Glucocorticoid regulation of the astrocyte transcriptome *in vitro* and *in vivo*

by

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Dedication

To my family, my parents and my sister, and my beloved wife Becky

In honor of all individuals who experience depression
and the communities that love them

Acknowledgements

During graduate school, I have learned that I am the product of an extraordinary combination of my efforts, circumstance, and the community around me. My personal growth and scientific training at Michigan are due in large part to the people who have supported and influenced me along the journey. I am truly grateful for the opportunities they have given me and the many ways they have defined my life during my time in Ann Arbor.

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Finally, I would like to thank my family for their unconditional love and support of me. Thank you to my parents for boldly supporting my scientific interests and learning about how to help me pursue my dreams. Thank you to my beautiful wife, Becky; of all my experiences during graduate school, the best have been being married to you and having adventures together.

Table of Contents

Chapter IV: Thesis Summary and Conclusions

List of Tables

List of Figures

Abstract

The broad goal of this thesis has been to gain understanding about the influence of cell type on transcriptional regulation in the brain by characterizing glucocorticoid-mediated regulation in astrocytes. Glucocorticoids are the primary mediator of the stress response facilitated through the hypothalamic-pituitary-adrenal axis. At the cellular level, glucocorticoids act as transcriptional regulators by binding their receptors and upregulating or downregulating target genes. All brain cell types (i.e. neurons and glia) express glucocorticoid receptors, yet most studies have interpreted data exclusively in terms of impact on neurons. We hypothesized that glucocorticoid mRNA regulation in the brain varies by cell type. We characterized glucocorticoid receptor-mediated mRNA regulation in mouse primary astrocyte cultures using microarrays, qPCR, and bioinformatics analyses. We identified numerous mRNAs regulated more than two-fold by the glucocorticoids dexamethasone and corticosterone, including 36 mRNAs demonstrating novel glucocorticoid regulation. Interestingly, glucocorticoids appear to selectively target astrocyte-enriched mRNAs in astrocyte *in vitro* in a time-dependent manner.

To test the physiological relevance of the observed glucocorticoid regulation in astrocytes *in vitro*, we measured a subset of regulated mRNAs by qPCR in two paradigms of *in vivo* corticosterone exposure in mice (acute/chronic). The majority of measured mRNAs regulated by glucocorticoids in astrocytes *in vitro* were also regulated

by corticosterone exposure *in vivo*. Acute corticosterone exposure regulated distinct mRNAs compared with chronic corticosterone exposure. Chronic corticosterone exposure *in vivo* also regulated additional mRNAs not regulated by acute corticosterone exposure *in vivo* or glucocorticoid treatment of astrocytes *in vitro*. In situ hybridization experiments revealed gene expression patterns for a subset of mRNAs regulated by chronic corticosterone exposure that were consistent with astrocyte localization. Together, these data indicate that astrocyte mRNAs can indeed be regulated by glucocorticoids and suggest that glucocorticoids selectively regulate mRNAs by cell type in the brain. Our findings contribute further knowledge to understanding the stress response in the brain and may have clinical relevance for further understanding psychiatric disorders, particularly depression because of the established associations between depression and hypercortisolemia as well as astrocyte pathology.

Chapter I

Introduction

Section 1: Stress hormone signaling and molecular mechanisms of glucocorticoid action

Stress: definition and biological purpose

"Reality is the leading cause of stress amongst those in touch with it."

— Jane Wagner

What is stress? In biology, stress is a universal concept based on the interplay of organism stability and external stimuli. At the core of cellular rules of life, organisms strive to obtain a dynamic equilibrium, referred to as homeostasis (from Greek, "same stand" or "being stable by being the same") (De Kloet et al., 2005). This equilibrium is constantly impacted or threatened by external stimuli, termed "stressors" in the literature. In human terms, stressors include both physical and psychological stimuli that impact homeostasis of the body and mind. In an effort to maintain homeostasis, organisms enact physiological and behavioral responses that attempt to mitigate, limit, or reverse the change or potential change induced by a given stressor. The classic

“stress response” is an adaptive process involving multiple neurotransmitter and hormonal signals that rapidly respond to stressors in an effort to return to homeostasis. These processes that underlie the stress response are collectively termed “allostasis” (from Greek *allo* meaning "variable;" thus, "remaining stable by being variable). Appropriate stress response also implies that the processes are well-regulated, rapidly responding to stressors and efficiently terminating once homeostasis has been restored.

The Stress Response - sympathetic NS, neural circuitry, HPA axis

Where does the activation signal for stress response come from and how does the brain carry out this response? Decades of neuroanatomical research have mapped out an intricate set of neural circuitry that mediates the stress response and is highly conserved across species. Sensory perception of stressors activates structures in the brainstem (visceral/sensory) and/or limbic system (psychological); these areas serve as integration points for incoming sensory information. The limbic system is traditionally defined as a set of structures adjacent to the thalamus in the center of the brain; these structures are associated with emotional processing and learning and memory and include the prefrontal cortex and cingulate cortex, hippocampus, and amygdala. There are two general neural circuits involved in mediating the subsequent stress response and restoring homeostasis; (1) the sympatho-adrenomedullary axis of the autonomic nervous system (ANS) and (2) the hypothalamic-pituitary-adrenal axis (HPA). In response to a stress signal, the brainstem first stimulates sympathetic reflex pathways of the ANS that stimulate end organs via the adrenal medulla and the spinal cord. The

result of these reflex pathways includes increased circulating levels of adrenaline and noradrenaline, increased heart rate, heightened awareness, and energy mobilization (reviewed in (Ulrich-Lai and Herman, 2009)). Additional brain regions are involved in regulating the sympathetic response to acute stress (e.g. locus ceruleus). This set of sympathetic reflexes is what is often referred to as the "fight-or-flight" response. These initial stress responses are generally short-lived due to subsequent reactions of the parasympathetic branch of the ANS that are generally opposite the sympathetic action. In addition to stimulating the ANS, both the brainstem and the limbic system have direct innervation to the paraventricular nucleus (PVN) of the hypothalamus located in the basal forebrain.

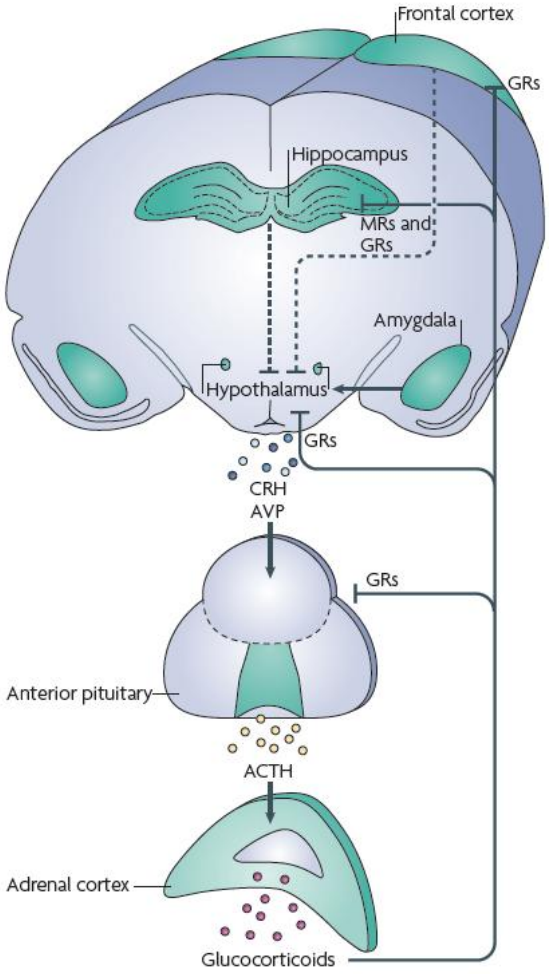

Figure 1.1: Overview of Hypothalamic-Pituitary-Adrenal Axis
In response to stress, the PVN neurons release neuropeptides (CRH/AVP) directly into the median eminence, a component of the portal blood system that supplies the hypothalamus and pituitary gland. These neuropeptides then stimulate the anterior pituitary to release adrenocorticotropic hormone (ACTH) into the general circulatory system. ACTH travels through the blood stream to the adrenal glands located adjacent to the kidneys. ACTH stimulates the adrenal glands to produce and release glucocorticoids, the primary mediator of HPA axis signaling. Glucocorticoids also engage in negative feedback mechanisms to self-regulate HPA axis signaling at multiple levels of the cascade (e.g. limbic structures, hypothalamus, pituitary). Used with permission from (Lupien et al., 2009).

The PVN is often thought to be the initial step of HPA axis signaling (endocrine circuit described in Figure 1.1). The PVN contains neurons that release the neuropeptides corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) into the median eminence. The median eminence is a venous structure that links brain systems with the pituitary gland and is one of the only locations of direct CNS-blood interaction. Once released, these neuropeptides act on the anterior pituitary to propagate HPA axis activity. Additional brain structures are involved in regulating the PVN in response to stress; in reality, most input from limbic structures relay through secondary structures such as the bed nucleus of the stria terminalis and additional hypothalamic nuclei (e.g. medial preoptic hypothalamus, arcuate nucleus, lateral hypthalamus, suprachiasmatic nucleus). The output of HPA axis activation is the release of glucocorticoids from the adrenal gland into the general blood supply. The glucocorticoid output of the HPA axis is cortisol in humans and corticosterone in rodents. Glucocorticoids then distribute throughout the body via the blood and induce a number of reactions that mobilize resources to respond to the stressor (e.g. increased heart rate, vasodilation, increased metabolic activity for muscles) and downregulate long-term processes (e.g. digestion, reproduction, growth processes). The timing of these effects is rapid, resulting in changes within seconds to minutes and enabling the organism to further respond to a given stimulus. In contrast to the rapid but short-lived ANS response, the HPA stimulation in response to stress results in long-lasting changes (e.g. minutes to hours) that can be adaptive and compensatory to deal with the current stressor and prepare for future stressor challenges.

