addition, hippocampal neuronal loss was evaluated following MR or GR blockade and KA insult to determine the role of ACRs in hippocampal vulnerability to injury *in vivo*. The results of this study provide further evidence for homo- and heterologous regulation of ACR expression and indicate that ACR mRNA regulation is altered by glutamate receptor stimulation.

**Materials and Methods**

**Subjects**

Young adult male Sprague Dawley rats (Harlan Sprague Dawley, Inc., Indianapolis, IN) weighing between 200-250 gm were maintained on a 12 hr light/dark cycle in an environment with constant temperature, humidity, and ad libitum access to food and water. All animal procedures were performed in accordance with the National Institutes of Health guidelines and approved by the University of Kentucky Institutional Animal Care and Use Committee.

**In vivo protocols**

All rats were injected s.c. twice daily, in morning and afternoon, with propylene glycol vehicle, MR antagonist spironolactone (SPIRO) (50 mg/kg) (Herman and Spencer, 1998) (Sigma, St. Louis, MO), or GR antagonist RU486 (25 mg/kg) (Ratka et al., 1989) (Sigma). Subjects were administered a final pretreatment injection at 900h on day three, followed by an i.p. injection of either saline vehicle or kainic acid (KA) (12 mg/kg)(Sigma) at 1100h. Subjects were monitored for seizure activity for 6h following KA injections, and only those displaying behavioral changes indicative of KA intoxication (Sperk et al., 1983) were included in the study. All animals were sacrificed on day 4 at 1200h, approximately 24h after seizure onset.

Animals processed for plasma CORT determination and in situ hybridization experiments were killed by rapid decapitation. Brains were rapidly removed, frozen in isopentane cooled to –40°C on dry ice, then stored at –80°C. Brains were later warmed to -20°C, sectioned to 16 um with a Bright-Hacker cryostat, thaw-mounted onto Ultrastick Slides (Becton Dickinson, Franklin
Lakes, NJ), and stored at -20°C until processing for in situ hybridization. Trunk blood samples were collected into heparinized tubes on ice, and plasma was separated by centrifugation and stored at -20°C.

Animals processed for cell counts received a lethal dose of sodium pentobarbitol (Butler, Columbus, OH)(150mg/kg) before undergoing transcardial perfusion with 0.9% saline followed by 4% paraformaldehyde. Brains were removed, post-fixed in 4% paraformaldehyde overnight, then placed in 30% sucrose at 4°C for 24h. Brains were then stored in 70% ethanol until embedded in paraffin.

Radioimmunoassay

Radioimmunoassay was performed to measure plasma CORT concentration. A double-antibody rat corticosterone kit (ICN Biochemicals, Costa Mesa, CA) was used to determine 125I-labeled CORT levels. Due to a skewed distribution of CORT values, we performed a log transformation of the data before analysis by two-way ANOVA. Data shown in Table 3.1 represent actual CORT values listed as mean ± standard error. Two-way ANOVA was used to analyze log-transformed CORT values for main effects (of antagonist groups and KA treatment) and their interactions. Pairwise comparisons were made to determine significance of individual group effects within treatments and vice versa using the Student-Newman-Keuls test, with significance set at p ≤ 0.05.

In situ hybridization

Antisense riboprobes specific for rat MR (550 bp, complementary to the coding region and 3’ untranslated region of rat MR mRNA) and rat GR (456 bp, complementary to the coding region and 3’ untranslated region of rat GR mRNA) were labeled according to previously published methods (Seroogy and Herman, 1996) by standard in vitro transcription using [35S]UTP. Tissue sections removed from a -20°C freezer were fixed in 4% paraformaldehyde for 10 min. Slides were then rinsed sequentially in the following solutions: 5 mM KPBS, 5 mM KPBS with 0.2% glycine, 5mM KPBS, 0.1 M triethanolamine with 0.25% acetic anhydride, 0.2X SSC, and graded alcohols. Labeled probes were added to a hybridization buffer containing 50% formamide, and diluted probe (50 ul containing 1x 10^6 c.p.m.) was applied to each slide. Slides were coverslipped, placed in moistened chambers, and incubated overnight at 55°C. Following
hybridization, slides were rinsed in 2X SSC and incubated in 0.2X SSC at 60°C for 1 hr. Slides were then dehydrated and exposed for 14 days to Kodak BioMAX xray film.

Autoradiographic images were digitized with NIH Image 1.62 software for Macintosh. Based on morphological criteria, regions CA1, CA3, and DG of the hippocampal formation were manually sampled for densitometric analysis. The mean gray level of an unhybridized (background) area from each section was subtracted from sampled regions to arrive at corrected gray level. The data were then expressed as a percentage of control (vehicle/saline) and analyzed by two-way ANOVA within each subregion. Pairwise comparisons were made using the Student-Neuman-Keuls test to determine significant effects of groups within treatments and vice versa, with significance set at p ≤ 0.05. Data in Figures 3.2 and 3.3 are presented as percentage of vehicle/saline control ± standard error.

Cell counts

Nissl-stained sections of perfused tissue were examined by light microscopy for undamaged neurons in the CA1 and CA3 pyramidal layers of dorsal hippocampus approximately 3mm posterior to bregma. Cell counts were not performed in dentate gyrus because no damaged granule cells were observed and because of the general insensitivity of this subregion to KA (Sperk et al., 1983). Neurons identified by round, healthy-looking somata and prominent nucleoli were counted by an observer blinded to the experimental design. In preparation for cell counts, perfused, paraffin-embedded, slide-mounted tissue was deparaffinized in xylene for 10 min, hydrated through graded alcohols, and immersed in cresyl violet Nissl stain for 5 min. Slides were then rinsed briefly in dH2O, dehydrated through graded alcohols, cleared in xylene for 5 min, and coverslipped with DPX mountant.

To obtain cell counts, numbers of neurons with identifiable nucleoli were recorded from a 100 μm² field. Counts were performed in three sections per subject, and within each section, three fields were counted from CA1 and from CA3 then averaged to calculate subregional means per section. Subregional means from each section were then averaged to determine mean numbers of live CA1 and CA3 neurons for each animal. Cell counts were analyzed for significant effects using two-way ANOVA. Post hoc comparisons were made using the Student-Neuman-Keuls test, with significance set at p ≤ 0.05. Data in Table 3.2 are presented as mean cell number per 100 μm² field ± standard error.
Results

Effects of ACR blockade and KA on CORT levels

Two-way ANOVA indicates that 24h post-insult, KA significantly increased CORT levels \[F(1,42) = 65.42; \ p \leq 0.05\] (Table 3.1). Post hoc comparisons indicate that KA-induced elevations in CORT levels were significant in all pretreatment groups. ACR antagonists also produced a significant main effect on CORT levels \[F(2,42) = 5.96; \ p \leq 0.05\]. In saline-treated animals, MR blockade with SPIRO increased basal CORT levels, while GR antagonist RU486 decreased basal CORT levels relative to vehicle-pretreated animals.

Table 3.1. Effects of adrenocorticosteroid receptor blockade and kainic acid on plasma corticosterone levels (ng/ml)

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>N</th>
<th>Mean CORT Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEHICLE</td>
<td>SALINE</td>
<td>11</td>
<td>78.35 ± 20.89</td>
</tr>
<tr>
<td>SPIRO</td>
<td>SALINE</td>
<td>7</td>
<td>137.01 ± 23.24</td>
</tr>
<tr>
<td>RU486</td>
<td>SALINE</td>
<td>6</td>
<td>23.93 ± 5.07</td>
</tr>
<tr>
<td>VEHICLE</td>
<td>KA</td>
<td>11</td>
<td>332.03 ± 59.42</td>
</tr>
<tr>
<td>SPIRO</td>
<td>KA</td>
<td>6</td>
<td>275.43 ± 53.35</td>
</tr>
<tr>
<td>RU486</td>
<td>KA</td>
<td>7</td>
<td>325.85 ± 69.87</td>
</tr>
</tbody>
</table>

SPIRO = MR antagonist spironolactone  
RU486 = GR antagonist mifepristone  
KA = excitotoxin kainic acid

\(^a p \leq 0.05\) as compared to vehicle/saline subjects (effect of antagonist)  
\(^b p \leq 0.05\) as compared to saline subjects within the same pretreatment group (effect of kainic acid)
Effects of ACR blockade and KA on MR and GR mRNA levels

Relative changes in ACR mRNA levels were determined by film densitometry following in situ hybridization with MR and GR-specific riboprobes (see Methods, Figure 3.1).

Figure 3.1. Photomicrographs illustrating the effects of saline (A,C) and kainic acid (B,D) on MR (A,B) and GR (C,D) mRNA levels in hippocampus of vehicle-pretreated rats. Panels A,B: Kainic acid decreases MR mRNA levels in CA1 and CA3. Panels C,D: Kainic acid decreases GR mRNA levels in dentate gyrus.
Pretreatment with ACR antagonists significantly affected MR mRNA levels in hippocampal subregions CA1 \([F(2,42) = 5.51; p \leq 0.05]\), CA3 \([F(2,42) = 6.58; p \leq 0.05]\), and DG \([F(2,42) = 4.80; p \leq 0.05]\) (Figure 3.2). MR antagonist SPIRO increased MR mRNA signal in CA1, CA3, and DG of saline-treated animals, while GR antagonist RU486 had no significant effects on basal MR mRNA levels. KA effects on MR mRNA levels were significant in CA1 \([F(1,42) = 83.43; p \leq 0.05]\) and CA3 \([F(1,42) = 60.62; p \leq 0.05]\). KA decreased MR mRNA levels in regions CA1 and CA3 of all pretreatment groups.

Figure 3.2. Effects of ACR blockade and KA treatments on MR mRNA levels in rat hippocampus. Semi-quantitative analysis expressed as percentage of control (vehicle/saline) corrected gray level. SPIRO significantly increased basal MR mRNA levels in CA1, CA3, and DG of saline-treated animals. KA significantly decreased MR mRNA levels in CA1 and CA3 of all pretreatment groups. \(^a p \leq 0.05\) as compared to vehicle/saline animals (effect of antagonist group). \(^b p \leq 0.05\) as compared to saline animals within same pretreatment group (effect of KA treatment).]
Two-way ANOVA demonstrates a significant group/treatment interaction effect on GR mRNA levels in region CA3 \([F(2,42) = 5.16; p \leq 0.05]\) (Figure 3.3). SPIRO and RU486 significantly increased basal GR mRNA in region CA3, but no significant interaction effects within post hoc comparisons were observed. Main effects of KA treatment on GR mRNA levels were significant in regions CA1 \([F(1,42) = 7.27; p \leq 0.05]\), CA3 \([F(2,42) = 24.72; p \leq 0.05]\), and DG \([F(2,42) = 91.25; p \leq 0.05]\). KA induced significant decreases in GR mRNA signal in CA1 of RU486 animals, in CA3 of SPIRO and RU486-treated animals, and in DG of all pretreatment groups.

![Figure 3.3. Effects of ACR blockade and KA treatments on GR mRNA levels in rat hippocampus. Semi-quantitative analysis expressed as percentage of control (vehicle/saline) corrected gray level. SPIRO and RU486 significantly increased basal GR mRNA levels in CA3 of saline-treated animals. KA decreased GR mRNA levels in CA1 of the RU486 pretreatment group, in CA3 of SPIRO and RU486 pretreatment groups, and in DG of all groups. \(^a p \leq 0.05\) as compared to vehicle/saline animals (effect of antagonist group). \(^b p \leq 0.05\) as compared to saline animals within same pretreatment group (effect of KA treatment).]

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Effects of ACR blockade and KA on hippocampal neuron viability

Neurons were counted to determine the effects of ACR antagonists on hippocampal neuron viability in CA1 and CA3 following KA challenge (see Methods) (Table 3.2). Neither ACR blockade nor KA significantly affected the number of CA1 pyramidal neurons. Kainic acid produced a significant main effect on the number of viable neurons in CA3 \([F(1,22) = 6.35; p \leq 0.05]\). The excitotoxin decreased live cell numbers in CA3 of SPIRO-pretreated subjects, but post hoc comparisons indicate no significant effects of antagonist pretreatment.

Table 3.2. Effects of adrenocorticosteroid receptor blockade and kainic acid on hippocampal neuron viability

<table>
<thead>
<tr>
<th>Region</th>
<th>Group</th>
<th>Treatment</th>
<th>N</th>
<th>Mean LIVE neurons per 100 (\mu m^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA1</td>
<td>VEHICLE</td>
<td>SALINE</td>
<td>4</td>
<td>63.54 ± 3.06</td>
</tr>
<tr>
<td></td>
<td>SPIRO</td>
<td>SALINE</td>
<td>4</td>
<td>62.46 ± 2.51</td>
</tr>
<tr>
<td></td>
<td>RU486</td>
<td>SALINE</td>
<td>3</td>
<td>65.00 ± 2.25</td>
</tr>
<tr>
<td></td>
<td>VEHICLE</td>
<td>KA</td>
<td>8</td>
<td>60.79 ± 2.16</td>
</tr>
<tr>
<td></td>
<td>SPIRO</td>
<td>KA</td>
<td>4</td>
<td>47.25 ± 14.81</td>
</tr>
<tr>
<td></td>
<td>RU486</td>
<td>KA</td>
<td>5</td>
<td>48.90 ± 8.36</td>
</tr>
<tr>
<td>CA3</td>
<td>VEHICLE</td>
<td>SALINE</td>
<td>4</td>
<td>39.67 ± 1.69</td>
</tr>
<tr>
<td></td>
<td>SPIRO</td>
<td>SALINE</td>
<td>4</td>
<td>34.63 ± 1.52</td>
</tr>
<tr>
<td></td>
<td>RU486</td>
<td>SALINE</td>
<td>3</td>
<td>31.39 ± 6.54</td>
</tr>
<tr>
<td></td>
<td>VEHICLE</td>
<td>KA</td>
<td>8</td>
<td>30.38 ± 2.82</td>
</tr>
<tr>
<td></td>
<td>SPIRO</td>
<td>KA</td>
<td>4</td>
<td>22.60 ± 3.92^a</td>
</tr>
<tr>
<td></td>
<td>RU486</td>
<td>KA</td>
<td>5</td>
<td>28.10 ± 5.20</td>
</tr>
</tbody>
</table>

SPIRO = MR antagonist spironolactone
RU486 = GR antagonist mifepristone
KA = excitotoxin kainic acid
^a\(p \leq 0.05\) as compared to SPIRO/saline subjects (effect of kainic acid)
Discussion

The present study characterizes the effects of MR and GR antagonists on plasma CORT levels and hippocampal ACR mRNA regulation under basal conditions and after excitotoxic challenge with kainic acid. We report increases in basal MR mRNA levels in CA1, CA3, and DG following MR blockade and demonstrate increases in basal GR mRNA levels in CA3 following both MR and GR blockade. Further, we report that administration of the ionotropic glutamate receptor agonist kainic acid reduces MR and GR mRNA levels in specific hippocampal subfields at 24h following insult, indicating differential regulation of ACR mRNAs during excitotoxic injury. Cell counts following KA administration provide evidence that the magnitude of ACR mRNA downregulation exceeds that of cell loss.