After enabling appropriate responses to a stressor, the HPA axis returns to baseline in part by utilizing the regulatory power of the output signal, glucocorticoids, in a negative feedback mechanism. Glucocorticoids can directly inhibit signaling in multiple upstream components of the HPA axis (e.g. hypothalamus, anterior pituitary). Glucocorticoids also impact limbic system structures (e.g. forebrain, hippocampus) that initiate the stress response, indirectly decreasing the initiating signals to the HPA axis. This relationship allows for ongoing self-tuning of the stress response in response to stressors, rebalancing the system back to baseline when appropriate. Overall, the complexity resulting from stress signaling at multiple locations in the brain and HPA axis modulation at multiple levels by glucocorticoids allows many neural circuits and signaling cascades to be influenced and regulated by stress.

Cellular mechanisms of glucocorticoid action; transcriptional regulation

At the cellular level, glucocorticoids carry out their role as HPA axis effectors primarily as transcriptional regulators (general pathway diagramed in Figure 1.2).

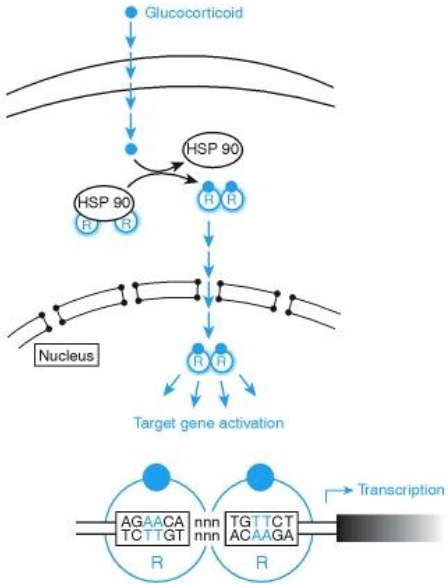

Figure 1.2: Cellular mechanisms of transcriptional regulation by glucocorticoids
As steroid molecules, glucocorticoids readily pass through the lipid-rich cell membrane and bind inactive receptors that are complexed with chaperone proteins in the cytoplasm. Binding of glucocorticoid ligand to specific protein receptors (MR/GR) activates the receptor protein (e.g. induces dimerization) followed by translocation of the activated receptors to the nucleus. In the nucleus, glucocorticoid receptors bind to DNA at stereotypic sequences known as Glucocorticoid Response Elements (GREs) that upregulate or downregulate transcription of target genes (e.g. termed transactivation/transrepression). Note that receptors can also translocate to the nucleus while in complex with chaperone proteins (Vandevyver et al., 2012). Used with permission from (Strachan and Read, 1999)

Glucocorticoids are known to regulate numerous mRNAs; cortisol has been estimated to directly or indirectly influence the expression of up to 20% of human genes (Chrousos, 2009). In addition to mediating HPA axis stress signaling, glucocorticoids have been implicated in a wide range of physiological processes, including development and metabolism (Lupien et al., 2009), immune response and cytokine signaling (Goujon et al., 1997; Liberman et al., 2007), circadian rhythms (Balsalobre et al., 2000), and apoptosis and cell cycle progression (Viegas et al., 2008). Extensive data has been generated on the mechanisms of glucocorticoid regulation (for historical connections, reviewed in (Rousseau, 1984)). Glucocorticoid receptors can regulate genes by binding directly to DNA at specific sequences known as Glucocorticoid Response Elements (GREs, Figure 1.2). Glucocorticoid receptors can also regulate genes by binding in concert with other transcription factors at what are termed composite GREs (i.e. GREs in proximity or adjacent to other transcription factor binding sites, such as AP-1) (Diamond et al., 1990). In addition, glucocorticoid receptors can regulate genes through protein-protein interactions without direct DNA binding (e.g. NFkB (Almawi and Melemedjian, 2002a), Stat5 (Stoecklin et al., 1997)). The process of primary glucocorticoid-mediated transcriptional regulation is temporally estimated to be on the order of minutes to hours. Because of their potent regulatory capacity, glucocorticoids are also associated with extensive secondary and tertiary regulatory events (i.e. genes initially regulated by glucocorticoids have been observed to regulate additional genes). Since the proteins that adrenal steroids directly bind to (e.g. glucocorticoid receptors) are well-known transcription factors, measuring mRNA expression changes is a relevant

functional output of glucocorticoid signaling.

The mechanisms of glucocorticoid regulation are influenced and modified by a number of factors in the brain and other tissues. First, glucocorticoids can act through two receptors, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). These receptors regulate transcription primarily as dimers that can be homomeric (MR/MR, GR/GR) or heteromeric (MR/GR); each of these protein complexes are thought to have different properties of ligand affinity and gene targeting. In the brain, each of these receptors also has overlapping and distinct gene expression patterns, adding anatomical complexity to their interactions. Second, these receptors interact with a diversity of chaperone proteins in the cytoplasm. Inactive glucocorticoid receptors are bound in complexes with chaperone proteins (e.g. heat shock proteins), and the composition of these complexes can vary by cell type and tissue (Smith and Toft, 2008). Third, activated glucocorticoid receptors can also interact with coregulators (coactivators/corepressors) and other transcriptional regulators (e.g. AP-1, NFKB), modulating their gene expression capacity and DNA binding sequence target (De Bosscher et al., 2003; Meijer et al., 2006). Coregulators can also modify the chromatin structure of target genes to enable or disable glucocorticoid receptor binding (reviewed for MR/GR in (Pascual-Le Tallec and Lombès, 2005)); for example, coactivators can induce histone acetylation to increase the availability of a target gene, whereas corepressors can inhibit binding through increased histone deacetylation. Fourth, the two receptors for glucocorticoids have differential affinities for different glucocorticoids. MR has a much higher affinity for the corticosteroid output of the HPA axis (i.e. cortisol

in humans, corticosterone in rodents) than GR (Rupprecht et al., 1993). The difference in affinity for glucocorticoids has led to the general associations of MR action with lower concentrations of corticosteroids (e.g. circadian rhythmicity of basal cortisol concentrations, initiation/detection of stress response) and GR action with higher concentrations of corticosteroids (e.g. stress response, HPA negative feedback mechanisms, recovery/memory processes) .

In an effort to distinguish specific effects of MR and GR, efforts have been made to identify and develop receptor-selective ligands to specify their functional influence. MR naturally binds mineralocorticoids (e.g. aldosterone, deoxycorticosterone) that do not bind to GR; MR-selective antagonists include spironolactone and eplerenone. The primary GR-selective agonist is dexamethasone, and the predominant GR antagonist is mifepristone (i.e. RU486), although RU486 also has progesterone receptor antagonism properties. Experiments in this thesis make use of receptor selective tools to examine GR-mediated gene expression effects.

Glucocorticoids also can act as non-genomic regulators. Membrane-bound receptor action is consistent with time scale of some observed glucocorticoid effects (e.g. temporally too short for transcriptional regulation), but specific mechanisms remain relatively uncharacterized at this time. Membrane MR is known to mediate fast effects of glucocorticoids in the brain (Joels et al., 2008). In general, membrane receptor-mediated effects are typically not blocked by antagonists in the same manner as transcriptional regulation (membrane glucocorticoid receptor action reviewed in (Tasker

et al., 2006)). These non-genomic actions are not investigated in this thesis, but these topics have been extensively reviewed in the literature (e.g. see (Stellato, 2004; Dallman, 2005; Evanson et al., 2010)).

Contextual factors of glucocorticoid regulation; transcriptional effects and cell selectivity

Many factors influence how glucocorticoids selectively regulate genes among different cell types (diagramed in Figure 1.3). Glucocorticoid availability varies by cell type due to the expression or absence of different enzymes that act on glucocorticoid ligands; for example, kidney cells express 11-B-hydroxysteroid (11B-HSD) type II enzyme that converts corticosteroids to an inactive state (i.e. to the mineralocorticoid ligand, aldosterone), preventing GR-mediated regulation in kidney cells (Edwards et al., 1988). GR binding can also be affected by the diversity of coregulators that complex with activated glucocorticoid receptors to influence properties of transcriptional regulation (reviewed in (Rosenfeld and Glass, 2001)); specific combinations of coregulators in a given cell type differentially influence the dynamics of glucocorticoid regulation.

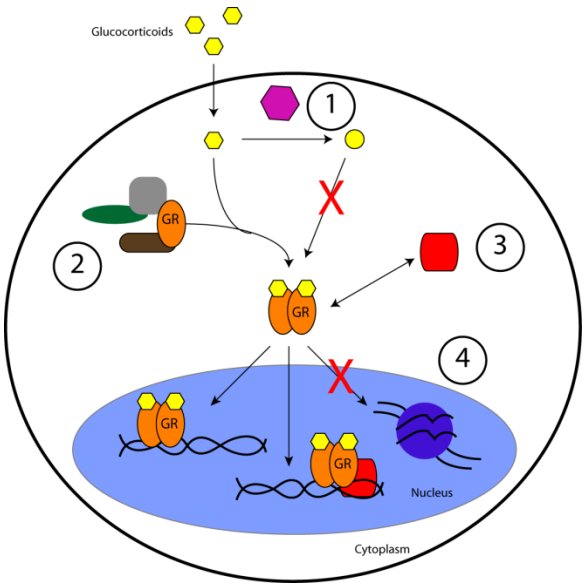

Figure 1.3: Factors influencing glucocorticoid receptor regulation by cell type
GR regulation is influenced by (1) expression of enzymes that inactivate corticosteroids, (2) set of chaperone proteins and coregulators, (3) expression of cofactors and transcription factors that interact with activated GR, and (4) basal and GR-regulated chromatin structure (e.g. genomic availability of genes to be regulated.