Effects of ACR blockade and KA on CORT levels

In this study, pretreatment with SPIRO significantly increased basal CORT levels (Table 3.1). There is evidence that MR blockade with antagonist RU28318 increases CORT levels in rat (Ratka et al., 1989; van Haarst et al., 1997), suggesting that the SPIRO-induced CORT increase is due to MR blockade and a resultant disinhibition of adrenocortical secretion. However, other studies using SPIRO have reported no significant effects on plasma CORT levels (Chabert et al., 1984; Herman and Spencer, 1998). The reason for this disagreement may be related to differing treatment protocols and durations.

Glucocorticoid receptor blockade with RU486 decreased basal CORT levels in this study. This effect concurs with data demonstrating RU486-mediated suppression of CORT induction 15 minutes following introduction to a novel environment (Ratka et al., 1989). Considering the relatively high CORT levels observed in our saline/vehicle-treated (control) subjects, the observed decrease in CORT levels may reflect RU486-mediated reduction of CORT levels subsequent to stress associated with either the euthanasia procedure or intraperitoneal injections received the previous day. Despite its efficacy as a GR antagonist, RU486 can also act as a partial GR agonist (Nordeen et al., 1995), which might explain a reduced stress response in saline animals. However, CORT response to KA is not attenuated in RU486-treated animals, suggesting that effects of RU486 on delayed negative feedback are minimal.
Kainic acid induced a massive increase in CORT secretion observable 24h after injection. Acute effects of KA include hyperactivity, “wet dog shakes”, and prolonged tonic-clonic convulsions. Dehydration, brain edema, and perivenous hemorrhages are also common 24-48h after KA injection (Sperk et al., 1983). It is therefore likely that the excitotoxin is a potent stressor on multiple levels and thus increases CORT secretion for an extended period after injection.

**ACR antagonists modulate MR and GR mRNA levels**

Treatment with the MR antagonist SPIRO increased basal MR mRNA levels in all observed hippocampal subregions by approximately 30-40% (Figures 3.1 and 3.2). These changes complement reports of MR mRNA upregulation by adrenalectomy (Herman et al., 1989a; Reul et al., 1989; Herman et al., 1993; Chao et al., 1998a) and provide additional evidence for homologous feedback regulation of MR. The MR also appears to regulate expression of GR. The present data demonstrate a significant SPIRO-induced increase in GR mRNA in CA3; GR mRNA levels are similarly elevated in CA1 and DG of SPIRO-pretreated subjects but do not reach our criteria for statistical significance. These data add to previous results from our laboratory showing significant increases in GR mRNA levels in CA1 and DG with SPIRO pretreatment (Herman and Spencer, 1998). In addition, the MR agonist aldosterone decreases GR binding in primary hippocampal cell cultures (O'Donnell and Meaney, 1994), and administration of aldosterone or low, MR-activating doses of CORT reverses ADX-induced increases in GR mRNA (Chao et al., 1998a). Together, these results and the current data suggest that MR functions as a tonic inhibitor of GR expression in hippocampus.

Preceding evidence also suggests that the activated glucocorticoid receptor negatively regulates ACR expression. MR mRNA levels decrease during periods of high CORT secretion (Herman et al., 1993) when the GR is most extensively activated (Reul and de Kloet, 1985). Further, glucocorticoid receptor agonist dexamethasone reverses ADX-induced increases in GR (Herman et al., 1989a; Reul et al., 1989) and MR (Herman et al., 1989a) mRNA levels and reduces GR mRNA expression in culture (Rosewicz et al., 1988). In the present experiment, RU486 significantly increased GR mRNA levels in CA3 (Figures 3.1 and 3.3), suggesting that glucocorticoid receptor blockade increases GR biosynthesis by homologous feedback mechanisms. However, RU486 did not increase MR mRNA levels, implying that glucocorticoid
receptor regulation of MR is limited to suppressing MR levels during periods of extensive GR activation.

SPIRO and RU486 are commonly used to achieve ACR blockade and are among the most specific antagonists available, but like many other antagonist drugs they may produce some nonspecific effects. SPIRO is considered relatively specific for the MR but has been demonstrated a weak GR antagonist in a transformed murine fibroblast cell line (Couette et al., 1992). RU486 is an extremely potent GR antagonist in vitro, binding the GR with approximately 18 times the affinity of CORT, but also has strong anti-progestin activity (Baulieu, 1989). RU486 may also act as a GR agonist under certain circumstances (Nordeen et al., 1995) and may not be sufficient to fully block GR activation in vivo. However, it should be noted that the currently reported antagonist effects correspond very well with the effects of adrenalectomy (Herman et al., 1989a; Reul et al., 1989; Herman et al., 1993; Chao et al., 1998a); the antagonist drugs increase hippocampal ACR mRNA expression in saline animals for the duration of the experiment.

Excitotoxic challenge with KA decreases MR and GR mRNA levels

The present data show dramatic reductions in hippocampal MR and GR mRNA levels following kainic acid insult (Figures 3.1, 3.2, and 3.3). KA-induced decreases in MR and GR binding have previously been demonstrated using whole dissected hippocampus from adrenalectomized rats (Lowy, 1992). Reductions in MR and GR binding are similar in magnitude to the currently reported mRNA decreases, are evident as early as one hour after systemic KA administration, and persist for at least 24 hours (Lowy, 1992). Therefore, the current data provide further evidence that MR and GR availability is reduced by KA and indicate that this reduction is likely achieved through genomic downregulation, rather than by protein degradation or receptor inactivation.

ACR downregulation by KA does not appear to be MR or GR-dependent. In the current study, KA reduces ACR mRNA expression in the presence of MR or GR blockade. In addition, a previous study has demonstrated that KA decreases ACR binding in adrenalectomized rats (Lowy, 1992); corticosterone depletion in adrenalectomized rats prevents ACR activation and their potential participation in the regulation of receptor expression. Furthermore, the pattern of ACR downregulation produced by KA is inconsistent with the pattern of regulation associated
with high CORT levels. High CORT levels decrease MR and GR mRNA levels by equal proportions in CA1, CA3, and DG (Herman et al., 1993). In contrast, KA regulation of ACR expression differs substantially by hippocampal subfield. Mineralocorticoid receptor expression is downregulated in KA-sensitive regions CA1 and CA3, while GR expression is reduced in the relatively KA-insensitive DG. These results suggest that KA regulation of ACR expression is not mediated by KA induction of CORT, but that differential ACR regulation by KA is achieved through other mechanisms related to KA susceptibility, such as glutamate receptor activation or Ca\(^{2+}\) influx.

Interestingly, increases in MR and GR mRNA that occur with ACR antagonist pretreatment are reversed by KA insult. Although KA does not affect MR mRNA levels in DG of vehicle-pretreated animals, the SPIRO-induced increase in this region is negated by KA treatment. Similarly, KA does not reduce GR mRNA levels in the pyramidal layers of vehicle-pretreated animals, but SPIRO and RU486-induced increases in CA3 are abolished with KA injection. These results suggest that homologous regulation of the receptors is suppressed or overridden by KA, functionally disconnecting autoregulatory feedback.

Hippocampal pyramidal neurons are extremely sensitive to KA toxicity; CA3 neurons are particularly vulnerable, while CA1 neurons are slightly less susceptible, to KA-induced damage and cell degeneration (Nadler et al., 1978). The potential impact of cell loss on mRNA levels as measured by \textit{in situ} hybridization is an important consideration that is presently undetermined. However, it is clear that in the hippocampal pyramidal layers, KA-induced decreases in MR mRNA levels exceed the proportion of neuronal loss indicated by cell counts – by approximately 25 to 50\% (see Figures 3.1, 3.2, and Table 3.2). Furthermore, DG granule cells are resistant to KA toxicity and exhibit no histological damage after systemic KA administration (Sperk et al., 1983). Therefore, the approximately 40-55\% reduction in GR mRNA levels observed in DG is not attributable to a decline in cell number.

KA-induced repression of ACR mRNA levels might influence neuronal viability during injury. The receptors have potent cellular effects, some of which could endanger neurons under compromising conditions. For example, MR-modulated excitation of pyramidal neurons (Reiheld et al., 1984; Joels and de Kloet, 1990, 1992) could be permissive to excitotoxic damage. MR blockade with SPIRO has been shown to inhibit CORT-mediated increases in susceptibility to KA-induced convulsions in mice (Roberts and Keith, 1994a, b). Thus, downregulation of the
MR in pyramidal cells may reduce excitability and vulnerability to excitotoxic challenge. Moreover, the GR has long been recognized as a potentially compromising influence on hippocampal neurons. GR activation has been shown to increase intracellular calcium levels in CA1 pyramidal cells (Kerr et al., 1992; Karst et al., 1994) and exacerbate the damaging effects of toxic insults including KA, oxidative stress, and ischemia (Sapolsky, 1985; Sapolsky and Pulsinelli, 1985; Sapolsky, 1986a; Sapolsky et al., 1988; Goodman et al., 1996; McIntosh and Sapolsky, 1996a). Downregulation of the GR in dentate gyrus might protect granule cells from effects of excitotoxicity such as extracellular glutamate accumulation, Ca\(^{2+}\) influx, and oxidative stress.

**MR blockade increases CA3 vulnerability to KA**

Kainic acid decreased the number of live CA3 neurons counted in SPIRO-pretreated subjects; however, KA had no significant effect on numbers of viable neurons in any other region or group (Table 3.2). These data indicate that MR blockade increases susceptibility to KA-induced degeneration in CA3. We have previously demonstrated that MR blockade with SPIRO decreases basal mRNA levels of the anti-apoptotic gene bcl-2 in the hippocampal pyramidal layers (McCullers and Herman, 1998) (Chapter Two). Thus, MR appears to play a neuroprotective role during neuronal injury that may occur through maintenance of viability-enhancing genes such as bcl-2.

The results of this study demonstrate that MR and GR mRNA levels respond to stress as conveyed not only by differential receptor activation, but also by ACR-independent signals generated by excitotoxic stress. Further, kainic acid regulation of ACR mRNA levels appears to override ACR-dependent feedback mechanisms. The present evidence for modulation of ACR mRNA expression during injury emphasizes the importance of ACR function for maintenance of neuronal viability, particularly considering the prevalence of HPA dysregulation in neurodegenerative conditions such as Alzheimer’s disease and in normal aging.
Chapter Four: Mifepristone protects CA1 hippocampal neurons following traumatic brain injury in rat

Introduction

Glucocorticoids modulate many cellular processes relevant to injury, including metabolism, ion balance, immune function, inflammation, neurotrophin expression, and cell death (Munck et al., 1984; McEwen et al., 1986; Reagan and McEwen, 1997; Joels and Vreugdenhil, 1998). Two steroid hormone receptors mediate glucocorticoid action: the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). Glucocorticoids bind GR with lower affinity but higher capacity relative to MR; thus, MR is extensively bound at very low (e.g. circadian nadir) glucocorticoid levels, while GR mediates the effects of high glucocorticoid levels (Reul and de Kloet, 1985; Reul et al., 1987). The MR and GR are abundantly expressed in hippocampus (Reul and de Kloet, 1985; Aronsson et al., 1988; Arriza et al., 1988; Reul et al., 1989), making this region a prime target for glucocorticoid action. Excessive activation of GR has been shown to increase hippocampal neuronal vulnerability to several forms of neuronal insult, including excitotoxicity, oxidative stress, and ischemia (Sapolsky, 1985; Sapolsky and Pulsinelli, 1985; Sapolsky, 1986a; Sapolsky et al., 1988; Goodman et al., 1996; McIntosh and Sapolsky, 1996a).

Moderate to severe traumatic brain injury (TBI) in rat produces neuronal damage and cell death in hippocampus (Cortez et al., 1989; Lowenstein et al., 1992; Goodman et al., 1994; Hicks et al., 1996). The significant cell death that occurs following TBI is believed to evolve in a biphasic sequence consisting of the primary mechanical insult coupled with a progressive secondary necrosis. Results of experimental TBI research have proposed several injury mechanisms including excitotoxicity (Choi, 1992; Hayes et al., 1992), ischemia (Okiyama et al., 1994), free radical formation (Braughler and Hall, 1992), elevated intracellular calcium (Young, 1992), edema (Wahl et al., 1988), proteolysis (Kampfl et al., 1997), and mitochondrial dysfunction (Sullivan et al., 1998; Sullivan et al., 1999). Glucocorticoids likely modulate some or all of the processes mediating secondary injury. One possible mechanism whereby glucocorticoids could influence neuronal vulnerability to injury is through regulation of the anti-apoptotic B-cell leukemia/lymphoma-2 gene, bcl-2. Several studies indicate that bcl-2 is induced by TBI and has neuroprotective effects after traumatic injury (Clark et al., 1997; Raghupathi et
al., 1998; Nakamura et al., 1999). Our laboratory has demonstrated that MR blockade with spironolactone decreases basal bcl-2 messenger RNA (mRNA) levels (McCullers and Herman, 1998) (Chapter Two), suggesting that MR activation maintains neuroprotective bcl-2 expression and may participate in regulation of neuronal viability after TBI.

The present study is designed to test the hypotheses that GR blockade will attenuate and MR blockade will exacerbate hippocampal cell loss following controlled cortical impact (CCI), an experimental model of TBI. Animals were pretreated with vehicle, the MR antagonist spironolactone (SPIRO), or the GR antagonist mifepristone (RU486) and subsequently subjected to either sham operation or injury by CCI. Using the optical fractionator method, CA1, CA3, and dentate gyrus (DG) hippocampal neurons were counted twenty-four hours after surgery to determine SPIRO and RU486 effects on cell survival after CCI. In addition, mRNA levels of the anti-apoptotic gene bcl-2, the pro-apoptotic gene bax, and the tumor-suppressor gene p53 (a transcriptional regulator of bcl-2 and bax) (Miyashita et al., 1994) were examined after MR or GR blockade and CCI to determine whether MR or GR regulation of these genes correlates with neuronal viability after traumatic brain injury.

Materials and Methods

Subjects

Young adult male Sprague Dawley rats (n=60) (Harlan Sprague Dawley, Inc., Indianapolis, IN) weighing 200-250g were maintained on a 12 hr light/dark cycle in an environment with constant temperature, humidity, and ad libitum access to food and water. All animal procedures were performed in accordance with the National Institutes of Health guidelines and approved by the University of Kentucky Institutional Animal Care and Use Committee. All efforts were made to minimize animal suffering and the number of animals used.

In vivo protocols

Subjects received subcutaneous injections of propylene glycol vehicle, MR antagonist spironolactone (SPIRO) (50 mg/kg) (Herman and Spencer, 1998) (Sigma, St. Louis, MO), or GR antagonist RU486 (25 mg/kg) (Ratka et al., 1989) (Sigma) twice daily, in morning and afternoon. Pretreatment was conducted for forty-eight hours to ensure adequate time for the altered
expression of ACR-regulated genes (Herman and Spencer, 1998). After a final injection was administered at 900h on day three, subjects were transported to a surgical procedure room. Between 1100h-1200h, animals were subjected to craniotomy only (sham operation) or a moderate unilateral cortical contusion (2mm). All animals were sacrificed on day four at 1200h, approximately twenty-four hours after surgery. 8-12 subjects were included in each pretreatment/surgery group. Half of the subjects in each condition were perfused for cell counts, and half were decapitated for in situ hybridization analysis, yielding n = 4-6 subjects per statistical group. Surgical procedures are briefly described below.