In addition, ligand-activated GRs also differentially bind DNA of target genes based on cellular context. GR binding of GRE sites in a human lung cell line were found to be present near many genes whose mRNA was regulated by glucocorticoids in that cell line, but GR binding was not present near genes known to be regulated by glucocorticoids in other tissues or types of cell lines (So et al., 2007). This finding may be due to differences in chromatin structure between cell types. Remarkably, additional studies indicate that up to 95% of *de novo* genomic binding by GR is based on chromatin accessibility, suggesting that cell-specific GR binding is primarily based on baseline chromatin accessibility (John et al., 2011). In addition, recent experiments using GR ChIP-seq methods have found that (1) the directionality of glucocorticoid regulation (upregulation/downregulation) is not sequence-dependent (i.e. GRE) as previously assumed and (2) additional factors beyond the glucocorticoid receptor determine the functional outcome of GR regulation (e.g. epigenetic regulators) (Uhlenhaut et al., 2012). Based on these recent findings, cellular context may thus be a critical factor in determining the character and outcome of glucocorticoid receptor-mediated mRNA regulation. Together, these data suggest that the cellular environment influences glucocorticoid signaling through the cellular expression and relative ratios of coregulators, chaperone proteins and/or chromatin structural arrangements (e.g. genes accessible vs. inaccessible to receptor binding and transcriptional regulation).

Since chromatin structure is an important determinant of which genes are glucocorticoid-sensitive in a given cell type, then the set of mRNAs expressed under basal conditions whose chromatin states permit them to be expressed in that cell type

may also be a factor in defining which genes can be regulated by glucocorticoids. For example, recent studies defining the heterogeneity of mRNA expression among cell types in the brain (i.e. neurons vs. astrocytes vs. oligodendrocytes (Cahoy et al., 2008)) could enable novel investigations into the impact of cell type on transcriptional regulation in a heterocellular tissue.

Glucocorticoid regulation in the brain; cell type complexity and physiological impacts

Studies of glucocorticoid regulation in neural contexts have identified numerous mRNAs regulated by glucocorticoids in the brain. Microarray studies have characterized tissue-level regulation in hippocampal slices, identifying hundreds of glucocorticoid-regulated genes (Datson et al., 2001) and demonstrating temporally dynamic gene expression changes due to glucocorticoid exposure (Morsink et al., 2006b). *In vitro* studies of glucocorticoid action in neural cell lines have also identified target genes (e.g. PC12 cells (Morsink et al., 2006a)), although the *in vivo* regulation of many of these genes remains to be confirmed. Genes regulated by glucocorticoids in the brain are involved in diverse signaling pathways. Glucocorticoids regulate genes that are associated with diverse functions, including neurotrophin signaling (e.g. BDNF (Suri and Vaidya, 2012), other neurotrophins (Barbany and Persson, 1992)), neurotransmission (e.g. tyrosine hydroxylase (Lewis et al., 1987), proenkephpalin (Weisinger, 1995), transcriptional regulation (e.g. NFKB (Almawi and Melemedjian, 2002b), SGK1), and receptor signaling (e.g. adenosine A1 receptors (Svenningsson and Fredholm, 1997)). Many other genes have been identified as glucocorticoid-sensitive in the brain on an individual gene basis as well (e.g. Cox2 (Yamagata et al., 1993), Per1/Per2 (So et al., 2009), GILZ (Ayroldi

and Riccardi, 2009)).

The physiological impacts of glucocorticoid regulation in the brain are diverse given the patterns of receptor expression and variety of pathways impacted. Both MR and GR receptors are expressed in the brain but have distinct neuroanatomical expression patterns. Specifically, MR is expressed primarily in the hippocampus and brainstem, while GR is expressed in most areas of the brain and highly expressed in limbic structures such as the prefrontal cortex and hippocampus. Since MR and GR can act in different isomer arrangements (i.e. homomers vs. heteromers) that have different regulatory properties, the differential expression of GR and MR RNAs in the brain has been interpreted that different ratios of MR:GR expression may influence functional regulation by brain region and cell type (reviewed in (De Kloet et al., 2009)). Most functional research on glucocorticoid action in the brain has focused on neuronal alterations at the tissue level and has focused on detrimental effects of prolonged glucocorticoid exposure. Chronic glucocorticoid exposure decreases cell volume, spine density, and dendritic arbor complexity in the hippocampus and the prefrontal cortex (McEwen, 1999); however, similar paradigms induce opposite effects in the amygdala and orbitofrontal cortex (Popoli et al., 2012). Glucocorticoids also impact cell viability, generally suppressing neurogenesis and promoting cell death (Sapolsky, 1999). In the hippocampus, glucocorticoids are able to modulate long-term potentiation (LTP) properties, decreasing LTP dynamic range and modifying learning and memory processes (reviewed in (McEwen and Sapolsky, 1995)).

How do glucocorticoids mediate these functional alterations at the cellular level? Several steps in neurotransmission can be affected by glucocorticoid signaling (reviewed in (Popoli et al., 2012)). First, glucocorticoids can increase glutamate levels at excitatory synapses. Previous studies found that this increased glutamate concentration may be neuronal in origin (i.e. presynaptic release) or a consequence of reversed glutamate transporters on glial cells. Acute stress or corticosteroid treatment causes a rapid increase in glutamate release in the hippocampus that is mediated by membrane-bound MR (Karst et al., 2005). Stress can also cause a delayed glutamate increase in the PFC that is dependent on intracellular GR (Musazzi et al., 2010). These opposing glutamate changes in these two brain regions likely arise in part due to the differential expression of the receptors by brain region (i.e. relatively low MR expression in PFC). Second, glucocorticoids impact glutamate-based synaptic currents based on postsynaptic glutamate receptor dynamics (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) and N-Methyl-D-aspartate receptor (NMDAR)). Acute stress or corticosterone treatment increases the concentration of both types of glutamate receptors at the postsynaptic membrane (AMPAR and NMDAR); as a result, acute stress or corticosterone treatment increases AMPA and NMDA currents in the PFC (Yuen et al., 2011). Acute stress also impairs LTP capacity in multiple brain regions (e.g. hippocampus, amygdala-PFC connection (Maroun and Richter-Levin, 2003)). In contrast, chronic stress causes loss of glutamate receptor subunits in PFC neurons, both on the cell surface and in terms of total protein expression (Yuen et al., 2012). Chronic stress also impairs LTP in multiple brain regions (e.g. thalamus-PFC, hippocampus-PFC connections (Cerqueira et al., 2007));

this reduction in LTP could be due to synapse changes due to glucocorticoid signaling (e.g. spine loss, atrophy, dendritic retraction) or decreased glutamatergic transmission in PFC neurons due to loss of postsynaptic glutamate receptors at the membrane. The impact of chronic stress on basal glutamatergic neurotransmission is less characterized; however, there are reports of decreased AMPA and NMDA currents in PFC with no change in AMPA or NMDA currents in the hippocampus or striatum (Yuen et al., 2012). These findings have been interpreted to implicate glucocorticoid signaling as a possible mechanism by which the PFC is particularly sensitive to the impact of chronic stress. Third, glucocorticoids impact glutamate reuptake transporters and glutamine metabolism in astrocytes. Glial glutamate transporter activity is known to influence stimulation of NMDARs and metabotropic glutamate receptors (mGluRs) but have relatively little impact on AMPAR currents (Zheng et al., 2008). Glucocorticoids can increase expression of GLT-1/EAAT2 in astrocytes *in vitro*, one of the two primary glutamate transporters expressed in astrocytes (Zschocke et al., 2005). Interestingly, chronic stress paradigms result in decreases in glutamate uptake in the hippocampus, suggesting a possible adaptive change in glutamate uptake mechanisms in response to prolonged stress (Fontella et al., 2004). In addition, chronic stress in rodents results in reduced glutamate-glutamine cycling in the PFC, despite no changes in expression of the enzyme involved in the conversion reaction (i.e. Glul) (Banasr et al., 2010). Finally, glucocorticoids can also impact neurotransmission through other indirect mechanisms. Membrane-bound glucocorticoid receptors can rapidly stimulate postsynaptic endocannabinoid production that subsequently results in inhibition of presynaptic neurotransmitter release (Chavez et al., 2010). Glucocorticoids can also

modulate mitochondrial functionality and calcium buffering capacity (Du et al., 2009); low concentrations of glucocorticoids tend to potentiate mitochondrial function, whereas high concentrations of glucocorticoids tend to attenuate mitochondrial function.

All of these many modes of glucocorticoid action are important for consideration in investigations of the functional consequences of glucocorticoids and stress in the brain. Additionally, cell type distinctions are relevant to glucocorticoid signaling in that the brain regions targeted by glucocorticoid action and negative feedback in the stress response (e.g. cortex, hippocampus) include all known brain cell types. Thus, understanding how glucocorticoids act in these locations requires knowledge of regulation in each cell type. Up to this point, most studies have focused on how glucocorticoids modulate neurons; we know relatively little about how glucocorticoids impact other brain cell types (e.g. astrocytes, oligodendrocytes, microglia). Given the involvement of both neurons and astrocytes in reported glucocorticoid-mediated alterations, knowing how glucocorticoids impact each cell type in the brain would likely inform our understanding of glucocorticoid action in the brain. A major goal of this thesis is to address how glucocorticoids modulate one of these cell types (i.e. astrocytes) both *in vitro* and *in vivo*.

Section 2: Astrocytes; definition and function

"…This connective substance forms in the brain, in the spinal cord, and in the higher sensory nerves a sort of putty (neuroglia), in which the nervous elements are embedded." – Virchow, 1856; first attributed use of term neuroglia/glia (Garcia-Marin et al., 2007)

Glia in a neurocentric world; classification and growing importance of astrocytes

The brain is composed of a diverse set of cell types that have distinct roles in supporting brain health and function. A basic categorization of cells in the brain is neurons and glia. Neurons have received the majority of attention in neuroscience research because of their well-accepted role in transmitting information in the brain. For most of the history of modern neuroscience, the field has had a "neuro-centric" perspective, viewing most phenomenon in relation to neurons with little regard for other cell types. While this view has been and continues to be fruitful in many aspects, recent data indicate it is insufficient in understanding brain health and disease because of the growing recognition of glial cells as active participants in brain signaling. This thesis section will discuss how glial cell types are defined, how astrocytes vary by morphology and brain region, astrocyte functionality, methods of investigating astrocytes, and transcriptional regulation in relation to astrocytes.