The moderate CCI injury used in these experiments results in significant loss of cortical tissue, blood brain barrier disruption, and loss of hippocampal neurons as previously described (Baldwin et al., 1997; Scheff and Sullivan, 1999), thus mimicking aspects of the pathology of human closed-head injury. Animals were anesthetized with isoflurane (2%) and placed in a stereotaxic frame (Kopf Instr., Tujunga, CA) prior to CCI injury. Using sterile procedures, the skin was retracted and a 6mm unilateral craniotomy was centered between bregma and lambda. The skull cap was removed without disruption of the underlying dura. The exposed brain was injured using a pneumatically controlled impacting device with a 5mm beveled tip that compressed the cortex 3.5 m/sec to a depth of 2mm. Following injury, Surgicel (Johnson and Johnson, Arlington, TX) was laid upon the dura, and the skull cap was replaced. A thin coat of dental acrylic was then spread over the craniotomy site and allowed to dry before the wound was stapled closed. Body temperature was monitored continuously throughout the surgical/recovery procedures and maintained at 37°C with a heating pad.

Animals processed for in situ hybridization experiments were killed by decapitation. Brains were rapidly removed, frozen in isopentane cooled to −40°C on dry ice, then stored at −80°C until sectioned. Trunk blood samples were collected on ice, and plasma was stored at -20 °C until plasma corticosterone levels were measured using commercial radioimmunoassay kits per manufacturers’ instructions (ICN Biochemicals, Costa Mesa, CA). Animals processed for cell counts received a lethal dose of sodium pentobarbitol (Butler, Columbus, OH) (150mg/kg) before undergoing transcardial perfusion with 0.9% saline followed by 4% paraformaldehyde. Brains were removed and post-fixed in 4% paraformaldehyde overnight before further processing.
Tissue processing

Frozen brains were removed from a –80°C freezer, warmed to –20°C, and sectioned to 16 μm with a Bright-Hacker cryostat. Sections were then thaw-mounted onto Ultrastick Slides (Becton Dickinson, Franklin Lakes, NJ) and stored at –20°C until processing for in situ hybridization. Perfused brains were processed as previously described (Baldwin et al., 1997). Briefly, brains were placed in a graded series of celloidin (Malinckrodt Baker, Inc., Paris, KY) dissolved in 50:50 ETOH:ethyl ether. After removal from 12% celloidin, the brains were placed in Peel-A-Way (Polyscience, Warrington, PA) disposable histology embedding molds with fresh 12% celloidin and exposed to chloroform vapors for 18 hours. Once the celloidin hardened, molds were removed. The embedded tissue was then attached to wooden blocks and stored in 70% EtOH until sectioning. The brains were cut in coronal sections (50 μm) with a Zeiss HM440E sliding microtome. Sections were collected from anterior to posterior beginning at the appearance of anterior hippocampus. In order to select a series in an unbiased manner such that every section had an equal probability of being selected, a die was thrown to determine the first section saved, after which every twelfth section was kept for staining with Giemsa. Six drops of 1% glacial acetic acid were added per 100 ml of Giemsa stain (Sigma, St. Louis, MO) diluted 1:4 with water. Following staining the sections were placed in cedar wood oil for 5 min to reduce background and subsequently washed in Hemo-D (Fisher Scientific, Pittsburgh, PA). The sections were then mounted on slides, covered with permount, and coverslipped.

Optical fractionator method

Bioquant Image Analysis software (R and M Biometrics, Memphis, TN) was used to estimate total cell number in regions of interest using the optical fractionator method of cell counting. The optical fractionator method involves the combining of optical dissector counting with fractionator sampling. This method of cell counting has several desirable attributes, such as being unaffected by tissue shrinkage and not requiring rigorous definitions of structural boundaries. For detailed explanation and discussion please see (West et al., 1991). The optical fractionator involves counting neurons with optical dissectors in a uniform sample that constitutes a known fraction of the structure being analyzed; the method systematically samples a known fraction of the section thickness, of a known fraction of sectional area, of a known
fraction of the sections that contain the region of interest. The equation is as follows (our specific parameters):

\[ N = \sum Q \cdot \frac{t}{h} \cdot \frac{1}{\text{asf}} \cdot \frac{1}{\text{ssf}} \]

Where \( N \) is the total number of neurons, and \( \Sigma Q \) is the total number of neurons counted in each region of interest in each animal. \( t \) is the measured section thickness (42-45 um), \( h \) is the height of the dissector (20 um), \( 1/\text{asf} \) is the counting grid area (100 um X 100 um)/ the dissector area (20 um X 20 um), and \( 1/\text{ssf} \) is the sampling section fraction (12). Criteria for counting neuronal nuclei were observed as previously described (Baldwin et al., 1997). Briefly, the plane of focus was moved 5 um down from the top of the section to obviate the problem of an uneven counting surface. Neuronal nuclei in focus at level –5 um, the top level of the dissector, were not counted, but nuclei in focus at –25 um, the bottom level of the dissector, were counted. Through the dissector, nuclei completely within the counting frame or touching the upper or right side of the frame were counted. Nuclei touching the left or lower sides of the frame were not counted (Baldwin et al., 1997). Coefficient of error (CE) and coefficient of variation (CV) were calculated to determine intra-animal variation and inter-animal variation, respectively (Tables 4.1 and 4.2).

<table>
<thead>
<tr>
<th>Table 4.1. Mean coefficient of error (CE)</th>
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<tbody>
<tr>
<td>CA1</td>
</tr>
<tr>
<td>---------------------</td>
</tr>
<tr>
<td>Vehicle</td>
</tr>
<tr>
<td>Spironolactone</td>
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<table>
<thead>
<tr>
<th>Table 4.2. Mean coefficient of variation (CV)</th>
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<tr>
<td>CA1</td>
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</tr>
<tr>
<td>Spironolactone</td>
</tr>
<tr>
<td>RU486</td>
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</tbody>
</table>

Cell count data were analyzed by two-way ANOVA within each subregion, with hemisphere and drug designated as independent variables. Pairwise comparisons were made using the Student-Neuman-Keuls test with significance set at \( p \leq 0.05 \). Data in Figure 4.1 are presented as neurons x \( 10^4 \) ± standard error.
In situ hybridization

Antisense riboprobes complementary to coding regions for bcl-2 (550 bp) (courtesy of Dr. D. Vaux, The Walter and Eliza Hall Institute, The Royal Melbourne Hospital, Victoria, Australia), bax (100 bp) (courtesy of Dr. J. Springer, University of Kentucky, KY, USA), and p53 (950 bp) (courtesy of Dr. G. Lozano, MD Anderson Cancer Center, Houston, TX, USA) mRNAs were generated for in situ hybridization. Probes were labeled according to previously published methods (Seroogy and Herman, 1996) by standard in vitro transcription using \[^{35}\text{S}U\text{TP}\]. Tissue sections removed from a −20°C freezer were fixed in 4% paraformaldehyde for 10 min. Slides were then rinsed sequentially in the following solutions: 5 mM KPBS, 5 mM KPBS with 0.2% glycine, 5 mM KPBS, 0.1 M triethanolamine with 0.25% acetic anhydride, 0.2X SSC, and graded alcohols. Labeled probes were added to a hybridization buffer containing 50% formamide, and diluted probe (50 ul containing 1x \(10^6\) c.p.m.) was applied to each slide. Slides were coverslipped, placed in moistened chambers, and incubated overnight at 55°C. Following hybridization, slides were rinsed in 2X SSC and incubated in 0.2X SSC at 60°C for 1 hr. Slides were then dehydrated and exposed for 14-18 days to Kodak BioMAX xray film.

Autoradiographic images were digitized with NIH Image 1.62 software for Macintosh. A standard curve was generated prior to image capture to ensure linearity of the signal. Based on morphological criteria, regions CA1, CA3, and DG of the hippocampal formation were manually sampled from both hemispheres for densitometric analysis. The mean gray level of an unhybridized (background) area from each hemisphere was subtracted from sampled regions to arrive at corrected gray level. Ipsilateral mRNA data were normalized as a percent of control (sham/vehicle) values from the ipsilateral hemisphere, and contralateral mRNA data were normalized as a percent of control (sham/vehicle) values from the contralateral hemisphere, with sham/vehicle levels expressed as one hundred percent. In situ hybridization data were then analyzed by two-way ANOVA within each hemisphere and subregion with drug and injury designated as independent variables. Post hoc comparisons were made using the Student-Newman-Keuls test, with significance set at \(p \leq 0.05\).
Results

Effects of adrenocorticosteroid receptor blockade and traumatic brain injury on hippocampal neuron viability

The numbers of hippocampal neurons surviving CCI at 24 hours in hippocampal subregions CA1, CA3, and DG were assessed using the optical fractionator method of cell counting (see Methods). Because sham operation does not cause cell loss and damage is confined to the ipsilateral hemisphere of injured rats, the numbers of neurons in sham/ipsilateral, sham/contralateral, and injured/contralateral hemispheres were statistically indistinguishable (i.e. unchanged by surgery) within each subregion (data not shown). Therefore, cell counts from injured/contralateral hemispheres were used as control values against which to compare injured/ipsilateral hemisphere values to determine the effects of CCI on neuronal survival. Effects of antagonist pretreatments were assessed as compared to vehicle within the ipsilateral and contralateral hemispheres.

Two-way ANOVA indicates a significant effect of hemisphere on numbers of neurons in CA1 [F(1,24) = 9.32; p ≤ 0.05], CA3 [F(1,24) = 38.17; p ≤ 0.05], and DG [F(1,24) = 5.12; p ≤ 0.05] (Figure 4.1). In CA1 of RU486-pretreated animals, numbers of surviving neurons were statistically indistinguishable in the ipsilateral and contralateral hemispheres. In contrast, numbers of hippocampal neurons in CA1 of vehicle and SPIRO-pretreated animals were significantly decreased by injury in the ipsilateral as compared to contralateral hemisphere. In CA3, injury significantly reduced the number of viable pyramidal neurons in the ipsilateral hemisphere in all pretreatment groups. Injury also significantly decreased the number of ipsilateral DG granule neurons counted in SPIRO and RU486-pretreated animals. Post-injury corticosterone levels fell within the range of approximately 15-80 ng/ml in all drug pretreatment groups, indicating that excessive corticosterone levels were not likely to contribute to the differential neuronal survival between groups.
Figure 4.1. Effects of MR blockade, GR blockade, and controlled cortical impact injury on hippocampal neuron viability as measured by the optical fractionator method (see Methods). Injury significantly decreased the number of viable neurons in CA1 and CA3 of vehicle-pretreated animals (n = 6). Pretreatment with RU486 protected CA1 pyramidal neurons from injury-induced cell loss (n = 5). Injury also decreased the number of viable neurons in CA3 and dentate gyrus of spironolactone (n = 4) and RU486-pretreated animals. [B p ≤ 0.05 as compared to contralateral hemisphere within same pretreatment group (effect of injury).]

Effects of adrenocorticosteroid receptor blockade and traumatic brain injury on bcl-2, bax, and p53 mRNA levels

The effects of MR or GR blockade and CCI on hippocampal bcl-2, bax, and p53 mRNA levels were determined using densitometric analysis following in situ hybridization with gene-specific radiolabeled riboprobes (see Methods, Figure 4.2). Unlike measures of cell loss, mRNA levels may be affected by injury both in the contralateral (unoperated) and ipsilateral (sham-operated or injured) hemispheres. Therefore, all injury-induced changes were assessed as compared to sham values from the same hemisphere. To this end, separate two-way ANOVAs were conducted comparing sham/contralateral to injured/contralateral values and comparing sham/ipsilateral to injured/ipsilateral values, with drug and injury as independent variables. In each hemisphere, effects of drug were assessed as compared to vehicle within the sham or injured treatment groups.
Figure 4.2. Photomicrographs illustrating (A) bcl-2, (B) bax, and (C) p53 mRNA levels in hippocampus of vehicle-pretreated, injured rats at 24h. Injury decreases bcl-2 mRNA levels in ipsilateral dentate gyrus (panel A) but has no significant effect on bax or p53 mRNA levels in vehicle-pretreated rats (panels B,C).
Two-way ANOVA demonstrates significant drug/injury interaction effects on contralateral bcl-2 mRNA levels in CA1 [F(2,27) = 3.47; p ≤ 0.05] and DG [F(2,27) = 5.67; p ≤ 0.05] (Figure 4.3). Post hoc comparisons indicate that bcl-2 signal was significantly decreased by RU486 and by SPIRO in contralateral CA1 and DG of sham-operated animals. Injury increased contralateral bcl-2 mRNA levels in DG of RU486 animals.

Drug/injury interaction effects on bcl-2 mRNA levels are observed in CA1 [F(2,27) = 5.01; p ≤ 0.05], CA3 [F(2,27) = 3.94; p ≤ 0.05], and DG [F(2,27) = 6.92; p ≤ 0.05] of the ipsilateral hemisphere (Figure 4.3). In sham-operated animals, ipsilateral bcl-2 mRNA levels were significantly decreased by SPIRO in DG and by RU486 in CA1 and DG. CCI decreased ipsilateral bcl-2 signal in DG of vehicle animals and increased ipsilateral bcl-2 signal in DG of RU486 animals.

Overall, bax mRNA levels were increased in DG [F(1,28) = 4.49; p ≤ 0.05] (Figure 4.4) contralateral to CCI. However, post hoc comparisons demonstrate no significant effects of drug or injury on bax mRNA levels, in any region or hemisphere.

Significant effects of injury on p53 mRNA levels are detected in contralateral CA1 [F(1,29) = 6.97; p ≤ 0.05] and DG [F(1,29) = 4.57] (Figure 4.5). Post hoc comparisons indicate that CCI decreased contralateral p53 signal in CA1 and DG of SPIRO-pretreated animals. No significant effects of drug or injury on p53 mRNA levels were observed in the ipsilateral hemisphere.
Figure 4.3. Effects of MR blockade, GR blockade, and controlled cortical impact injury on bcl-2 mRNA levels in hippocampus. Semi-quantitative analysis expressed as percentage of control (sham/vehicle; see methods) corrected gray level; n = 5-6 per group. Contralateral bcl-2 mRNA levels were decreased by spironolactone and RU486 in CA1 and dentate gyrus of sham-operated animals. Ipsilateral bcl-2 levels were decreased by spironolactone in dentate gyrus and by RU486 in CA1 and dentate gyrus of sham-operated animals. Injury increased contralateral bcl-2 mRNA levels in dentate gyrus of RU486 animals. CCI decreased ipsilateral bcl-2 signal in dentate gyrus of vehicle animals and increased ipsilateral bcl-2 signal in dentate gyrus of RU486 animals. [\(^{\text{A}}\) p ≤ 0.05 as compared to vehicle animals within the same surgery condition (effect of drug). \(^{\text{B}}\) p ≤ 0.05 as compared to sham-operated animals within same drug pretreatment group (effect of injury).]
Figure 4.4. Effects of MR blockade, GR blockade, and controlled cortical impact injury on bax mRNA levels in hippocampus. Semi-quantitative analysis expressed as percentage of control (sham/vehicle; see methods) corrected gray level; n = 4-6 per group. Post hoc comparisons indicate no significant effects of antagonist pretreatment or injury on bax mRNA levels in any region or hemisphere.
Figure 4.5. Effects of MR blockade, GR blockade, and controlled cortical impact injury on p53 mRNA levels in hippocampus. Semi-quantitative analysis expressed as percentage of control (sham/vehicle; see methods) corrected gray level; n = 5-6 per group. Injury decreased contralateral p53 mRNA levels in CA1 and dentate gyrus of SPIRO-pretreated animals. Post hoc comparisons indicate no significant effects of antagonist pretreatments. [B p ≤ 0.05 as compared to sham-operated animals within same drug pretreatment group (effect of injury).]
Discussion

The present study demonstrates that GR blockade with RU486 prevents CA1 neuron loss 24h after CCI injury. The data do not suggest that regulation of viability-related genes bcl-2, bax, or p53 is involved in the selective preservation of CA1 neurons by RU486. Furthermore, MR blockade with SPIRO did not exacerbate CCI-induced neuron loss in hippocampus.