“Glia” is the comprehensive label for non-neuron brain cells, which were originally described as passive cells that simply supported neuron function. However, even from the beginning of modern neuroscience, glia have been viewed with intrigue and curiosity. A founding father of modern neuroscience, Santiago Ramón y Cajal was awarded the 1906 Nobel Prize for his work on neuron connectivity, but he also spent time characterizing glial cell morphology and hypothesizing glia-specific functionality in processes such as sleep, memory, and blood circulation (Garcia-Marin et al., 2007). Decades of research and ongoing studies have now established glial cells as dynamic components of brain function, playing active roles in many processes (e.g. metabolism, neurotransmission, immune function, blood-brain barrier signaling). Glial cells are classified in two main groups: microglia and macroglia. Microglia are macrophage-like cells that regulate the inflammatory response of the neural tissue and are located throughout the brain. Macroglia are generally subdivided into four specialized cell types: ependymal cells, Schwann cells, oligodendroglia, and astroglia. Ependymal cells are involved in the production of cerebrospinal fluid (CSF) and are located in thin layers lining cavities in the CNS (i.e. ventricles, spinal cord). Schwann cells and oligodendrocytes are responsible for the formation of myelin, an essential component facilitating action potential propagation down the neuron axon via saltatory conduction. Both Schwann cells and oligodendrocytes modulate axon excitability and are primarily distinguished by their location; Schwann cells are in the peripheral nervous system, and oligodendroglia are in the central nervous system. Historically, astroglia referred to a heterogeneous cell population that expressed glial fibrillary acidic protein

(Gfap) initially termed astrocytes. This classification actually included authentic astrocytes, but also included marginal glia, radial glia in the developing brain, Bergmann cells in the cerebellar cortex, Mueller cells in the retina, pituicytes in the neurohypophysis, and tanycytes in the hypothalamus. Given the diversity of cells expressing Gfap, this Gfap-based definition of astroglia has since been modified, particularly in light of work identifying subpopulations of Gfap-negative astrocytes (Walz and Lang, 1998). Gfap is still often used as a marker of astrocyte identity, and additional proteins are now used to characterize astrocytes (e.g. S100B, Aldh1l1). Astrocytes are located throughout the brain and are known to facilitate intercellular interactions based on their associations with blood vessels, neurons, and other glial cell types. Although this thesis focuses particularly on astrocytes, the types of questions raised and investigated are likely also applicable to other types of glia.

Astrocytes: origins and evolution

The term “astrocyte” (literally “star cell”) was first introduced in 1893 to describe the spindly cell shape (von Lenhosse´k, M. 1893). Like neurons and oligodendrocytes, astrocytes originate from the ectoderm. Contrary to long-held notions that astrocytes arise from homogeneous precursors, recent studies have discovered that astrocytes initially arise from specific sets of precursors that are non-overlapping and produce distinct astrocyte populations (in spinal chord (Rowitch, 2004), in brain (Tsai et al., 2012)). Astrocytes develop in concert with neurons (reviewed in (Gotz and Huttner, 2005)), deriving information from neuronal signals and actively participating in

synaptogenesis (Christopherson et al., 2005) and synapse elimination (Stevens, 2008).

Astrocytes are estimated to be the most abundant glial cell in the brain and can be categorized into subpopulations based on their morphology and location in the brain. Mature astrocytes have been classically categorized into two types based on morphology, fibrous astrocytes and protoplasmic astrocytes. Compared with protoplasmic astrocytes, fibrous astrocytes display fewer processes that are longer but less complex (i.e. branched less frequently and branch at more acute angles). Fibrous astrocytes occur mainly in the white matter of the brain and spinal cord (Garcia-Marin et al., 2007) and are arranged in an overlapping lattice structure. Protoplasmic astrocytes that are typically found in gray matter demonstrate non-overlapping arrangements where each astrocyte appears to occupy a unique domain, an anatomy referred to as "tiling" (Halassa et al., 2007b). Astrocytes are also proliferative and can generate additional astrocytes; indeed, recent studies indicate that astrocytes actively turn over in the brain throughout life (Ge et al., 2012), suggesting cell cycle regulation in astrocytes continues to be important beyond development. Adult astrocytes appear to functionally vary by brain region. For example, astrocytes in the brain stem are more sensitive to extracellular pH changes compared to astrocytes in the cerebral cortex (Kasymov et al., 2013), while astrocytes in the hippocampus are more vulnerable to apoptosis from lack of oxygen and glucose compared to astrocytes in the cortex (Zhao and Flavin, 2000).

Recent findings indicate that astrocytes are more morphologically complex than was

initially appreciated. This evolving understanding is well described in a recent review by Freeman (Freeman, 2010). Most studies of astrocyte morphology used Gfap immunostaining, but this marker only labels primary astrocyte processes that account for only ~15% of the total volume of a typical astrocyte (Bushong et al., 2002) (Figure 1.4). Based on more recent studies, the field now estimates that "a single mature rodent astrocyte can cover a spatial domain in the brain that ranges between 20,000 and 80,000 mm3 (Bushong et al., 2002; Halassa et al., 2007b), wrap multiple neuronal somata (Halassa et al., 2007b), associate with 300 to 600 neuronal dendrites (Halassa et al., 2007b), and contact ~100,000 individual synapses (Bushong et al., 2002; Oberheim et al., 2006). In humans, [each of] these numbers increase dramatically, with a single astrocyte occupying a volume in the brain that is almost 30 times the volume in rodents and associating with ~2,000,000 synapses (Oberheim et al., 2006)" (from (Freeman, 2010)). This astounding complexity places the astrocyte in a unique context to coordinate neuronal signaling in the brain.

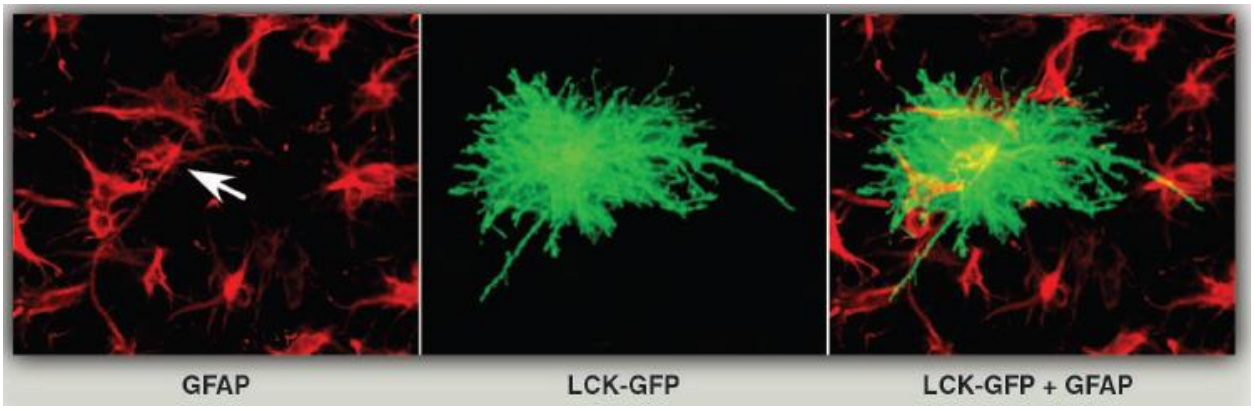

Figure 1.4: Complexity of Astrocyte morphology
"Astrocytes in an organotypic slice preparation labeled with the common marker GFAP (red) overlaid with a single astrocyte transfected with the membrane marker Lck-GFP (green fluorescent protein). Arrow indicates GFAP label of Lck-GFP–marked astrocyte." Note extensive processes identified with membrane marker not observed with Gfap staining [From (Freeman, 2010). Reprinted witth with permission from AAAS.]

Evidence across species points to increasing importance of astrocytes for cognitive function. For example, higher order species have an increased number of astrocytes compared with lower order species; humans have the highest astrocyte:neuron ratio of all (Nedergaard et al., 2003) (Figure 1.5). There is also increasing structural complexity of astrocytes; primates have larger astrocytes with more complex processes compared with rodents (Oberheim et al., 2006). Human astrocytes are also known to have enhanced functionality compared with astrocytes in other animals; for example, human astrocytes propagate calcium signals significantly faster than rodent astrocytes (Oberheim et al., 2006). Remarkably, recent data demonstrates that cross-species transplantation of human astrocytes derived from stem cells results in enhanced memory task performance in recipient mice (Han et al., 2013). Given the possible influence of astrocytes based on their morphology and their increased numbers in the brain across species, understanding astrocyte functions and the molecular mechanisms that regulate them may give insight into both health and disease states of the human brain.

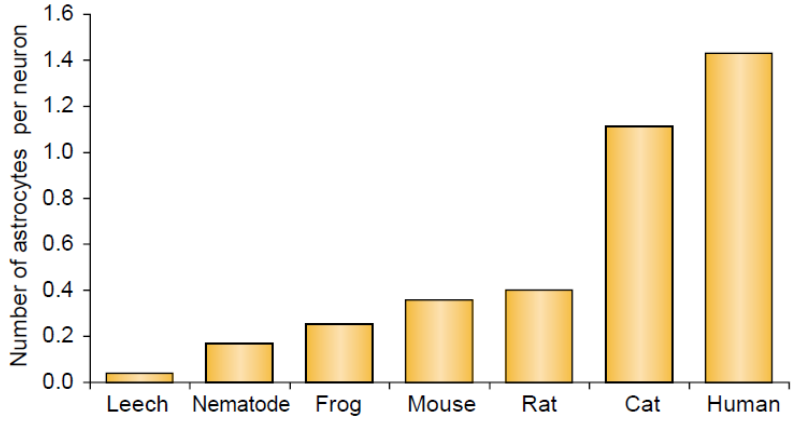

Figure 1.5: Cross-species comparison of astrocytes:neuron ratio. Values for mice, rats, cats, and humans are representative for cortex. With permission from (Nedergaard et al., 2003)

Astrocyte function: overview

What do astrocytes do? In a word, astrocytes are multifunctional; several areas are worth highlighting in forming a conceptual foundation of astrocyte function: (1) neurotransmission regulation, (2) electrical coupling and gap junctions, (3) gliotransmission, (4) metabolism and the blood brain barrier (5) astrogliosis and immune function. These functions are summarized in the diagram below (Figure 1.6) and further described in the subsequent text (Maragakis and Rothstein, 2006).

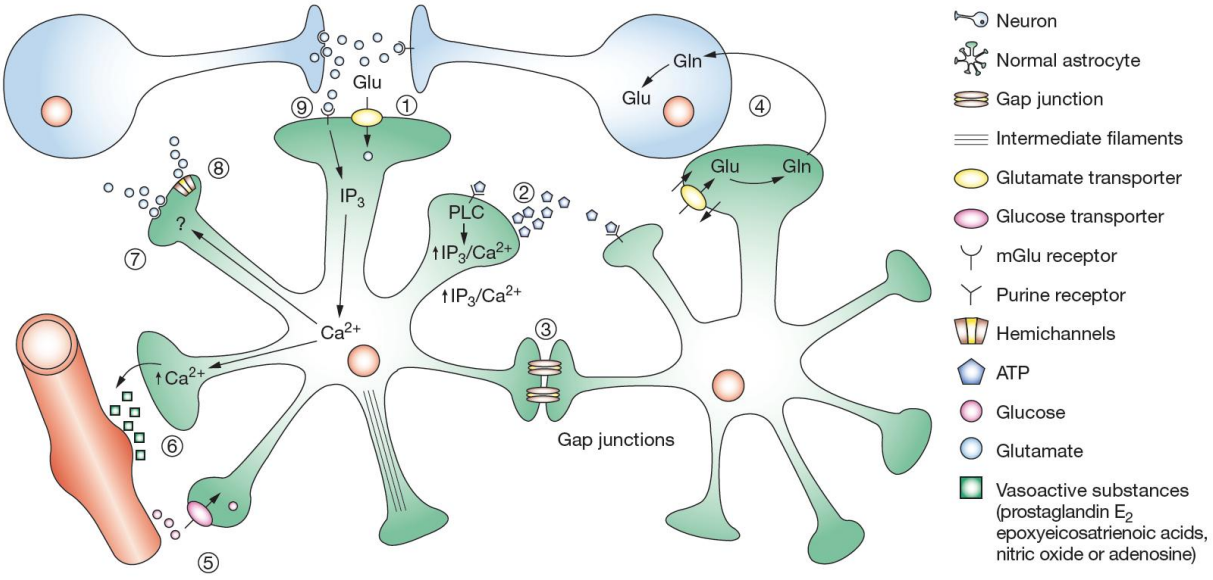

Figure 1.6: Overview of astrocyte functionality
Astrocytes are integral to neurotransmission in multiple ways, including clearance of neurotransmitters such as glutamate from the synapse and cycling neurotransmitters back into neurons (e.g. glutamate-glutamine metabolism and transport) (labeled #1/#4). Astrocytes also release their own gliotransmitters, both onto neurons as well as other astrocytes and glial cells (labeled #2/#8). In contrast to chemical coupling at the synapse, astrocytes have extensive electrical coupling via gap junctions, forming dynamic networks that passage molecules between astrocytes and also from other cells. This passage includes interactions with the vasculature at the blood brain barrier, transporting metabolites to supply energy needs of neurons (labeled #3/#6). With permission from (Maragakis and Rothstein, 2006)

Neurotransmission; glutamate uptake and processing, tripartite synapse

Astrocytes contribute to neurotransmission by clearing glutamate and other neurotransmitters from the synapse. Astrocytes account for >90% of the glutamate removal from the synaptic cleft (Nadler, 2012). The two main glutamate transporters expressed in astrocytes are GLT-1 (i.e. EAAT2, *Slc1a2*) and GLAST (i.e. EAAT1, *Slc1a3*); these transporters also have distinct neuroanatomical expression patterns, with GLAST robustly expressed in the cerebellum and olfactory system while GLT-1 is more ubiquitously expressed throughout the brain (Lehre et al., 1995). Once glutamate is taken up from the synapse, astrocytes can convert glutamate to glutamine via glutamine synthetase, an enzyme uniquely expressed in astrocytes in the brain. This conversion relies on a nitrogen source (e.g. ammonia) from the blood supply. The non-neuroactive glutamine is then released by System N transporters on astrocytes and taken up by System A transporters on neurons (Bröer and Brookes, 2001). These relationships allow for high fidelity of excitatory neurotransmission and an exchange of nitrogen metabolites between neurons and astrocytes.

Astrocytes also actively respond to both excitatory and inhibitory neurotransmitters. In response to glutamatergic neurotransmission, glutamate receptors at the astrocyte cell membrane (e.g. mGluR5) respond to neuronal activity with an elevation of their internal Ca2+ concentration. Increases in astrocyte Ca2+ concentrations trigger the release of compounds (e.g. ATP/adenosine) from glia that in turn induce feedback regulation of neuronal activity and synaptic strength (Araque et al., 1999). Although well-characterized in the literature, this mechanism remains somewhat controversial; recent

results indicate that the expression of glutamate receptors responsible for this phenomenon in astrocytes (mGluR5) changes as a function of development and may not extend to adult tissues (e.g. mGluR5 expressed in young mice but minimal expression in adult mice (Sun et al., 2013)). Astrocyte calcium signaling can also stimulate presynaptic intake of extracellular K+, resulting in neuronal hyperpolarization that can rapidly modulate neuronal network activity (Wang et al., 2012). Astrocytes also express GABA transporters and can affect inhibitory neurotransmission as well, although the specific mechanisms are not as well characterized due in part to the expression of identical GABA transporters on both neurons and glial cells. One study has reported a differential inhibition by GABA antagonists on glial GABA transporters versus neuronal GABA transporters (Schousboe et al., 1979); the physiological relevance of this finding and the exact roles of astrocytes in inhibitory neurotransmission remain to be investigated.

In general, astrocytes express many of the same types of neurotransmitter receptors as neurons, making them responsive to signals via glutamate, adenosine, ATP, GABA, histamine, norepinephrine, and acetylcholine (reviewed in (Verkhratsky, 2009)). This responsiveness and active involvement in neurotransmission has elevated the astrocyte in theoretical discussions of the synapse and given rise to the term “tripartite synapse” (presynaptic neuron, postsynaptic neuron, astrocyte adjacent to synaptic cleft; Figure 1.7). Given these diverse and numerous roles of astrocytes, stimuli that alter the function of astrocytes could thus also affect neurotransmission.

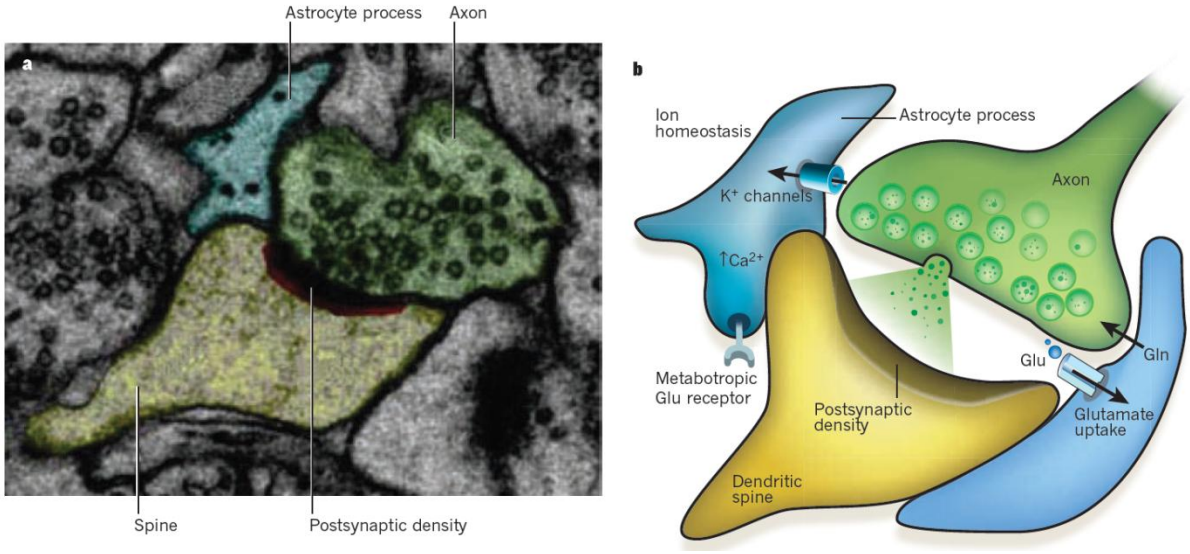

Figure 1.7: Anatomy of tripartite synapse. (A) Electron micrograph of presynaptic neuron, postsynaptic neuron, and adjacent astrocyte. (B) Diagram of tripartite synapse. Presynaptic neuron labeled in green, postsynaptic neuron labeled in yellow, and astrocytes are labeled in blue. With permission from (Eroglu and Barres, 2010).

Gliotransmission: impact of astrocyte-derived signals

Astrocytes actively respond to neurotransmitter release from neurons, but they also can initiate their own signals through exocytosis of molecules. Termed “gliotransmission”, this process enables astrocytes to signal adjacent neurons and glial cells. The mechanisms and functions of gliotransmission are areas of active research. Astrocytes can release several transmitters (reviewed in (Halassa et al., 2007a)), including glutamate, D-serine, and ATP. Astrocytes are known to exert neuroprotective effects on neurons by way of releasing growth factors (e.g. NGF (Gadient et al., 1990), FGF ligands (Albrecht et al., 2002), BDNF (Jean et al., 2008)).

Excitatory neurotransmission can be directly regulated by astrocyte gliotransmission.