Hippocampal neuron loss 24 hours after controlled cortical impact injury

The present study uses the optical fractionator method of cell counting, a procedure that yields an unbiased estimation of total cell number, to evaluate survival of hippocampal neurons 24h after CCI injury (Figure 4.1). Counts revealed that CCI significantly decreased the number of ipsilateral CA1 pyramidal neurons in vehicle and SPIRO-pretreated rats by approximately 35%. In contrast, no significant cell loss occurred in RU486-pretreated animals, demonstrating protection of CA1 neurons by RU486. Approximately 40-55% of CA3 neurons were lost at 24h. We have previously reported a similar amount of cell loss in CA3 after the same level of injury using the optical dissector method of cell counting. The same report demonstrated that no significant cell loss occurs in CA3 beyond 24 hours post-injury, indicating that neuronal cell death in this region is rapid following TBI (Baldwin et al., 1997). In the current study, CCI also reduced the number of granule cells in DG by approximately 20-30%. While injury did not significantly decrease the number of DG neurons in the vehicle-pretreated group, post-hoc comparisons indicate that the amount of cell loss in vehicle-pretreated subjects was not statistically different from that observed in SPIRO and RU486-pretreated subjects. The appearance of the data suggests that the actual extent of cell loss in vehicle-pretreated animals may have been obscured by high variability in the number of contralateral cells counted in this region.

RU486 protection is specific to CA1

The selective defense of CA1 neurons suggests either that RU486 preferentially protects this neuronal population or that CCI injury in CA1 is more responsive to treatment. Differential action of RU486 might be achieved through the heterogeneous distribution of GR across subfields (Herman, 1993). The high density of GR expressed in CA1 relative to other
hippocampal regions might explain, in part, the efficacy of RU486. Relatively little GR is expressed in CA3, perhaps lessening the impact of RU486 on this subregion. However, GR levels are substantial in DG, a region not protected by RU486, suggesting that additional factors are involved in determining neuronal susceptibility to injury.

Previous studies predict that CCI may affect neurons differently according to subregion, allowing for selective intervention. Subpopulations of hippocampal neurons are selectively vulnerable to different forms of insult; CA1 neurons are most severely damaged by ischemia (Kirino, 1982; Pulsinelli et al., 1982; Schmidt-Kastner and Freund, 1991), while CA3 neurons are most vulnerable, and DG granule cells least vulnerable, to kainate excitotoxicity (Nadler et al., 1978; Kohler and Schwarcz, 1983). Ischemia (Okiyama et al., 1994) and excitotoxicity (Choi, 1992; Hayes et al., 1992) are among the many components of TBI which can lead to neuronal death. Differential vulnerability in hippocampal neuron populations could therefore govern the severity of CCI injury or the length of time over which cell death occurs, allowing for more successful treatment in specific regions. CCI results in very rapid cell loss in CA3 (Baldwin et al., 1997). Death of CA1 neurons as induced by ischemia is slower in comparison, occurring over a period of hours to days (Kirino, 1982; Pulsinelli et al., 1982). It is possible that CCI injury is either delayed or less severe in CA1 as compared to CA3, such that intervention with RU486 is able to increase or prolong neuronal survival. Clarification of this issue will require further elucidation of the mechanisms governing CCI-induced cell death and a time course characterization of CA1 neuron survival after CCI.

A variety of factors could account for differential vulnerability to TBI by region. For example, CA3 neurons do not express neuronal calcium-binding proteins calbindin-D$_{28k}$ or parvalbumin (Sloviter, 1989), which may reduce CA3 Ca$^{2+}$ buffering capacity as compared to CA1. In addition, ionotropic glutamate receptors are heterogeneously distributed in hippocampus; NMDA and AMPA subunits are preferentially expressed in CA1 and DG, while kainate receptors are mainly found in CA3 (Greenamyre et al., 1985; Tarazi et al., 1996). Glutamate receptor configuration may dictate sensitivity to the large amounts of glutamate released during injury. Subregional differences in afferent input, intracellular signaling, control of ion homeostasis, antioxidant defenses, and neurotrophic factor expression are among other factors that could also differentially influence cell viability.
Glucocorticoid receptor blockade and neuronal viability

GR-activating concentrations of glucocorticoids exacerbate many types of neuronal insult including kainic acid toxicity, oxidative stress, amyloid-beta peptide toxicity, and ischemia (Sapolsky, 1985; Sapolsky and Pulsinelli, 1985; Sapolsky, 1986a; Sapolsky et al., 1988; Goodman et al., 1996; McIntosh and Sapolsky, 1996a). The GR may heighten neuronal sensitivity to insult through several mechanisms. Glucocorticoids can inhibit glucose transport (Horner et al., 1990) and may increase neuronal vulnerability to insult through energy depletion (Sapolsky, 1986b). Elevated glucocorticoid concentrations can also result in excitatory amino acid accumulation (Stein-Behrens et al., 1992; Stein-Behrens et al., 1994b), increased intracellular Ca\(^{2+}\) concentrations (Kerr et al., 1992; Elliott and Sapolsky, 1993; Karst et al., 1994), and free radical formation (McIntosh and Sapolsky, 1996a, b), all of which may compromise cells by triggering Ca\(^{2+}\)-dependent proteolysis, lipid peroxidation, oxidative stress, and mitochondrial dysfunction.

Inactivation of GR by glucocorticoid removal or GR blockade has been shown to improve survival of rodent hippocampal neurons following several types of neurotoxic challenge. Metyrapone, an inhibitor of adrenal glucocorticoid synthesis, reduces loss of CA1 neurons after ischemia (Smith-Swintosky et al., 1996) and loss of CA1 and CA3 neurons after insult with kainic acid, an excitotoxic glutamate analog (Stein and Sapolsky, 1988; Smith-Swintosky et al., 1996). RU486 reverses glucocorticoid-mediated vulnerability to glutamate in a cultured murine hippocampal cell line (Behl et al., 1997b). Further, RU486 (Antonawich et al., 1999) and removal of endogenous glucocorticoids by adrenalectomy (Sapolsky and Pulsinelli, 1985; Morse and Davis, 1990) attenuate loss of CA1 neurons following ischemia. However, studies have indicated that while adrenalectomy and RU486 improve CA1 viability in gerbil at 4 days following ischemia, the protection is lost at 7 days (Morse and Davis, 1990; Antonawich et al., 1999). Thus it is possible that RU486 protection of CA1 neurons after CCI is a transient effect. Additional time points will be required in order to determine if RU486 completely prevents CA1 cell loss or merely delays neuronal loss after injury.

Metyrapone and adrenalectomy studies suggest that RU486 may exert its neuroprotective effects by blocking the deleterious effects of GR. RU486 is a potent inhibitor of GR activation that both impedes and competes with glucocorticoid binding to the GR. Complexes of RU486 bound to the GR can bind glucocorticoid response elements found in target gene promoter
regions but do not facilitate transcription, c.f.(Cadepond et al., 1997). While RU486 generally acts as an anti-glucocorticoid, it may also act as a GR agonist under certain circumstances (Nordeen et al., 1995). It is therefore possible that RU486 influences GR activation in an unpredicted manner after CCI. However, we have consistently observed in the current subjects (McCullers, 2001a) (Chapter Five) and in a previous study (McCullers and Herman, 2001) (Chapter Three) that in vivo pretreatment with RU486 increases GR mRNA levels. This effect concurs with GR mRNA upregulation after adrenalectomy (Herman et al., 1989a; Reul et al., 1989) and provides evidence for the functional blockade of GR in this study. In addition to its anti-glucocorticoid effects, RU486 is also a potent progesterone receptor antagonist. However, modest hippocampal expression of progesterone receptor (Kato et al., 1994) and evidence that progesterone is neuroprotective after TBI (Roof and Hall, 2000) suggest that progesterone receptor blockade is unlikely to underlie RU486 protection of CA1 neurons after CCI.

Potential mechanisms of RU486 neuroprotection

Subfield-specific modulation of Ca\textsuperscript{2+} homeostasis may influence neuronal viability after TBI. Studies of CA1 neurons in hippocampal slices demonstrate enhanced afterhyperpolarization (Joels and de Kloet, 1989; Kerr et al., 1989), Ca\textsuperscript{2+} action potentials (Kerr et al., 1989), and Ca\textsuperscript{2+} currents (Kerr et al., 1992; Karst et al., 1994) with increased GR activation. These reports indicate that GR influences Ca\textsuperscript{2+} influx in CA1 neurons and suggest that excessive GR activation could lead to Ca\textsuperscript{2+} neurotoxicity. In agreement with this hypothesis, prolonged exposure to high glucocorticoid concentrations increases basal intracellular Ca\textsuperscript{2+} levels (Elliott and Sapolsky, 1993) and prolongs intracellular Ca\textsuperscript{2+} elevation after kainic acid treatment (Elliott and Sapolsky, 1992) in hippocampal neuron cultures. Characterizations of Ca\textsuperscript{2+} channel subunit mRNA expression at low and high glucocorticoid levels are also consistent with the hypothesis that predominant MR occupation leads to restricted Ca\textsuperscript{2+} influx, while additional GR occupation enhances Ca\textsuperscript{2+} influx (Nair et al., 1998). In the present study, RU486 blockade of GR should result in predominant MR activation. RU486 may therefore shift expression of Ca\textsuperscript{2+} channel subunits and other Ca\textsuperscript{2+} regulatory genes to a neuroprotective configuration that could prevent excessive intracellular Ca\textsuperscript{2+} accumulation in CA1 neurons.

RU486 could also improve CA1 neuronal viability via non-genomic action. RU486 is reported to have antioxidant effects that may mediate neuroprotection in CA1 pyramidal cells.
Previous studies have shown that RU486 prevents oxidation of the low-density lipoprotein (Parthasarathy et al., 1994) and inhibits oxidative modification of proteins by pre-existing lipid peroxides in cell-free systems (Carpenter et al., 1996). Furthermore, Behl, et al. (1997a) have demonstrated neuroprotective antioxidant effects of RU486 in cellular and organotypic slice cultures. Pretreatment with $10^{-5}$ M RU486 protects rat primary hippocampal cultures from beta amyloid toxicity, mouse clonal hippocampal HT22 cells from beta amyloid, H$_2$O$_2$, and glutamate insults, and organotypic rat hippocampal slices from H$_2$O$_2$-induced cell death (Behl et al., 1997a). Intracellular peroxide accumulation and DNA degradation were also prevented by RU486 in HT22 cells. Experiments in monkey kidney CV$_1$ cells suggest that the protective effects of RU486 are independent of GR or PR antagonism or activation (Behl et al., 1997a). Structural similarity to other antioxidant compounds suggests that the dimethylaminophenyl side chain moiety of RU486 is likely responsible for the antioxidant properties of RU486 (Parthasarathy et al., 1994; Carpenter et al., 1996; Behl et al., 1997a).

**Expression of apoptosis-related genes after corticosteroid receptor blockade and injury**

The present data demonstrate decreased hippocampal bcl-2 mRNA levels in SPIRO and RU486-pretreated rats 24h after sham-operation, as compared to vehicle-pretreated subjects (Figure 4.3). These results extend our previous finding that SPIRO decreases bcl-2 mRNA levels in hippocampus (McCullers and Herman, 1998) (Chapter Two). This evidence for reduction of bcl-2 mRNA levels by MR and GR antagonists suggests that both receptor types, perhaps functioning as heterodimers (Trapp et al., 1994), are required for optimal maintenance of bcl-2 expression. However, the antagonist-induced decreases in bcl-2 mRNA levels observed in sham animals do not appear to predict increased hippocampal neuron vulnerability to injury. Injury-induced cell loss is not increased in antagonist-injected subjects in any region (as compared to vehicle-injected controls); on the contrary, RU486 treated subjects clearly show protection despite decreased bcl-2 mRNA levels observed in sham animals. These data fail to support the hypothesis that decreased bcl-2 expression subsequent to ACR blockade will increase neuronal vulnerability to CCI. It should be noted, however, that the effects of ACR blockade on bcl-2 mRNA levels are very different in sham versus injured animals. Among injured animals, ACR blockade did not reduce bcl-2 mRNA levels (as compared to vehicle-injected controls) 24 hours post-injury in any hippocampal region. Thus, CCI injury abrogates the differential
decrease of bcl-2 mRNA levels in antagonist-pretreated groups, perhaps accounting for the lack of correlation between sham bcl-2 levels and injury induced cell loss. Nonetheless, it is possible that a shorter period of antagonist pretreatment may be sufficient for RU486 protection of CA1 neurons while avoiding the potential undesired effect of bcl-2 mRNA downregulation.

The present study demonstrates decreased ipsilateral bcl-2 mRNA levels in DG of vehicle-pretreated subjects following CCI injury. Decreased bcl-2 expression has also been demonstrated in fluid percussion (Dong et al., 2001) and in vitro (Morrison et al., 2000) models of TBI. In contrast, Clark et al. have reported increases in hippocampal bcl-2 mRNA levels 24h following CCI with the addition of 30 minutes moderate hypoxemia following injury (Clark et al., 1997). Increased bcl-2 mRNA levels appear to be associated with neuroprotection after CCI with hypoxia (Clark et al., 1997), and Bcl-2 overexpression in transgenic mice decreases hippocampal tissue loss after CCI (Nakamura et al., 1999). In addition, decreased bcl-2 mRNA levels have been associated with increased kainate-induced hippocampal cell loss (Gillardon et al., 1995; McCullers and Herman, 2001) (Chapter Three). However, decreased bcl-2 mRNA levels observed in DG of injured animals in the current study were not associated with significant neuronal loss following CCI injury. As previously noted, a high degree of variability in contralateral DG cell counts may have obscured significant effects of CCI on DG neuron loss. Even so, the process of secondary neuronal degeneration is very complex and involves numerous factors, and decreased bcl-2 expression, though perhaps an endangering factor, need not dictate neuronal loss following injury.

Hippocampal bax mRNA levels were unchanged by MR/GR antagonist pretreatments or CCI (Figure 4.4). These findings agree with results obtained using an in vitro model of TBI (Morrison et al., 2000). The ratio of anti-apoptotic proteins such as Bcl-2 to pro-apoptotic proteins such as Bax is believed to determine cell sensitivity to apoptotic signals (Oltvai et al., 1993). The current data suggest that transcriptional regulation of this ratio would be accomplished by modulation of bcl-2, and not bax, following traumatic brain injury.

Levels of p53 mRNA in the present study were not significantly elevated in vehicle-pretreated animals at the 24 hour time point; however, our data do not preclude changes earlier in time. For instance, another group has demonstrated increases in p53 mRNA levels 6 hours following fluid percussion brain injury which return to sham levels by 24 hours (Napieralski et al., 1999). The current report also demonstrates that injury reduced contralateral p53 signal in
CA1 and DG of SPIRO animals (Figure 4.5). We have previously shown that SPIRO attenuates kainic acid-induced increases in p53 mRNA levels (McCullers and Herman, 1998) (Chapter Two), but the effects of p53 downregulation on neuronal survival are presently unclear.

Hippocampal cell death is associated with functional (Lowenstein et al., 1992) and neurobehavioral (Hicks et al., 1993; Smith et al., 1994) deficits following TBI. By preventing CA1 neuron loss after CCI, RU486 could potentially improve behavioral outcome after injury. RU486 may be particularly effective in promoting neuron survival due to its combined anti-glucocorticoid effects and antioxidant properties. While glucocorticoids have been used clinically to control edema after TBI, the value of this treatment is unclear. Some evidence indicates that glucocorticoids can improve recovery after central nervous system injury (Hall et al., 1992), but many studies show no improvement with glucocorticoid administration (Tornheim and McLaurin, 1978; Cooper et al., 1979; Giannotta et al., 1984; Brain, 1996). In contrast, many studies have demonstrated exacerbation of neuronal insult by glucocorticoids (Sapolsky, 1985; Sapolsky and Pulsinelli, 1985; Sapolsky, 1986a; Sapolsky et al., 1988; Goodman et al., 1996; McIntosh and Sapolsky, 1996a) and improved neuronal viability with antiglucocorticoid treatments (Sapolsky and Pulsinelli, 1985; Stein and Sapolsky, 1988; Morse and Davis, 1990; Smith-Swintosky et al., 1996; Behl et al., 1997b; Antonawich et al., 1999). Considering the benefits of glucocorticoid removal and blockade in other models of neuronal injury, the antioxidant properties of RU486, and the current data demonstrating CA1 neuron protection, RU486 appears to have potential as a useful treatment for head injury.
Chapter Five: Traumatic brain injury regulates adrenocorticosteroid receptor mRNA levels in rat hippocampus

Introduction

The mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR), collectively referred to as adrenocorticosteroid receptors (ACRs), are ligand-activated transcription factors that mediate glucocorticoid action in brain. The MR binds glucocorticoids with high affinity (kD ~ 0.5-1 nM) and is extensively occupied at basal glucocorticoid levels (Reul and de Kloet, 1985). The GR binds glucocorticoids with a five-fold lower affinity, resulting in greater GR activation with increasing glucocorticoid levels (i.e. during stress and at the peak of circadian hormone secretion)(Reul and de Kloet, 1985; Spencer et al., 1990). This two-receptor system allows for differential regulation of MR and GR-coordinated gene expression in response to changes in circulating glucocorticoid concentration. The binding of glucocorticoid ligand allows receptor translocation to the cell nucleus, where the MR and GR can regulate transcription either by direct DNA binding to hormone response elements in gene promoter regions or by protein-protein interactions with other transcription factors [see (Yamamoto, 1985; De Kloet et al., 1998)].

Considerable evidence suggests that excessive GR activation by CORT increases hippocampal vulnerability to several forms of neuronal insult including excitotoxicity, oxidative stress, and ischemia (Sapolsky, 1985; Sapolsky and Pulsinelli, 1985; Sapolsky, 1986a; Sapolsky et al., 1988; Goodman et al., 1996; McIntosh and Sapolsky, 1996a). Furthermore, blockade of GR reduces hippocampal neuron vulnerability to traumatic brain injury (TBI) (McCullers et al., 2001b) (Chapter Four), indicating the potential deleterious effects of GR activation with this type of injury. The degree of GR activation experienced by cells is largely determined by circulating glucocorticoid concentration and the amount of available GR; therefore, regulation of GR expression and the magnitude of HPA response are crucial determinants of glucocorticoid influence on neuronal viability after injury.

To examine the respective roles of MR and GR in regulating ACR expression and glucocorticoid secretion following TBI, adult male Sprague Dawley rats were pretreated with vehicle, the MR antagonist spironolactone (SPIRO), or the GR antagonist mifepristone (RU486). Subjects were subsequently sham-operated or injured using the controlled cortical impact (CCI)
model of TBI. Twenty-four hours post-injury, ACR mRNA levels were measured to determine if glucocorticoid signaling is altered through regulation of ACR expression following injury. As the acute hypothalamic-pituitary-adrenal (HPA) response to CCI has not been characterized, additional studies were performed to assess CORT and ACTH secretion over the 24-hour period following injury.

**Materials and Methods**

**Subjects**

Young adult male Sprague Dawley rats (Harlan Sprague Dawley, Inc., Indianapolis, IN) weighing 200-250g were maintained in an environment with constant temperature, humidity, ad libitum access to food and water, and a 12-hour light/dark cycle, with lights on between 600h-1800h. All measures were taken to minimize animal pain or discomfort. All animal procedures were performed in accordance with the National Institutes of Health guidelines and approved by the University of Kentucky Institutional Animal Care and Use Committee.

**In vivo protocols**

Subjects (n = 5-7 per design group) received subcutaneous injections of propylene glycol vehicle, MR antagonist spironolactone (SPIRO) (50 mg/kg) (Herman and Spencer, 1998) (Sigma, St. Louis, MO), or GR antagonist RU486 (25 mg/kg) (Ratka et al., 1989) (Sigma) in morning (900h) and afternoon (1600h) for two days. On day three, animals were administered a final pretreatment injection at 900h and transported to a surgical procedure room. Between 1100h-1200h, animals were subjected to craniotomy (sham operation) or a moderate unilateral cortical contusion, described below. Subjects were killed on day 4 at 1200h, 24h after surgery.

A separate set of animals (n = 6 per group) was used for analysis of plasma ACTH and CORT levels during the 24 hours following surgery. Baseline blood samples were drawn by tail nick between 900-1000 hours, after which animals were transported to a surgical procedure room. Subjects then underwent sham operation or moderate unilateral cortical contusion between 1200-1300 hours. Sham and CCI surgeries were performed under identical conditions on separate days. Blood samples were collected at 1.5, 6, 12, 18, and 24 hours after the start of surgery. All samples were collected by tail nick, with the exception of the 24-hour point, which
was obtained by collection of trunk blood at the time of sacrifice. Blood sampling was initiated 1.5 hours after the start of surgery to allow all subjects to wake from anesthesia, and six hour intervals were observed thereafter to assess circadian hormone levels at times including lights off (1800 h) and lights on (600h).

Surgical procedures were performed as previously described (Baldwin et al., 1997). Briefly, animals were anesthetized with isoflurane (2%) and placed in a stereotaxic frame (Kopf Instr., Tujunga, CA). Sterile procedures were used to retract the skin and perform a 6mm unilateral craniotomy midway between bregma and lambda. The underlying dura was not disrupted during removal of the portion of skull. A pneumatically controlled impacting device with a 5mm beveled tip was used to injure the exposed brain, compressing the cortex at 3.5m/sec to a depth of 2mm. Following injury, Surgicel (Johnson and Johnson, Arlington, TX) was laid upon the dura, and the skull cap was replaced. After a thin coat of dental acrylic was spread over the craniotomy site and allowed to dry, the wound was stapled closed. Body temperature was monitored continuously throughout the surgical/recovery procedures and maintained at 37°C with a heating pad.

Animals processed for these experiments were killed by decapitation. Brains were rapidly removed, frozen in isopentane cooled to –40°C on dry ice, then stored at –80°C until sectioned. Trunk blood samples were collected on ice, and plasma was stored at –20°C. Frozen brains were removed from a –80°C freezer, warmed to –20°C, and sectioned to 16 um with a Bright-Hacker cryostat. Sections were then thaw-mounted onto Ultrastick Slides (Becton Dickinson, Franklin Lakes, NJ) and stored at –20°C until processing for in situ hybridization.

**In situ hybridization**

Antisense riboprobes complementary to coding and 3’ untranslated regions for rat MR mRNA (550 bp, courtesy of P. Patel and S. Watson, Univ. Michigan) and rat GR mRNA (456 bp, courtesy of K. Yamamoto, UCSF) were labeled according to previously published methods (Seroogy and Herman, 1996) by standard in vitro transcription using [35S]UTP. Slide-mounted tissue sections were removed from a -20°C freezer and fixed in 4% paraformaldehyde for 10 min. Slides were then rinsed in 5 mM KPBS, 5 mM KPBS with 0.2% glycine, 5mM KPBS, 0.1 M triethanolamine with 0.25% acetic anhydride, and 0.2X SSC, then dehydrated in graded alcohols. Labeled probes were diluted in hybridization buffer containing 50% formamide, and
50 ul of diluted probe (containing 1x 10^6 c.p.m.) was applied to each slide. Slides were then coverslipped, placed in moistened chambers, and incubated overnight at 55^oC. Following hybridization, slides were rinsed in 2X SSC and incubated in 0.2X SSC at 60^oC for 1 hr. After dehydration in graded alcohols, slides were exposed for 14-18 days to Kodak BioMAX xray film.

Densitometric analysis was performed using NIH Image 1.62 software for Macintosh. A standard curve was generated using ^14^C standards prior to image capture to ensure signal linearity. Based on morphological criteria, regions CA1, CA3, and DG of the hippocampal formation were manually sampled from the ipsilateral (operated) and contralateral (unoperated) hemispheres. The mean gray level of an unhybridized area (background) from each hemisphere was subtracted from sampled regions to compute corrected gray level. Ipsilateral mRNA data were normalized as a percent of control (sham/vehicle) values from the ipsilateral hemisphere, and contralateral mRNA data were normalized as a percent of control (sham/vehicle) values from the contralateral hemisphere, with sham/vehicle levels expressed as one hundred percent. Each region of each hemisphere was then analyzed by two-way analysis of variance with drug and injury designated as independent variables. Post hoc comparisons were made using the Student-Newman-Keuls test, with significance set at p ≤ 0.05.

**Radioimmunoassay**

Plasma ACTH and corticosterone concentrations were determined using (^125^I-labeled) rat ACTH (Diasorin, Stillwater, MN, USA) and corticosterone (ICN Biochemicals, Costa Mesa, CA) kits per manufacturers’ instructions. The limits of detection for each kit are 15 pg/ml and 8 ng/ml, respectively.

A skewed distribution of CORT levels observed in subjects pretreated with antagonist drugs was normalized by log transformation of the data prior to analysis for significant main effects by two-way analysis of variance. Post hoc comparisons were made using the Student-Newman-Keuls test, with significance set at p ≤ 0.05. Data presented in Table 5.1 are actual CORT values listed as mean concentration ± standard error. Time course values for ACTH and CORT were analyzed for significant main effects using a repeated measures analysis of variance. Post hoc comparisons were made using the Student-Newman-Keuls test, with significance set at p ≤ 0.05. Data graphed in Figures 5.1 and 5.2 represent mean hormone concentration ± standard error.
Results

Effects of ACR blockade and CCI on plasma ACTH and corticosterone levels

ACTH and CORT levels were measured 24 hours following surgery to assess the effects of ACR blockade and CCI on HPA activation. Two-way ANOVA demonstrates a significant drug/injury interaction effect on plasma CORT levels at 24h \( F(2,29) = 8.02; p \leq 0.05 \) (Table 5.1). Post hoc comparisons indicate that SPIRO significantly increased CORT secretion in sham-operated animals. Controlled cortical impact significantly decreased CORT levels in SPIRO and RU486-pretreated animals as compared to sham-operated animals within the same drug pretreatment groups. RU486 animals also had significantly lower CORT levels after injury as compared to vehicle/injured animals.

Table 5.1. Effects of adrenocorticosteroid receptor blockade and controlled cortical impact on plasma corticosterone levels (ng/ml)

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>n</th>
<th>Mean CORT Level</th>
</tr>
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<tbody>
<tr>
<td>VEHICLE</td>
<td>SHAM</td>
<td>7</td>
<td>80.5 ± 29.9</td>
</tr>
<tr>
<td>SPIRO</td>
<td>SHAM</td>
<td>6</td>
<td>324.5 ± 77.2 (^a)</td>
</tr>
<tr>
<td>RU486</td>
<td>SHAM</td>
<td>6</td>
<td>121.0 ± 16.2</td>
</tr>
<tr>
<td>VEHICLE</td>
<td>INJURED</td>
<td>5</td>
<td>81.7 ± 33.6</td>
</tr>
<tr>
<td>SPIRO</td>
<td>INJURED</td>
<td>6</td>
<td>51.9 ± 26.0 (^b)</td>
</tr>
<tr>
<td>RU486</td>
<td>INJURED</td>
<td>5</td>
<td>14.7 ± 6.8 (^{a,b})</td>
</tr>
</tbody>
</table>

SPIRO = MR antagonist spironolactone  
RU486 = GR antagonist (mifepristone)

\(^a\) p < 0.05 compared to vehicle-pretreated subjects in same surgery group (effect of antagonist)

\(^b\) p < 0.05 compared to sham-operated subjects in same pretreatment group (effect of injury)
Plasma ACTH and corticosterone levels: time course after injury

Blood samples taken 24 hours after surgery suggest the dynamic regulation of CORT secretion over the post-injury period. To examine the kinetics of HPA activation following CCI, additional animals were subjected to sham operation or injury, after which sequential blood samples were drawn by tail nick over the 24-hour post-surgery period. Plasma ACTH levels exhibited a significant injury/time interaction as determined by repeated measures analysis of variance [F(5,41) = 7.89; p ≤ 0.05] (Figure 5.1). Plasma ACTH concentrations were increased to approximately 200 pg/ml at 1.5 hours and at 18 hours post-surgery in injured animals. ACTH concentrations in sham-operated animals were indistinguishable from baseline at all time points.

Plasma CORT concentrations also demonstrate a significant injury/time interaction as indicated by repeated measures analysis of variance [F(5,50) = 5.29; p ≤ 0.05] (Figure 5.2). Corticosterone levels are significantly increased in injured rats at 1.5 hours (109 ± 12 ng/ml) and at 6 hours (53 ± 4 ng/ml) following surgery, but returned to baseline by 12 hours and remained unchanged for the rest of the 24-hour period. Corticosterone concentrations in sham-operated animals were indistinguishable from baseline at all time points.
Figure 5.1. Twenty-four hour time course analysis of plasma ACTH levels (pg/ml) following controlled cortical impact. Injury increased ACTH levels at 1.5 and 18 hours after surgery (p ≤ 0.05). ACTH levels in sham-operated animals were indistinguishable from baseline at all time points.