Glutamate released by astrocytes can act on presynaptic receptors to enhance synaptic transmission. Astrocytes can also modulate glutamatergic neurotransmission via regulation of D-serine, a coactivator for the NMDA receptor; the enzyme involved in D-serine production is produced exclusively in astrocytes. The concentration of D-serine present at a synapse determines the availability of NMDA receptors to participate in NMDA receptor-mediated plasticity (e.g. LTP/LTD). The importance of this mechanism has been observed in multiple physiological scenarios. For example, astrocytes in the hypothalamus respond to lactation by reducing ensheathment of synapses in the supraoptic nucleus (Montagnese et al., 1990). With less corresponding D-serine available for NMDA receptor activation, this scenario results in decreases in synaptic potentiation in lactating animals relative to virgin animals.

ATP and its degradation product, adenosine, are also major components of astrocyte gliotransmission. Astrocyte-derived ATP can potentiate synaptic output by increasing AMPA receptor surface expression on the postsynaptic membrane (Gordon et al., 2005). Conversely, ATP is hydrolyzed in the extracellular space to adenosine, which can act on presynaptic A1 adenosine receptors to inhibit neurotransmission (Pascual et al., 2005). The inhibitory action of adenosine has also been shown to be activity-dependent; greater presynaptic activity results in increased release of ATP from astrocytes that degrades to adenosine and thus inhibits further presynaptic release, acting in a negative feedback loop. Interestingly, astrocyte-derived adenosine can also act on neighboring synapses, providing a mechanism to selectively enhance a neuronal signal by inhibiting adjacent synapses (Halassa et al., 2009a). By controlling the level

of extracellular adenosine, astrocytes can coordinate the strength of networks of synaptic connections. Adenosine has also been described as an important signal among astrocytes, modulating calcium flux among astrocyte networks (Stout et al., 2002). At the whole organism level, astrocyte adenosine signaling has recently been implicated as an important component of sleep and wakefulness (Halassa et al., 2009b), highlighting the behavioral relevance of astrocyte gliotransmission.

Electrical coupling; gap junctions

In contrast to the chemical coupling of neuronal synapses and gliotransmission, astrocytes also maintain extensive electrical coupling with other astrocytes and glial cells in the brain via gap junctions. Gap junctions are composed of connexin proteins and allow for diffusion of ions and small molecules between cells. Astrocytes primarily express 2 types of connexins, connexin-43 and connexin-30. Connexin proteins form multi-subunit structures called connexons (6 connexins together); these connexons can form either hemichannels on the cell membrane (allow diffusion between cytoplasm and extracellular space) or gap junctions with another cell (two connexons between cells). Cx43 hemichannels in astrocytes have been shown to be important for ATP signaling. Gap junctions operate by passive diffusion, and the functionality varies according to the connexin composition (e.g. differences in permeability and selectivity). Between Cx43 and Cx30, both preferentially pass positively charged ions (e.g. sodium), but Cx30 has a smaller pore size and excludes larger molecules than Cx43 (e.g. lucifer yellow dye) (e.g. (Orthmann-Murphy et al., 2007). The complete character of which ions and molecules pass through gap junctions made up of these

connexins remains largely uncharacterized. Most gap junction signaling in astrocytes has been historically attributed to Cx43 based on its greater overall expression across the brain, but astrocyte connectivity may in reality be more complex based in part on the differential anatomical patterns of Cx43 and Cx30 expression. For example, dye transfer studies using Cx30 knockout mice have attributed 25% of astrocyte-astrocyte coupling to Cx30 in the hippocampus (Gosejacob et al., 2011), but recent results indicate Cx30 accounts for up to 70% of astrocyte-astrocyte coupling in the thalamus (Griemsmann et al., 2012). These anatomical distinctions presumably impact intercellular communication dependent on astrocyte gap junctions. One well-characterized astrocyte phenomenon dependent on gap junctions is calcium waves. As mentioned earlier, local changes in calcium concentrations in astrocytes can mobilize internal stores of calcium that in turn diffuse across gap junctions to other astrocytes; these calcium changes can act as a signal across a network of astrocytes. Calcium waves are rapid and thought to enable astrocytes to tune individual neurons on a network-level (e.g. inhibit or increase firing of adjacent neurons).

The connexin composition of gap junctions also depends on the cells involved in the gap junction. Both Cx43 and Cx30 can form homomeric gap junctions in astrocyte-astrocyte gap junctions but not heteromeric gap junctions (i.e. not Cx43-Cx30). Both astrocyte connexins are also involved in electric coupling with oligodendrocytes, the myelinating glial cell in the brain. Astrocyte-oligodendrocyte gap junctions are formed by specific pairings of connexins (i.e. Cx43-Cx32, Cx30-Cx47). Although the content passed between astrocytes and oligodendrocytes is currently unknown, the presence of

these gap junctions appears critical for myelination; selective knockout of connexins in mice to remove astrocyte-oligodendrocyte coupling results in progressive neurodegeneration and death (Magnotti et al., 2011; Tress et al., 2012).

Metabolic roles: vascular interactions, energy and neurotropic support

Astrocytes are intricately associated with the cerebral vasculature, serving as a bridge at the blood-brain barrier between the endothelial cells and neurons (reviewed in (Bélanger et al., 2011)). The blood-brain barrier regulates the flux of ions, fluid, and metabolites in and out of the brain. Astrocytes contact the vasculature with specialized protrusions known as perivascular endfeet; these protrusions serve as exchange points with the blood supply and contain specific types of transporters, including high concentrations of multiple potassium channels (e.g. Kir4.1) and water channels (i.e. aquaporin 4). In normal brain activity, neurons respond to neurotransmitters by influxing sodium ions and effluxing potassium ions; neuronal activity also relies on glucose metabolism, which generates water as a byproduct. Astrocytes buffer the ionic environment by influxing potassium ions, which then diffuse along the electrochemical gradient in the astrocyte network to locations of lower potassium ion concentration (a process referred to as potassium buffering). Potassium ions can also be deposited in the perivascular space via the K+ channels at astrocytic endfeet (Bay and Butt, 2012). The net ion gain from potassium ion uptake results in osmotic water uptake in astrocytes and subsequent cell swelling. In response to neural activity, the water produced by glucose metabolism in neurons and accumulated in astrocytes by potassium buffering is spatially limited and needs to be removed, and the high density

of AQP4 water channels in perivascular astrocytic endfeet facilitates redistribution and excretion of the water from the brain into the blood. Astrocytes are also able to secrete factors that influence the blood-brain barrier as well as respond to signals from the endothelial cells (Cortese, 2008). Through chemical signaling, astrocytes can modulate blood-brain barrier structure and function, resulting in changes to both physical and transport/permeability properties.

Astrocytes play important roles in supplying neurons with energetic compounds and neurotrophic molecules derived from the blood. Of the glucose entering the brain, studies have estimated that 50% is taken up directly by neurons and the remaining 50% is taken up by glial cells, mainly astrocytes . Although neurons can derive glucose directly from the blood supply, they also receive glycolytic compounds from astrocytes. Evidence for what is known as the “astrocyte-neuron lactate shuttle” hypothesis suggests that astrocytes can supply neurons with lactate, enabling neurons to meet additional, activity-dependent metabolic needs (Hertz, 2004). In concert with excitatory neurotransmission, glutamate uptake in astrocytes stimulates glucose utilization and lactate production. This lactate is then transported to neurons and can be used in the citric acid cycle to produce ATP and thus mobilize cellular energy. While many studies have found evidence for this hypothesis, the concept remains controversial (Chih et al., 2001).

Astrogliosis; neuron protection, inflammatory signaling, and proliferation

Astrocytes are prominent players in response to cellular injury and inflammation in the

brain. CNS insults such as infection, trauma, ischemia and neurodegeneration induce astrocytes to enter an activated state termed “astrogliosis” based on changes in gene expression and morphology. Astrogliosis is associated with the concept of glial scar formation, a structural response to severe CNS injury based on proliferation of astrocytes that overlap to fill and contain the injury site (Fawcett and Asher, 1999). Although astrogliosis occurs in glial scarring, recent work has characterized additional mild and moderate injury responses involving astrocytes that do not involve glial scarring (Sofroniew, 2009), suggesting a continuum of reactive astrocyte action that is highly contextual based on injury type and location in the brain. The general functions of reactive astrocytes can be broadly categorized as (1) protecting neural cells and (2) regulating inflammation in the CNS. Astrocytes can inhibit or limit neuron injury and cell death through a number of signaling mechanisms (e.g. glutathione metabolism vs. oxidative stress (Dringen et al., 2001)). Experimental ablation or attenuation of reactive astrogliosis in transgenic mice results in larger lesion sizes as well as increased demyelination and cell death in response to neural injury (Bush et al., 1999). Recent studies have also demonstrated that astrocytes interact with microglia and can exert both pro-inflammatory and anti-inflammatory effects on neurons and glial cells. Reactive astrocytes can limit the spread of infection and movement or infiltration of inflammatory cells in response to traumatic injury or immune responses (e.g. experimental autoimmune encephalomyelitis, EAE (Voskuhl et al., 2009)), but reactive astrocytes also have been associated with enhancement of inflammation. Many different signaling molecules have been associated with initiating or modulating reactive astrogliosis, including various cytokines (e.g. interleukins), neurotransmitters (e.g.

glutamate), and other molecules (e.g. ATP, nitric oxide (NO), reactive oxygen species (ROS), ammonia, amyloid-beta) (Sofroniew, 2009). Glial scarring results from induced proliferation of astrocytes, but recent data indicates astrocytes can proliferate under basal conditions as well (Ge et al., 2012). Regulation of astrocyte cell cycle is thus of interest for understanding mechanisms of basal astrocyte cell division and glial scarring.

Additional functionality: signaling cascades common to multiple CNS cell types

In addition to the aforementioned astrocyte-specific functions and many others, astrocytes also express signaling molecules and receptors found generically in CNS cell types (e.g. growth factors, GPCRs, transcription factors). Although these molecules are not often associated with astrocytes, if considered at all, they are presumed to have similar regulation and function in astrocytes as that observed in other cell types. This presumption may or may not be correct; certain pathways may display unique functions based on cellular context, and additional studies will need to assess whether such presumptions are true regardless of cell type. At this point, how different or similar signals common to multiple CNS cell types function in astrocytes is not well characterized. Further investigations of both generic as well as cell-specific pathways in astrocytes would likely enable better understanding of the complex intercellular nature of the brain.

Investigating astrocytes: experimental approaches and the astrocyte transcriptome

Research studies of astrocytes and particularly astrocyte functions have made extensive use of primary astrocyte cell culture models. The benefits of these models

include selective isolation of the cell of interest, extensive control of experimental variables relative to the intact brain, and relatively convenient and scalable sample generation. These models have contributed numerous findings on astrocyte function (e.g. (Khelil et al., 1990; Ruzicka et al., 1995; McClennen and Seasholtz, 1999)) and continue to be refined (Foo et al., 2011). Primary astrocytes culture methods alone cannot, however, address how astrocytes interact with other cell types *in vivo*. Questions about astrocyte function *in vivo* are being informed by knowledge from astrocyte culture studies as well as novel transgenic mouse models (e.g. Gfap-GFP, Aldh1l1-GFP) (Barres, 2008).

Given the relative lack of functional knowledge of astrocytes compared to neurons and other brain cell types, a current limitation of many cell culture and animal studies is that they require prior knowledge of an astrocyte function to investigate. One way in which the field is seeking to address this shortcoming is by further defining astrocytes by their gene expression patterns relative to other cell types in the brain. The logic of these studies is based on the notion that a cell and corresponding cellular function are defined in part by the set of mRNAs and proteins that are expressed in that cell. Knowing which mRNAs are expressed can implicate the presence of cell-independent mechanisms (e.g. common across cell types) as well as cell-dependent mechanisms (e.g. specific functionality in that cell type). Gene expression profiling experiments by the Barres lab using meticulous cell type isolation techniques on intact brain tissue have produced convincing evidence that there are indeed cell-type-specific mRNA expression patterns in the CNS between astrocyte, oligodendrocytes, and neurons (Cahoy et al.,

2008). The existence of specific gene expression profiles is somewhat predicted given the long history of brain cell type markers, but the scale of gene expression enrichment in terms of number of mRNAs (e.g. thousands enriched per cell type) is large enough to permit many cellular differences. These recent findings also enable more thorough investigations into cell-type-specific transcriptional regulation and functional consequence, which may be particularly relevant in cellularly diverse tissues like the brain.

Section 3: Clinical connections – stress hormones, astrocytes, and depression

Developing a deeper understanding of both HPA axis mechanisms and astrocyte functionality may have clinical relevance in the realm of mental health. Alterations of glucocorticoid signaling and astrocytes have each been individually associated with depression (i.e. major depressive disorder, MDD); how glucocorticoid signaling and astrocytes influence each other is currently not well known, an area this thesis attempts to address. The following section discusses the impact of depression on individuals and society, the association of HPA axis alterations with depression, emerging data indicating changes in astrocytes in depression, and the precedence for glucocorticoid regulation in astrocytes.

Depression; definition and challenges

"That's the thing about depression: A human being can survive almost anything, as long as she sees the end in sight. But depression is so insidious, and it compounds daily, that it's impossible to ever see the end. The fog is like a cage without a key."

- Elizabeth Wurtzel, Prozac Nation (Wurtzel, 1994)

Definition of major depressive disorder (MDD)

Depression (major depressive disorder, unipolar depression) is clinically defined by the continuous presence of a collection of symptoms affecting mood for at least 2 weeks. According to the DSM-IV criterion, a patient should have at least five of the following persistent symptoms listed below to be diagnosed with depression, including both depressed mood and anhedonia.

(1) Depressed mood – most of the day, nearly every day, as indicated by either subjective report (e.g., feels sad or empty) or observation made by others (e.g., appears tearful).

(2) Anhedonia - markedly diminished interest or pleasure in all, or almost all, activities most of the day (as indicated by either subjective account or observation made by others)

(3) Changes in appetite/weight – significant weight loss when not dieting or weight gain (e.g., a change of more than 5% of body weight in a month), or decrease or increase in appetite.

(4) Changes in sleep – insomnia or hypersomnia

(5) Changes in movement – psychomotor agitation or retardation (observable by others, not merely subjective feelings of restlessness or being slowed down)

(6) Fatigue or loss of energy

(7) Feelings of worthlessness or excessive or inappropriate guilt (may be delusional, not merely self-reproach or guilt about being sick)

(8) Diminished ability to think or concentrate, or indecisiveness (either by subjective account or as observed by others)

(9) Recurrent thoughts of death (not just fear of dying), recurrent suicidal ideation without a specific plan, or a suicide attempt or a specific plan for committing suicide

Given that the definition has a temporal component, depression is often described in terms of "episodes", a period of time where symptoms are present but potentially not permanent. Indeed, the goal of treatment for depressed patients is to relieve these symptoms so as to end an episode. Episodes can be of varying duration on the order of weeks to years, and patients can experience multiple episodes over their lifetime.

Depression; prevalence, comorbidity

Depression is a highly prevalent disease in present society and appears to be increasing in occurrence. Public health studies of depression estimate that 8-10% of Americans experience a depressive episode in a given year, and up to 15-20% of the population experiences a depressive episode in their lifetime (Kramer, 2006). Depression demonstrates a strong genetic component as well. Depression has a heritability of 35-40%, the same level of inheritance as high cholesterol, high blood

pressure, and diabetes (Kramer, 2006). Depression also has the unfortunate distinction of often affecting people early in life. Fifty percent of children and adolescents and 20 percent of adults report some symptoms of depression, and many depressed children will experience depression again as adults (Leahy, 2013). Depression often manifests in a vicious feed-forward cycle; odds of additional episodes are likely and increase incrementally with every episode experienced. 50%-67% of patients who have ever been depressed will be depressed in a given year in the future. After a second episode, the two-year recurrence rate is 75%; after a 5th episode, the 6-month recurrence rate is 30% (Kramer, 2006). The diagnosis rates of depression are increasing over time (Kessler and Walters, 1998; Kessler et al., 2001). Children born after 1960 are significantly more likely to suffer from depression in childhood or adolescence than kids born before 1960 (Klerman and Weissman, 1989). One interpretation of these data is that the prevalence of depression is increasing, although such an increase could also be due to improvements in diagnosing depression and simply identifying more cases over time as well. Together, these data paint a societal picture of depression as a major public health concern, a disease that affects many people of all ages and whose incidence seems to be on the rise.

If depression was an isolated medical condition, it would still be a major public health problem; unfortunately, depression often influences or occurs with other diseases and behaviors. Comorbidity of depression and other diseases suggests a compounding effect of vulnerability and personal cost. Depression is known to weaken the immune system, making the body more susceptible to other medical illnesses. Many illnesses

have an increased prevalence associated with depression (e.g. cardiovascular disease (Steffens et al., 1999), diabetes (Anderson et al., 2001), rheumatoid arthritis (Ang et al., 2005), migraine (Kramer, 2006)). The combination of depression and these other conditions is often much more disabling that disease alone. These instances of comorbid depression are not included in depression calculations, meaning general statistical estimations likely underestimate the total number of people dealing with depression. Depression also is a risk factor for early death, particularly among older individuals. Depression increases the likelihood of death by 40% for people over age 65, placing depression as a risk factor for death of the elderly at the same level as high blood pressure, smoking, stroke and congestive heart failure (Kramer, 2006).

Depression also confers behavioral vulnerability as well. The proportion of incarcerated individuals with mental health diagnoses is much higher than the general population. Prisons and jail houses have three times as many people with mental health disorders than hospitals (Kramer, 2006). Depressed individuals are five times more likely to abuse drugs (Leahy, 2013). Perhaps most tragic are the intimate correlations between depression and suicide; people who are depressed are 30 times more likely to kill themselves than people who are not depressed (Paykel, 1992). Based on these data, depression can also be described as a significant factor for addition health problems and risky behavior.

Depression: economic costs = national crisis?

By any economic metric, the costs of depression to society are tremendous and growing

(Leahy, 2013). Eighty percent of people in a depressive episode are impaired in their daily functioning (Pratt and Brody, 2008). Depression is the leading cause of medical disability for people ages 14 to 44 (Stewart et al., 2003). Among individuals in a depressive episode, fifty percent of the loss of work productivity is due to absenteeism (repeated absence at work) and short-term disability (Kessler et al., 1999). Depression impairs productivity even when people are at work, a phenomenon termed "presenteeism" (i.e. substandard performance, hindered ability to concentrate, decreased efficiency). Individuals with depression are seven times more likely to be unemployed than people without depression (Lerner et al., 2004). Conservative estimates put the annual cost of depression (lost productivity and increased medical expenses) to individuals in the US at $83 billion each year (Greenberg et al., 2003). These trends are seen worldwide across many countries and diverse cultural backgrounds. An extensive study by the World Health Organization, World Bank, and Harvard School of Public Health found that MDD accounted for 20% of all disability-adjusted life years (DALYs) lost by women in developed countries (>3x next illness). In terms of chronic disease of midlife, depression was already the most burdensome in the world by 1990. Predictions from that study estimate that depression was the #2 most disabling disease in world and is predicted to be the #1 most disabling disease in developing regions by 2020 (Kramer, 2006).

While the current societal impact of depression is striking, the true cost of depression on the lives of individuals is only now emerging from longitudinal data. A recent large-scale study following 5000 American families over 40 years found that children or adults who

suffer from depression on average have lower incomes (decreased potential 20% annually), lower educational attainment, and work fewer days each year (average 7 weeks less). The subsequent lifetime loss of income for each family who has a depressed family member is estimated to be $300,000 (Smith and Smith, 2010). Strictly in terms of individual income, this economic loss of 2.1 trillion dollars for all people with depression, an amount that does not include the costs associated with medical care (Smith and Smith, 2010).