Figure 5.2. Twenty-four hour time course analysis of plasma corticosterone levels (ng/ml) following controlled cortical impact. Injury increased corticosterone secretion at 1.5 and 6 hours after surgery (p ≤ 0.05); levels returned to baseline by 12 hours after injury. Corticosterone levels in sham-operated animals were indistinguishable from baseline at all time points.
Effects of ACR blockade and CCI on ACR mRNA levels

Relative changes in mRNA levels were measured by film densitometry following in situ hybridization with specific complementary antisense riboprobes (see Methods) (Figure 5.3).

Figure 5.3. Photomicrographs illustrating the effects of sham operation (A,C) and controlled cortical impact (B,D) on MR (A,B) and GR (C,D) mRNA levels in hippocampus of vehicle-pretreated rats. Panels A,B: Controlled cortical impact has no significant effects on MR mRNA levels in vehicle-pretreated animals. Panels C,D: Controlled cortical impact decreases ipsilateral GR mRNA levels in dentate gyrus and increases contralateral GR mRNA levels in CA1 and DG of vehicle-pretreated subjects.
In the hemisphere contralateral to injury, two-way ANOVA demonstrates significant main effects of injury on MR mRNA levels in CA1 \([F(1,28) = 13.10; p \leq 0.05]\), CA3 \([F(1,28) = 32.39; p \leq 0.05]\), and DG \([F(1,28) = 4.31; p \leq 0.05]\) (Figure 5.4). Post hoc comparisons indicate that injury induces significant contralateral increases in MR mRNA levels in CA1 of SPIRO-pretreated animals and in CA3 of SPIRO and RU486 animals; comparisons in DG did not reach statistical significance. No significant effects of antagonist pretreatment were observed in contralateral MR mRNA levels.

In the injured hemisphere, two-way ANOVA demonstrates significant main effects of injury on MR mRNA levels in CA1 \([F(1,28) = 6.55; p \leq 0.05]\) and CA3 \([F(1,28) = 42.18; p \leq 0.05]\) (Figure 5.4). Post hoc comparisons indicate that cortical impact significantly increased ipsilateral MR mRNA levels in CA1 of RU486-pretreated animals and in CA3 of SPIRO and RU486 animals. A significant main effect of drug on MR mRNA levels is observed in ipsilateral CA1 \([F(2,28) = 3.68; p \leq 0.05]\), but post hoc comparisons did not distinguish between individual pretreatment groups.

Contralateral GR mRNA levels exhibited significant drug/injury interactions in CA1 \([F(2,28) = 7.75; p \leq 0.05]\) and DG \([F(2,28) = 5.70; p \leq 0.05]\) (Figure 5.5). In sham-operated animals, SPIRO and RU486 increased contralateral GR signal in CA1 and DG. Cortical injury increased contralateral GR mRNA levels in CA1 and DG of vehicle-pretreated animals and decreased contralateral GR mRNA levels in CA1 of RU486 animals.

Main effects of drug/injury interaction were observed in ipsilateral GR mRNA levels in subregions CA1 \([F(2,28) = 6.53; p \leq 0.05]\), CA3 \([F(2,28) = 3.71; p \leq 0.05]\), and DG \([F(2,28) = 4.29; p \leq 0.05]\) (Figure 5.5). RU486 significantly increased ipsilateral GR signal in CA1, CA3, and DG of sham-operated animals, and SPIRO increased ipsilateral GR signal in CA1. Cortical impact significantly decreased ipsilateral GR mRNA levels in CA1 and CA3 of SPIRO and RU486-pretreated animals and in DG of all antagonist pretreatment groups.
Figure 5.4. Effects of MR blockade (SPIRO), GR blockade (RU486), and controlled cortical impact (CCI) injury on MR mRNA levels in hippocampus. Semi-quantitative analysis expressed as percentage of control (sham/vehicle) corrected gray level. Injury and antagonist pretreatments interact to increase contralateral and ipsilateral MR mRNA levels in CA1 and CA3. $^{[B]} \ p \leq 0.05$ as compared to sham-operated animals within same pretreatment group (effect of CCI).
Figure 5.5. Effects of MR blockade, GR blockade, and controlled cortical impact (CCI) injury on GR mRNA levels in hippocampus. Semi-quantitative analysis expressed as percentage of control (sham/vehicle) corrected gray level. Antagonist pretreatment increases GR mRNA levels in sham-operated animals, but antagonist-induced increases in GR mRNA levels are prevented by injury. Contralateral GR mRNA levels are increased in CA1 and DG of vehicle-pretreated animals, and ipsilateral GR mRNA levels are decreased in DG of all pretreatment groups after injury. $[^A] p \leq 0.05$ as compared to vehicle-pretreated animals within same surgical treatment (effect of antagonist pretreatment). $[^B] p \leq 0.05$ as compared to sham-operated animals within same pretreatment group (effect of CCI).
Discussion

CCI modulates HPA activation

The extent of HPA activation after TBI may influence neuronal survival, given evidence for glucocorticoid exacerbation of several forms of neuronal injury (Sapolsky, 1985; Sapolsky and Pulsinelli, 1985; Sapolsky, 1986a; Sapolsky et al., 1988; Goodman et al., 1996; McIntosh and Sapolsky, 1996a). In the first experiment of this study, vehicle-pretreated animals demonstrated equivalent plasma CORT levels 24 hours after sham operation and injury. The levels were slightly higher than would be expected in unoperated rats but were not of a magnitude to saturate GR binding at the 24-hour time point. Another study assessing ACTH and CORT levels in rats following closed head injury reported similar results at 24 hours (Shohami et al., 1995).

Clinical studies have reported increased HPA activation in humans after head injury (King et al., 1970; Pentelenyi and Kammerer, 1984). To determine if differential activation of the HPA axis occurs during the first 24 hours after experimental CCI, a time course analysis of plasma CORT and ACTH levels was performed in sham-operated versus injured rats. Blood sampling over the 24-hour period following surgeries confirmed the differential activation of the HPA axis in animals injured by CCI. Increased levels of ACTH and CORT were observed 1.5 hours following injury, after which a prolonged, low level of CORT secretion was observed for at least 6 hours after injury.

Plasma ACTH and CORT levels six hours after surgery (time of day = 1800h) indicate a disruption of normal circadian rhythms. Diurnal peaks in rodent ACTH and CORT secretion normally occur near the onset of darkness (Ruhmann-Wennhold and Nelson, 1977; D'Agostino et al., 1982; Kwak et al., 1992). ACTH levels in CCI-injured animals were indistinguishable from baseline at 1800h, the time of lights-off, and plasma CORT concentrations, although slightly elevated at 1800h, did not approach typical peak levels of 150-200 ng/ml (D'Agostino et al., 1982; Kwak et al., 1992). Sham-operated animals also lacked ACTH and CORT rhythms, exhibiting unchanged plasma hormone levels across the 24-hour period after surgery. Alteration of circadian rhythms has been reported after surgery in rats (Levine et al., 1980) and in humans (King et al., 1970; McIntosh et al., 1981; Pentelenyi and Kammerer, 1984), but the exact cause for disruption in these subjects, whether by anesthesia or the trauma of craniotomy, remains to be determined.
Injured animals demonstrated an unusual peak in ACTH levels 18h after the onset of surgery (time of day = 600h). While the significance of this increase is unclear, we speculate that increased ACTH levels could result from decreased sensitivity to glucocorticoid-mediated feedback. Decreased sensitivity to feedback normally occurs at the peak of the circadian rhythm and after stress (Dallman, 1992) and could be precipitated by the stress of injury or alteration of circadian rhythms.

ACR antagonists modulate plasma corticosterone levels

Blockade of MR-mediated feedback inhibition of the HPA axis may account for increased plasma CORT levels observed in SPIRO/sham subjects (Table 5.1). Our laboratory has previously demonstrated increased basal CORT secretion with SPIRO administration (McCullers and Herman, 2001) (Chapter Three), although some studies report no effect of SPIRO on morning CORT levels (Chabert et al., 1984; Herman and Spencer, 1998; Yau et al., 1999). Activation of MR is required to maintain CORT secretion at the low basal levels normally observed in rats in the morning (Spencer et al., 1998). Furthermore, studies using the MR antagonist RU28318 have consistently demonstrated that MR blockade increases adrenocortical secretion (Ratka et al., 1989; van Haarst et al., 1997; Spencer et al., 1998). The mechanism whereby CCI interacts with ACR antagonists to reduce CORT secretion is presently unknown. The inhibitory influence of the hippocampus over HPA activation (Herman et al., 1989b; Jacobson and Sapolsky, 1991) invites speculation that this structure may be involved. It is possible that aberrant hippocampal activation subsequent to injury in combination with altered ACR signaling might culminate in decreased HPA secretion, but support for this hypothesis will require further study.

ACR antagonists regulate GR mRNA levels

SPIRO pretreatment did not alter MR mRNA levels in sham-operated animals (Figure 5.4). Previously we have observed that SPIRO pretreatment (followed by i.p. saline injection) increases MR mRNA levels by approximately 30-40% in CA1, CA3, and DG (McCullers and Herman, 2001) (Chapter Three), and many preceding studies suggest that MR is subject to auto-regulation (Herman et al., 1989a; Reul et al., 1989; Herman et al., 1993; Chao et al., 1998a). The lack of MR mRNA upregulation observed in the SPIRO/sham-operated group may be
attributable to significantly increased CORT secretion in these subjects (Table 5.1). MR mRNA levels are negatively regulated by GR activation at high glucocorticoid levels (Herman et al., 1989a; Herman et al., 1993). Thus, enhanced GR activation due to elevated CORT levels in SPIRO/sham animals may attenuate the transcriptional effects of MR blockade.

Both MR antagonist SPIRO and GR antagonist RU486 increased GR mRNA levels in sham-operated animals (Figure 5.5). We have observed similar effects with administration of these antagonists in other experiments (Herman and Spencer, 1998; McCullers and Herman, 2001) (Chapter Three). These data, together with reports that GR agonist dexamethasone (Herman et al., 1989a; Reul et al., 1989), MR agonist aldosterone, or low, MR binding doses of CORT (Chao et al., 1998a) each reverse ADX-induced increases in GR mRNA provide compelling evidence for regulation of hippocampal GR mRNA expression by both MR and GR.

CCI increases MR mRNA and decreases GR mRNA levels

Injury increased MR mRNA levels in CA1 and CA3 of MR and GR antagonist-pretreated animals (Figure 5.4). Increased MR mRNA levels in SPIRO-pretreated subjects may be attributable to MR auto-regulation (Herman et al., 1989a; Reul et al., 1989; Herman et al., 1993; Chao et al., 1998a); however, previous experiments in our laboratory have not indicated MR mRNA upregulation with RU486 pretreatment (McCullers and Herman, 2001) (Chapter Three). These results suggest that TBI influences ACR-mediated regulation of MR expression. It is interesting to note that CORT levels are significantly lower in these groups as compared to sham-operated animals (Table 5.1). These data raise the possibility that injury-induced CORT secretion could influence MR mRNA levels through auto-regulatory processes.

Injury did not alter ipsilateral GR mRNA expression in CA1 and CA3 of vehicle-pretreated animals (Figure 5.5). However, injury abolished autoregulatory effects of SPIRO and RU486, sharply decreasing ipsilateral GR mRNA levels (as compared to sham) by approximately 50-80% in antagonist-pretreated subjects. These results indicate that CCI suppresses or interferes with GR autoregulatory processes in hippocampus. Our laboratory has reported similar effects of excitotoxic insult with kainic acid (McCullers and Herman, 2001) (Chapter Three).

While it is clear that GR auto-regulation (Herman et al., 1989a; Reul et al., 1989) is disrupted after injury, the extent to which GR mRNA levels may be further decreased due to cell loss is presently ambiguous. Nonetheless, decreases in GR signal in the ipsilateral DG of all
pretreatment groups surpass the magnitude of cell loss experienced in this region (McCullers et al., 2001b) (Chapter Four). We have observed similar GR mRNA decreases of approximately 40-55% in DG of vehicle, SPIRO, and RU486 pretreated animals 24h after excitotoxic insult with kainic acid, at a dose which does not cause cell loss in DG (McCullers and Herman, 2001) (Chapter Three). Thus, multiple types of neuronal injury result in the significant down-regulation of GR mRNA levels in DG.

The extent to which cell loss affects measurement of mRNA levels by in situ hybridization is an important consideration that is presently undefined. In parallel subjects, CCI resulted in the loss of approximately 40% of CA1 and CA3 pyramidal neurons and 10% of DG granule neurons 24 hours after injury, while no cell loss was observed in the contralateral hemisphere of injured rats or in sham-operated subjects (McCullers et al., 2001b) (Chapter Four). If comparable loss is assumed in this study, the lack of demonstrable GR mRNA decrement in injured, vehicle-treated groups indicates that the in situ hybridization technique underestimates cell loss. The lack of correlation between cell loss and mRNA level is also seen for MR, where levels are significantly higher on the injured side. It is possible that GR mRNA is upregulated in CA1 and CA3 to compensate for signal that would otherwise be decreased by cell loss; however, the increases in MR mRNA argue against this explanation, as greater induction of MR mRNA would have to be assumed. In conjunction with the differential effects of antagonist pretreatment, it is likely that the observed levels of GR and MR are due to injury-related gene regulation rather than simple cell loss. This notion is supported by the large and disproportionate loss of GR mRNA in DG, the region showing least cell death following CCI.

Traumatic brain injury initiates a multitude of cellular and molecular cascades that influence gene expression. Although it is difficult to isolate specific factors of the many that are likely to influence neuronal GR expression, results from the current study and others (Lowy, 1990, 1992; McCullers and Herman, 2001) (Chapter Three) suggest a role for glutamate in the regulation of hippocampal GRs. Extracellular glutamate levels increase in hippocampus after CCI injury (Faden et al., 1989) and kainic acid injection (Liu et al., 1997), and similar patterns of GR mRNA downregulation are observed in hippocampus after both types of injury (McCullers and Herman, 2001) (Chapter Three). Thus, excessive glutamate receptor activation may lead to interference with antagonist-mediated autoregulatory effects and to the preferential downregulation of GR mRNA in DG. Glutamate receptor activation and alterations in calcium
signaling that accompany CCI (McIntosh et al., 1998c) could potentially regulate GR mRNA levels via activation of transcription factors (Cabral et al., 2001), expression of neurotrophic factors (Sarrieau et al., 1996), or altered neurotransmitter release (Seckl et al., 1990). The results of this study also indicate that glucocorticoid secretion is increased immediately following CCI, potentially exposing hippocampal neurons to endangering levels of hormone. Considering the negative effects of excessive GR activation on injured hippocampal neurons, injury modulation of HPA activation and ACR expression may influence hippocampal neuron viability after traumatic injury.
Chapter Six: Discussion

Adrenocorticosteriod receptor regulation of hippocampal neuron viability

The present thesis examines the respective roles of MR and GR on hippocampal neuron vulnerability to injury. To isolate individual receptor effects on neuronal survival, MR and GR antagonists were administered in subjects prior to excitotoxic challenge with KA or traumatic injury by CCI. Cell counts performed twenty-four hours following insult tested the hypotheses that MR blockade would increase and GR blockade would decrease hippocampal neuron vulnerability to each form of injury. In addition, expression of bcl-2 and other viability-related genes was examined following injury to determine if ACR antagonist effects on viability correlate with regulation of the selected genes. Finally, MR and GR mRNAs were measured after injury to test the hypothesis that glucocorticoid signaling is altered following injury via regulation of hippocampal ACR expression.

Adrenocorticosteriod receptor regulation of hippocampal neuron survival

The results of these experiments support the hypotheses that MR protects and GR endangers challenged hippocampal neurons. In support of the latter, GR antagonist RU486 prevented the loss of CA1 neurons twenty-four hours following CCI injury (Chapter Four). The selective protection of CA1 neurons may be due to regional differences in CCI injury or density of GR expression (see Chapter Four). The potential for RU486 neuroprotection against KA excitotoxicity could not be evaluated due to the absence of measurable KA-induced injury in vehicle and RU486-pretreated subjects (Chapter Three). The current work predicts that RU486 would protect hippocampal neurons subjected to a more severe KA lesion.

Pretreatment with SPIRO, an MR antagonist, increased vulnerability to KA excitotoxicity in hippocampal field CA3 (Chapter Three). While these results may suggest selective MR protection of this region, the comparable distribution of MR in CA1 and CA3 suggests that differential cell loss is more likely attributable to the selective vulnerability of CA3 neurons to KA insult (Nadler et al., 1978). Whether MR blockade increases susceptibility to KA by increasing injury severity or by lowering the neuronal threshold for cell death remains unclear. MR blockade might exacerbate neuronal injury by disrupting cellular Ca2+ homeostasis.
Adrenalectomy, which prevents endogenous CORT production and subsequent MR activation, increases Ca\(^{2+}\) currents in CA1 neurons, while selective activation of MR results in very small Ca\(^{2+}\) currents (Karst et al., 1994). Predominant MR activation may therefore reduce harmful Ca\(^{2+}\) influx (Karst et al., 1994), whereas MR blockade may contribute to pathologic rises in intracellular Ca\(^{2+}\). Alternatively, MR blockade may lower the neuronal “set point” for cell death by modulating expression of pro- or anti-apoptotic factors, such as members of the bcl-2 family. Following TBI, SPIRO did not exacerbate neuronal loss in CA1 and CA3. However, these regions suffered substantial (35-40%) injury-induced neuronal loss in vehicle and SPIRO-pretreated subjects. Because CCI alone was sufficient to destroy vulnerable cells, compromising effects of MR blockade may not have been evident at this level of injury.

**Adrenocorticosteriod receptor regulation of viability-related gene expression**

The current data indicate potential for ACR influence on neuronal survival via regulation of the anti-apoptotic gene bcl-2. MR blockade with SPIRO decreased basal mRNA levels of the survival-promoting gene bcl-2 in CA1 (Chapter Two), suggesting that SPIRO would increase hippocampal vulnerability to insult in this region. However, subjects receiving KA injection following SPIRO pretreatment exhibited no differential cell loss in CA1 (Chapter Three). In addition, RU486 decreased bcl-2 levels in CA1 and DG of animals subjected to sham-CCI, yet RU486 had a dramatic neuroprotective effect on CA1 neurons after CCI injury (Chapter Four). Thus, ACR-induced decreases in bcl-2 mRNA expression observed in uninjured (saline-injected or sham-operated) animals did not consistently predict increased vulnerability following injury.

Experiments in the present thesis do not provide support for bax, p53, BDNF, or NT-3 involvement in ACR modulation of hippocampal survival. Adrenocorticosteriod regulation of hippocampal bax, BDNF, or NT-3 mRNA levels was not observed in any of the current experiments (note that BDNF and NT-3 mRNA levels were not assessed in the CCI model). In addition, ACR blockade had no significant effects on hippocampal p53 mRNA levels in CCI-injured hemispheres (Chapter Four). Kainic acid-induced increases in p53 mRNA levels were attenuated by MR blockade in CA3, suggesting the potential for enhanced resistance to apoptosis in this region, yet MR blockade increased CA3 vulnerability to KA excitotoxicity (Chapter Three). Therefore, ACR-mediated effects on p53 expression do not appear to have significant influence on hippocampal neuron survival.
**Adrenocorticosteriod receptor regulation of hippocampal neuron survival - potential mechanisms**

**Adrenocorticosteriod receptor modulation of calcium homeostasis**

Altered Ca\(^{2+}\) homeostasis is proposed to be a key cellular mechanism underlying glucocorticoid neurotoxicity. Voltage-dependent Ca\(^{2+}\) currents are strongly regulated by corticosteroids with U-shaped dose dependency; in CA1 of hippocampus, Ca\(^{2+}\) current amplitude is large in the absence of corticosteroids, small under conditions of predominant MR activation, and large with the additional occupation of GR at higher glucocorticoid levels (Kerr et al., 1992; Karst et al., 1994; Werkman et al., 1997). Evidence suggests that neuronal Ca\(^{2+}\) influx is enhanced by glucocorticoid modulation of L-type Ca\(^{2+}\) currents (Landfield and Eldridge, 1994; Joels, 2001). In combination with depolarizing stimuli such as KA-induced seizures or excessive levels of extracellular glutamate, GR-mediated increases in intracellular Ca\(^{2+}\) levels may enhance neuronal vulnerability to Ca\(^{2+}\)-related injury mechanisms (Landfield and Eldridge, 1991; Joels, 2001).

In addition to increasing Ca\(^{2+}\) influx, GR activation reduces Ca\(^{2+}\) extrusion in hippocampal neurons (Elliott and Sapolsky, 1993; Joels, 2001). Consistent with these findings, high CORT levels reduce expression of PMCA1 (Bhargava et al., 2000), a plasma membrane Ca\(^{2+}\) ATPase responsible for translocating calcium from the cytosol to the extracellular space (Garcia and Strehler, 1999). Overexpression of PMCA isoform 4 in pheochromocytoma 12 cells reduces vulnerability to Ca\(^{2+}\)-mediated cell death, suggesting that regulation of PMCA expression may influence cellular survival of insults involving pathological increases in intracellular calcium (Garcia et al., 2001). In agreement with this hypothesis, KA administration decreases hippocampal expression of PMCA isoforms 1 and 2 (Garcia et al., 1997b). Further reduction in PMCA levels prompted by GR activation would be expected to intensify injury-induced rises in intracellular Ca\(^{2+}\) levels and exacerbate neuronal degeneration.

Adrenocorticosteriod receptor blockade with MR or GR antagonists may influence hippocampal neuronal viability by altering Ca\(^{2+}\) conductances or extrusion mechanisms. Blocking MR activation with SPIRO should result in the predominant activation of GR. It is well established that voltage-dependent Ca\(^{2+}\) currents increase with GR activation at high CORT levels (Kerr et al., 1992; Karst et al., 1994; Werkman et al., 1997). However, it is currently
unclear whether exclusive GR activation is sufficient to induce large Ca\(^{2+}\) currents in hippocampal neurons. Electrophysiological studies in hippocampal slices have reported mixed results; one study indicates that GR agonist RU28362 increases Ca\(^{2+}\) currents in CA1 neurons (Kerr et al., 1992) while another indicates that it does not (Karst et al., 1994). If exclusive GR activation is indeed sufficient to increase Ca\(^{2+}\) currents, then SPIRO blockade of MR may endanger hippocampal neurons by enhancing Ca\(^{2+}\) influx, particularly under conditions of GR-saturating CORT levels. In terms of the current experiments, this scenario is consistent with the increased vulnerability of CA3 neurons to KA toxicity following SPIRO pretreatment (Chapter Three).

Glucocorticoid receptor blockade with RU486 should result in the predominant activation of MR. Exclusive MR occupation yields relatively small Ca\(^{2+}\) currents (Karst et al., 1994) and may thus protect hippocampal neurons from exposure to excessive Ca\(^{2+}\) influx. In addition, GR blockade should prevent GR-mediated repression of PMCA isoforms, improving Ca\(^{2+}\) extrusion mechanisms. These beneficial effects of GR blockade on neuronal Ca\(^{2+}\) homeostasis are consistent with the neuroprotective effects of RU486 on CA1 neurons following TBI (Chapter Four).

Adrenocorticosteriod receptor suppression of inflammation

Glucocorticoids inhibit synthesis, release, and/or function of many factors involved in the inflammatory response, including: cytokines, such as interleukin (IL)-1\(\beta\), IL-2, IL-3, IL-5, IL-6, IL-12, and tumor necrosis factor alpha (TNF-\(\alpha\)); chemokines such as IL-8; and inflammatory mediators and enzymes such as histamine, bradykinin, and nitric oxide (Cato and Wade, 1996; Steer et al., 1997; Sapolsky et al., 2000). Glucocorticoids also repress expression of cyclooxygenase 2 (COX-2) (Cato and Wade, 1996; Sapolsky et al., 2000), an enzyme that catalyzes prostaglandin formation (Rivest, 1999). Further, glucocorticoids inhibit expression of adhesion molecules, alter lymphocyte trafficking, and reduce activation and proliferation of B and T cells (Cato and Wade, 1996; Sapolsky et al., 2000). Glucocorticoid inhibition of KA-induced AP-1 (Unlap and Jope, 1995a) and NF-\(\kappa\)B (Unlap and Jope, 1995b) DNA binding has been demonstrated, suggesting that the potent anti-inflammatory and immunomodulatory action of glucocorticoids may be mediated in part through repression of AP-1 and NF-\(\kappa\)B activity (De Bosscher et al., 2000).
Inflammation has been proposed to influence neuronal survival following several forms of insult including KA-mediated excitotoxicity and traumatic brain injury. Kainic acid increases glial expression of several cytokines in hippocampus and cortex including IL-1\(_\beta\) (Minami et al., 1991; Yabuuchi et al., 1993; Eriksson et al., 2000). Administration of IL-1ra, an endogenous antagonist of IL-1, protects neurons against KA-induced degeneration in CA1 and CA3, suggesting a deleterious role for IL-1\(_\beta\) in excitotoxic neuronal death (Panegyres and Hughes, 1998). Hippocampal and cortical mRNA levels of IL-1\(_\beta\) also increase following experimental TBI (Fan et al., 1995). Administration of IL-1 receptor antagonist IL-1ra has been shown to reduce cortical lesion volume (Toulmond and Rothwell, 1995), attenuate hippocampal neuron loss, and improve cognitive function following lateral fluid percussion (Sanderson et al., 1999), providing further evidence that IL-1\(_\beta\) may contribute to injury-mediated neurodegeneration. As glucocorticoids decrease IL-1 production (McEwen et al., 1997), ACR regulation of this cytokine should enhance neuronal survival after injury and would not appear to explain the endangering effects of GR.

Other studies raise the possibility that mediators of inflammation may play a neuroprotective role after neuronal injury. Mice lacking IL-6 demonstrate increased susceptibility to KA-induced damage in hippocampus, suggesting that IL-6 can improve survival after excitotoxic injury (Penkowa et al., 2001). In addition, selective inhibitors of COX-2 exacerbate KA-induced neuronal degeneration in hippocampus (Baik et al., 1999) and worsen motor performance following TBI (Dash et al., 2000), indicating a protective role for COX-2. Glucocorticoids decrease IL-6 production (McEwen et al., 1997) and inhibit COX-2 expression (Masferrer et al., 1992), raising the possibility that the anti-inflammatory effects of glucocorticoids may contribute to neuronal vulnerability to injury.

Glucocorticoids may also increase neuronal vulnerability to injury via suppression of TNF-\(\alpha\), a pro-inflammatory cytokine that is rapidly induced after several types of brain injury including KA excitotoxicity (Minami et al., 1991; de Bock et al., 1996) and TBI (Taupin et al., 1993; Fan et al., 1996; Knoblach et al., 1999). The role for TNF-\(\alpha\) in mediating neuronal survival is controversial; several studies indicate that inhibition of TNF-\(\alpha\) reduces neurological deficits and hippocampal damage following TBI (Shohami et al., 1996; Shohami et al., 1997; Knoblach et al., 1999). However, increasing evidence suggests that TNF\(\alpha\) may have neuroprotective effects under certain circumstances following central nervous system insult. Pretreatment with TNF-\(\alpha\)
protects cultured hippocampal neurons against oxidative insults (Barger et al., 1995; Mattson et al., 1997), excitatory amino acid toxicity (Cheng et al., 1994), and injury induced by glucose deprivation (Cheng et al., 1994). In addition, TNF-deficient mice exhibit long-lasting motor deficits following CCI brain injury as compared to wild-type (despite increased memory and motor deficits within the acute posttraumatic period), suggesting that TNF may facilitate long-term recovery after brain injury (Scherbel et al., 1999). Further, mice lacking TNF receptors are more susceptible to neuronal loss induced by focal cerebral ischemia and KA administration (Bruce et al., 1996). TNF-α appears to protect neurons by stabilizing cellular Ca^{2+} homeostasis and stimulating antioxidant pathways via activation of NF-κB (Cheng et al., 1994; Barger et al., 1995; Bruce et al., 1996; Mattson et al., 1997; Furukawa and Mattson, 1998). Glucocorticoids may counter these potentially neuroprotective effects through suppression of TNF-α release from monocytes and macrophages (Joyce et al., 1997; Steer et al., 1997) and inhibition of NF-κB activation (De Bosscher et al., 2000).

Glucocorticoids have been proposed to protect the organism by limiting inflammatory responses that, left unchecked, might threaten cellular homeostasis (Munck et al., 1984). In this view, it is possible that glucocorticoids prevent excessive activation of mediators of inflammation that would otherwise contribute to neuronal degeneration after injury. However, it is also possible that glucocorticoid suppression of the beneficial effects of inflammation, such as Ca^{2+} regulation and the expression of antioxidants (Wong and Goeddel, 1988; Cheng et al., 1994), may contribute to neuronal degeneration. Administration of RU486 would be expected to attenuate the anti-inflammatory effects of GR activation, as RU486 has been shown to reverse GR suppression of TNFα release from monocytes (Steer et al., 1997). Thus, RU486 repression of anti-inflammatory glucocorticoid effects may contribute to the improved survival of hippocampal neurons after injury.

Adrenocorticosteroid receptor regulation of hippocampal neurotrophic factor expression

Despite negative findings reported in the current work, the possibility for glucocorticoid modulation of neuronal viability via regulation of neurotrophic factors can not be dismissed. Due to the twenty-six hour time period between antagonist injection and decapitation, ACR antagonist effects on BDNF or NT-3 mRNA levels may not have been detected. Glucocorticoid regulation of BDNF expression is rapid and transient; hippocampal BDNF mRNAs are reduced
within one hour after the onset of stress, recover substantially by four hours, and return towards baseline levels within twenty-four hours (Smith et al., 1995b). Similarly, hippocampal nerve growth factor mRNA levels peak approximately three hours after CORT injection, fall below baseline levels by six hours, and return close to control levels by 24 hours (Lindholm et al., 1994), emphasizing the transitory nature of growth factor induction. In addition, neurotrophin levels were not assessed in sham and CCI-injured animals in the current work, and ACR effects on numerous other trophic factors remain to be investigated.

Several previous studies validate the possibility that ACR regulation of neurotrophic factors may influence hippocampal vulnerability to injury. Although reports are not entirely consistent, there is general agreement that in specific hippocampal subfields, CORT and stress decrease hippocampal BDNF mRNA levels, while adrenalectomy increases BDNF mRNAs and decreases NT-3 mRNAs in hippocampus (Chao and McEwen, 1994; Smith et al., 1995a, b; Schaaf et al., 1997; Chao et al., 1998b). Further, CORT and dexamethasone have been shown to increase nerve growth factor mRNA levels (Lindholm et al., 1994; Scully and Otten, 1995). There is also evidence to indicate that glucocorticoids regulate neurotrophic factor expression following injury. Adrenalectomy attenuates BDNF mRNA induction and nearly abrogates NGF mRNA induction by KA, and GR agonist dexamethasone enhances NGF mRNA induction by KA (Barbany and Persson, 1993). In cultured hippocampal neurons, KA-induced increases in BDNF mRNAs are negated by dexamethasone (Cosi et al., 1993). Furthermore, serum deprivation-induced death of cultured hippocampal neurons is accelerated by CORT and associated with decreases in BDNF mRNA levels and protein (Nitta et al., 1999). Addition of BDNF to serum-deprived cultures attenuates negative CORT effects, supporting the hypothesis that reduced synthesis of BDNF contributes to CORT-mediated endangerment of hippocampal neurons (Nitta et al., 1999). A recent study has also demonstrated that glucocorticoids influence neurotrophic factor expression following TBI. Adrenalectomy enhances fluid percussion injury-induced increases in BDNF mRNA levels, an effect that is reversed with administration of CORT (Grundy et al., 2000). Adrenalectomy also prevents and CORT replacement permits the induction of NGF mRNA levels following fluid-percussion injury, indicating the essential role of CORT in the NGF response (Grundy et al., 2001).

Despite evidence for glucocorticoid regulation of neurotrophic factor expression following injury, the potential impact of this regulation on hippocampal neuronal viability is presently
unclear. High glucocorticoid levels decrease BDNF mRNAs and increase pyramidal neuron vulnerability to injury (Sapolsky, 1994; Smith et al., 1995b), suggesting reduced BDNF expression as a potential mechanism for glucocorticoid toxicity. Reports demonstrating an association between decreased BDNF mRNA levels and increased neuronal degeneration support this hypothesis (Hicks et al., 1999; Nitta et al., 1999). In contrast, the attenuation of KA-induced increases in BDNF expression by adrenalectomy is not consistent with a protective role for glucocorticoid removal (Barbany and Persson, 1993). The neuroprotective value of BDNF is also uncertain, as exogenous administration of BDNF has been shown to exacerbate CA3 neuronal loss following KA administration (Rudge et al., 1998). Furthermore, glucocorticoids potentiate NGF expression and maintain NT-3 mRNA levels, in seeming disagreement with the profile for neuronal endangerment by glucocorticoids. The literature suggests that blockade of GR with RU486 should enhance KA- and TBI-mediated induction of BDNF expression, while decreasing injury-induced NGF expression. However, the potential effects MR blockade on injury-induced neurotrophin expression are less clear because it has not been established whether GR activation alone is sufficient for neurotrophin regulation. Clarification of these issues will require further study.

Summary

The present thesis provides evidence supporting the hypotheses that MR protects and GR endangers hippocampal neurons following specific types of neuronal injury. The current experiments did not support the hypotheses that ACR effects on neuronal survival involve the regulation of bcl-2, bax, or p53 expression. Rather, the widespread effects of glucocorticoids on several other systems crucial to neuronal survival suggest that glucocorticoids influence hippocampal neuron viability via more global mechanisms. Glucocorticoids exert direct effects on cellular metabolism, Ca\(^{2+}\) homeostasis, inflammatory processes, and neurotrophic factor expression; in addition, these targets are largely interrelated and capable of altering one another (Figure 6). For instance, glucocorticoid effects on neurotrophin expression, cytokine production, and cellular metabolism can influence intracellular Ca\(^{2+}\) levels (Sapolsky, 1990; Mattson and Scheff, 1994; Tymianski and Tator, 1996; Jiang and Guroff, 1997). In turn, Ca\(^{2+}\) influx can modulate neurotrophin expression (Finkbeiner, 2000), regulate cytokine production (Hotchkiss and Karl, 1996), and disrupt mitochondrial production of ATP (Green and Reed, 1998). It
becomes clear that glucocorticoid effects on hippocampal neuron viability are not likely carried out via isolated signaling pathways, but rather they result from the integration of many factors associated with the injury response.

Figure 6. Potential mechanisms for glucocorticoid modulation of hippocampal neuron survival after injury. Excessive activation of the glucocorticoid receptor may impair antioxidant defenses, suppress neuroprotective inflammatory responses, decrease neuronal energy stores, increase voltage-dependent Ca\(^{2+}\) currents, and compromise ATP-dependent processes responsible for maintaining membrane potential. All of these factors may culminate to increase oxidative stress and intracellular Ca\(^{2+}\) levels, potentially leading to neuronal death.
Injury regulation of adrenocorticosteroid receptor mRNA levels

Experiments in the current thesis reveal that hippocampal ACR mRNA levels are dramatically regulated by excitotoxic and traumatic injury (Chapters 6 and 8). Kainic acid decreased MR mRNA levels by approximately 40% in CA1 and CA3 and decreased GR mRNA levels by approximately 40% in DG (Chapter Three). Traumatic injury did not influence MR mRNA levels but had powerful effects on GR levels; CCI decreased GR levels by approximately 30% in DG of the injured hemisphere and increased GR levels by approximately 20% in CA1 and DG of the contralateral hemisphere (Chapter Five). These data support the hypothesis that glucocorticoid signaling is altered through regulation of ACR expression following injury.

Injury regulation of adrenocorticosteroid receptor mRNA levels – potential mechanisms

As discussed in Chapters Three and Five, altered ACR expression following KA administration and CCI is not attributable to CORT-mediated auto-regulatory mechanisms. Still, the downregulation of dentate gyrus GR mRNA levels by both types of injury suggests the possibility of a common regulatory mechanism. Potential candidates for this role include glutamate receptor activation and Ca\(^{2+}\) signaling, as both KA-induced seizures and TBI lead to the accumulation of extracellular glutamate and increased intracellular Ca\(^{2+}\) levels (Ben-Ari, 1985; Faden et al., 1989; Sun et al., 1992; McIntosh et al., 1998c).

Glutamate regulation of adrenocorticosteroid receptor mRNA levels

Receptor expression patterns provide no evidence for differential action of ionotropic glutamate receptors in DG, as NMDA and AMPA glutamate receptor subtypes are preferentially expressed in CA1, and KA receptors are mainly located in stratum lucidum of CA3 (Foster et al., 1981; Represa et al., 1987). However, metabotropic glutamate receptor subtype 1 (mGluR1) is strongly expressed in granule neuron cell bodies and the molecular layer of dentate gyrus (Shigemoto et al., 1997), and mGluR1 expression is increased in dentate gyrus following systemic KA injection (Blumcke et al., 2000). The literature provides support for mGluR activation as a viable candidate mechanism for regulation of GR expression after injury. Group 1 mGluRs, which include mGluR1 and mGluR5, stimulate formation of cyclic adenosine monophosphate (cAMP) (Aramori and Nakanishi, 1992), which has been shown to decrease GR
expression in multiple cell lines. The cAMP analog 8-bromo-cAMP decreases GR binding in mouse AtT20 anterior pituitary corticotrope tumor cells, and forskolin, a drug that stimulates adenylate cyclase, decreases GR binding and mRNA levels (Sheppard et al., 1991). In addition, treatment with dibutyryl cAMP, another cAMP analog, decreases GR protein levels in rat C6 glioma cells, B104 neuroblastoma cells, and in rat primary hippocampal and cortical cultures (Sipe, 2000). Thus, activation of mGluR1 represents a possible mechanism whereby injury may decrease GR expression in DG via cAMP-dependent pathways.

**Calcium regulation of adrenocorticosteriod receptor mRNA levels**

Intracellular Ca\(^{2+}\)-dependent signaling may also mediate injury-induced changes in ACR expression. Calcium influx (Young, 1992; Fineman et al., 1993; Tymianski and Tator, 1996) has been hypothesized to regulate changes in gene expression induced by both kainate (Pennypacker and Hong, 1995) and TBI (McIntosh et al., 1997; Morrison et al., 2000). Furthermore, Ca\(^{2+}\) regulation of GR expression has been demonstrated in vitro in AtT-20 cells (Sheppard, 1994). However, the lack of Ca\(^{2+}\) accumulation in DG following KA injection (in contrast to significant Ca\(^{2+}\) accumulation in CA3) (Friedman, 1997) and the high density of Ca\(^{2+}\)-binding proteins in DG (Sloviter, 1989) indicate that GR downregulation does not simply correspond to overall Ca\(^{2+}\) load. Rather, Ca\(^{2+}\)-mediated regulation of GR expression might occur via distinct Ca\(^{2+}\) signaling pathways linked to specific routes of Ca\(^{2+}\) influx, such as L-type voltage-dependent Ca\(^{2+}\) channels (Bading et al., 1993). In support of this hypothesis, treatment with BAY K8644, which selectively promotes Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels, decreases GR binding in AtT-20 cells (Sheppard, 1994).

**Injury regulation of adrenocorticosteroid receptor mRNA levels – potential influence on hippocampal neuron viability**

**Potential effects of GR downregulation on hippocampal circuitry**

Ample evidence supports the hypothesis that GR compromises survival of CA1 and CA3 pyramidal neurons after injury, yet GR activation does not appear to compromise DG granule neuron survival after administration of KA or TBI. Nonetheless, alterations in dentate neuron excitability may influence downstream synaptic transmission and the survival of pyramidal
neurons, as DG is the major recipient of perforant path projections from the entorhinal cortex and delivers the majority of excitatory input into CA3 (Amaral and Witter, 1989) (see Figure 1.5). Electrophysiological studies demonstrating GR attenuation of CA1 neuron excitability (Kerr et al., 1989) raise the possibility that decreased GR expression in DG may enhance KA and TBI-induced granule neuron hyperexcitability (Lowenstein et al., 1992; Sloviter, 1992; Santhakumar et al., 2000), potentially contributing to excitotoxic stress in CA3 and CA1. However, a recent report demonstrates that in contrast to CA1, GR activation does not suppress synaptically evoked field potentials in dentate gyrus (Stienstra and Joels, 2000), indicating that decreased GR expression may not influence excitability in this region. In addition, GR blockade, which should have a maximal effect if any on glucocorticoid-modulated synaptic transmission, does not compromise CA3 or CA1 survival after TBI (Chapter Four). It therefore does not appear likely that altered GR expression influences pyramidal neuron survival via modulation of DG synaptic transmission.

Alternatively, downregulation of GR expression may facilitate rearrangement of DG circuitry after excitotoxic and traumatic brain injury. This hypothesis is supported by studies investigating reactive synaptogenesis in response to entorhinal cortex lesion in the rat. Glucocorticoids inhibit axon sprouting (Scheff and Cotman, 1982) and influence synaptic replacement (Scheff et al., 1986) in DG following delivery of entorhinal cortex lesion. In addition, an approximately 33% bilateral decrease in GR mRNA levels has been observed in DG one day following unilateral entorhinal cortex lesion (O'Donnell et al., 1993). The authors of this study proposed that decreased GR expression might serve to attenuate glucocorticoid inhibition of lesion-induced synaptogenesis (O'Donnell et al., 1993). Strikingly, the patterns of GR downregulation following entorhinal cortex lesion, kainic acid insult, and TBI are very similar (Chapters Three and Five), raising speculation that decreased expression of GR in dentate gyrus may have a permissive effect on restructuring of DG circuitry following multiple types of injury. Kainic acid is known to induce mossy fiber sprouting (Davenport et al., 1990), but the extent of DG remodeling induced by TBI remains to be investigated.

**Potential effects of GR downregulation on neurogenesis**

In addition to facilitating rearrangement of residual DG circuitry, downregulation of GR expression may influence DG neurogenesis after injury. Neurogenesis of DG granule cells
continues well into adulthood in the rat and is inhibited by corticosterone in a concentration-dependent manner (Cameron and Gould, 1994). Kainic acid (Gray and Sundstrom, 1998) and TBI (Dash et al., 2001) have both been shown to enhance DG neurogenesis. GR downregulation in dentate gyrus after KA and traumatic brain injury may therefore facilitate injury-induced neurogenesis and the subsequent restoration of functional DG circuitry.
Summary

The present thesis supports the hypotheses that MR activation enhances and GR activation endangers neuronal survival in specific hippocampal subregions following excitotoxic or traumatic brain injury. Experiments in the current work demonstrate a neuroprotective effect of GR blockade after experimental TBI in vivo, as pretreatment with RU486 prevented CCI-induced neuronal loss in hippocampal subregion CA1 (Chapter Four). The present data also provide evidence that MR protects hippocampal neurons following excitotoxic injury, as MR blockade with SPIRO increased CA3 cell loss following systemic KA injection (Chapter Three). In addition, this work demonstrates KA and TBI-induced downregulation of GR mRNA levels in dentate gyrus, which may facilitate the recovery of functional DG circuitry after injury (Chapters Three and Five). Adrenocorticosteroid receptors clearly influence hippocampal neuron survival after injury, and future study of ACR regulation under challenging conditions could contribute to the improved treatment of seizure, stroke, traumatic injury, and neurodegenerative diseases.
Appendix

List of Abbreviations

mineralocorticoid receptor (MR), glucocorticoid receptor (GR), adrenocorticosteroid receptor (ACR), mifepristone (RU486), hypothalamic-pituitary-adrenal (HPA), corticotropin-releasing hormone (CRH), adrenocorticotropin-releasing hormone (ACTH), paraventricular nucleus of the hypothalamus (PVN), heat shock protein (HSP), glucocorticoid response element (GRE), activator protein-1 (AP-1), cyclic AMP response element binding protein (CREB), nuclear factor-κB (NF-κB), negative glucocorticoid response element (nGRE), calcium (Ca$^{2+}$), corticosterone (CORT), plasma membrane calcium pump isoform 1 (PMCA1), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), excitatory postsynaptic potential (EPSP), entorhinal cortex (EC), subicular complex (S), nerve growth factor (NGF), apoptosis-inducing factor (AIF), apoptotic protease activating factor-1 (Apaf-1), inhibitor of CAD (ICAD), caspase-activated deoxyribonuclease (CAD), traumatic brain injury (TBI), controlled cortical impact (CCI), anti-apoptotic B-cell leukemia/lymphoma-2 gene (bcl-2), messenger RNA (mRNA), interleukin (IL), tumor necrosis factor alpha (TNF-α), cyclooxygenase 2 (COX-2), metabotropic glutamate receptor subtype 1 (mGluR1), cyclic adenosine monophosphate (cAMP)
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