Current treatments for depression; antidepressant mechanisms and the need for better treatments

The high personal and economic costs of depression make effective medical treatment critical. Fortunately, depression in many cases can be managed, and there are a number of established therapeutic interventions. Treatment strategies typically incorporate multiple interventions, often including both psychotherapy (e.g. cognitive-behavioral therapy, CBT) and antidepressant medication. The combination of these two approaches is reported to have higher success for severe depression than each individually (Thase et al., 1997). Modern antidepressants trace their beginnings to two serendipitous discoveries in the 1950s (Pletscher, 1991). Patients treated with an experimental drug for tuberculosis, iproniazid, were unexpectedly found to demonstrate an improved mood, leading to its use as a mood enhancer. Subsequent pharmacological studies have characterized Iproniazid as a monoamine oxidase inhibitor (MAO-I). A separate line of clinical research on antihistamines yielded drugs that also had mood improving outcomes such as imipramine, subsequently classified as

a tricyclic antidepressant. Mechanistically, these types of compounds target monoamine metabolism (e.g. MAO-Is) and reuptake (e.g. tricyclics), resulting in increased levels of serotonin, dopamine, and norepinephrine at their respective synapses. The impact of these drugs was the foundation of the monoamine hypothesis of depression (first mentioned by Schildkraut in 1965), which posits that hypoactivity of monoamine neurotransmission underlies depression pathology. This hypothesis has been highly influential in the field of depression research since its inception, resulting in the creation of more selective medications targeting monoamine neurotransmission that are the primary initial treatment options to this day (e.g. selective serotonin reuptake inhibitors (SSRIs)). Certainly, manipulations of the monoamine system alter mood and depressive states; however, observations of the treatment of patients with depression suggest that the monoamine hypothesis may be at best incomplete. First, monoamine-based antidepressants demonstrate a well-established lag time of efficacy; new patients are often told that the drugs take a few weeks to a month to take effect. Given that the modulation of monoamine neurotransmission occurs rapidly upon taking the drugs, this time lag in mood modulation suggests the impact of these antidepressants upon mood may be a secondary effect; the treatment may be altering circuitry that eventually adapts but is not directly targeting the source of pathology. Second, not all patients respond to monoamine-based antidepressants; in fact, in the largest open-label study of antidepressants, less than a third of subjects achieved remission (Trivedi et al., 2006). 40-50% of depressed patients do not respond to an initial treatment plan with these drugs (ongoing non-responsiveness termed “treatment-resistant” depression), indicating that other molecular pathways are responsible for depression. Additional

hypotheses are thus needed to identify and characterize the underlying cellular and molecular defects in depression. Such hypotheses should be based on clinical observations from patients with depression, preferably from biological measures.

Associations between the HPA axis, glucocorticoids, and depression

Although there are many different molecular mechanisms that have been implicated in the pathology of depression, almost all of them relate in some way to stress and specifically alterations in glucocorticoid signaling. From clinical literature, depressive episodes often occur due to a highly stressful event or period, referred to as a "trigger." One of the most commonly described biological symptoms of depression is hypercortisolemia, an elevated baseline concentration of the glucocorticoid cortisol in the blood and presumably the brain (Figure 1.8, (Wong et al., 2000)). Hence, enhanced understanding of glucocorticoid action has the potential to inform our understanding of the cellular and molecular basis of depression.

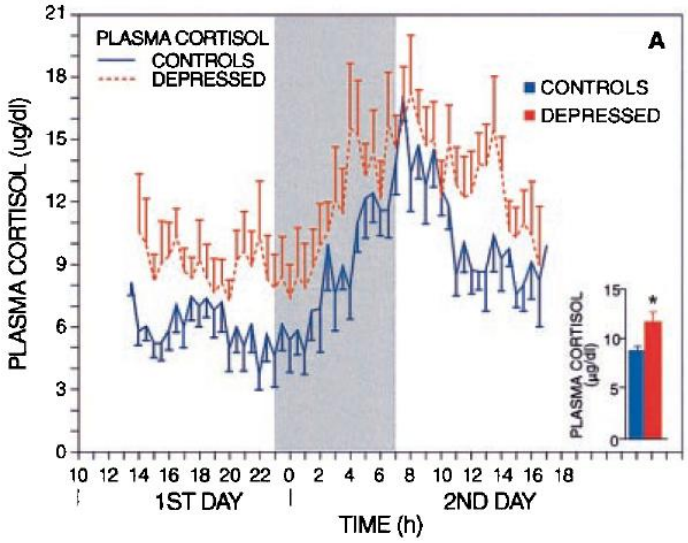

Figure 1.8: Patients with depression have elevated plasma cortisol levels. Representative data comparing blood plasma cortisol levels between patients with depression (red) and non-depressed controls (blue) across the daily cycle. Used with permission from (Wong et al., 2000).

Molecular impacts of glucocorticoids in pathology; from adaptive to harmful

Although the HPA axis (see Section 1 on stress and the HPA axis) is intended for adaptation to stressors, glucocorticoid action can change from protective to harmful under conditions of extreme or chronic stress. Inadequate or excessive and prolonged HPA axis stimulation (which leads to persistently elevated glucocorticoids) may make the cost of reinstating homeostasis too high, a condition that is termed ‘‘allostatic load’’ (McEwen, 2003). A number of hypotheses involving glucocorticoid receptors have been put forth to explain how this phenomenon could occur; these hypotheses are relevant to normal physiology (e.g. aging) and pathology (e.g. depression) (reviewed in (Oitzl et al., 2010)). In the glucocorticoid cascade hypothesis, chronic stress induces a cycle of excess glucocorticoid release and a compensatory downregulation of glucocorticoid receptors in the brain; the ultimate result of this feed-forward mechanism is cellular damage to the brain (e.g. decreased hippocampal volume, decreased dendritic arborization in neurons). In the balance hypothesis, an imbalance in the MR:GR ratio in specific brain regions (e.g. limbic system) is thought to alter signaling in neural circuits, resulting in alterations in behavior and HPA axis signaling. The MR:GR ratio can be affected by behavioral, genetic, and epigenetic factors. This hypothesis is partially supported by postmortem findings of altered MR:GR gene expression ratios in human depression (Medina et al., 2012). Results from experiments using transgenic mice associate changes in MR and GR levels with increased depressive-like behaviors. Overexpression of GR in forebrain neurons in mice results in increased depressive-like behaviors and emotional lability (Wei et al., 2004), while overexpression of MR in forebrain neurons in mice results in decreased anxiety-like behavior and decreased GR

expression in the hippocampus (Rozeboom et al., 2007). One interpretation of these data is that increasing steroid receptor availability may increase the magnitude of glucocorticoid-mediated regulation; alternatively, increasing one receptor may disrupt signaling to the other receptor. Additional hypotheses focus on behavioral and environmental factors, such as epigenetic changes in stress responsiveness due to early life experience or stress (maternal mediation hypothesis) and contextual influences due to differential response to environmental changes (e.g. environment in development vs. adulthood) (predictive adaptation hypothesis). Behavioral and environmental factors that modify stress signaling likely impact glucocorticoid signaling as well. These hypotheses are not mutually exclusive, and a combination of them likely occurs in conditions of chronic stress and disease involving excesses of glucocorticoids.

Stress signaling and animal models of depression

Extensive work on HPA axis alterations in relation to depression has been carried out using animal models of depression. Most of these animal models cause a persistent activation of the mammalian stress response, often measured by an increase in glucocorticoids concentration in the blood plasma. Originally created to experimentally test hypotheses concerning the basis of depressive illness, animal models of depression were defined by a behavior that could be modified and subsequently normalized by treatment with antidepressants (i.e. monoamine-based medications); these models are not “depression” in an animal *per se* but exhibit biological underpinnings parallel to human depression. This crude definition has enabled quantifiable study of antidepressant effects and related molecular pathways, although

understanding the specific mechanisms of antidepressant action remains incomplete. Behavioral tests that fit this definition include (corresponding depressive-like behavior); open field test (decreased time in center), open arm maze (decreased time in open arms), forced swim test (decreased latency to floating), sucrose preference test (decreased preference for sugar vs. saline), and spatial memory tests such as the Morris water maze (decreased performance) (reviewed in (Willner, 1997; Cryan and Holmes, 2005)). The models with the highest experimental validity are the chronic unpredictable stress model (animal exposed to various stressors over period of time) and the social defeat model (animal repeatedly exposed to dominant stranger animal). Both of these models are based on stress-inducing protocols in which rodents exhibit hypercortisolemia that can be normalized by antidepressant treatment. Extensive reviews on the validity of animal models of depression have been done by Willner (Willner, 1984, 1997). In addition, chronic glucocorticoid administration also results in increased depressive-like behaviors (Murray et al., 2008), suggesting that excess glucocorticoids alone can lead to changes in these behaviors. While the focus of this thesis is on molecular mechanisms as opposed to behavioral experiments, findings in animal models are ongoing inspirations of molecular investigations and demonstrate behavioral relevance.

HPA axis and depression; use in diagnostics and treatments

The association between stress hormones and depression led to clinical efforts to use glucocorticoid signaling as a diagnostic test to aid in depression diagnosis. The dexamethasone suppression test (DST) consists of treatment with a low dosage of

dexamethasone and subsequent measurements of plasma levels of ACTH and cortisol (Carroll, 1982). Under basal conditions, dexamethasone interaction with GR will decrease the levels of both ACTH and cortisol. If the levels of ACTH and cortisol are not significantly decreased by dexamethasone treatment, then GR function is assumed to be desensitized (presumably due to persistently elevated cortisol levels in depression). Subsequent versions of the DST incorporated a CRH co-treatment that increased test accuracy as a diagnostic for depression. Initial studies that made use of the DST as a diagnostic tool with depression were initially promising but were not consistently accurate, although the test is still used in research studies of HPA axis function. HPA axis components have also been used as therapeutic targets in depression. For example, GR antagonists have been used to treat psychotic depression (Belanoff et al., 2001). Since CRH stimulation initiates HPA axis signaling, more recent studies have found CRH receptor antagonists to have antidepressant properties that are under further investigation for therapeutic potential (Holsboer and Ising, 2008). Although the HPA axis is not the standard target of developing diagnostics and therapies in depression at this time, the integral relationship of stress, glucocorticoids, and depression make the HPA axis an important consideration for all such efforts.

Additional hypotheses regarding cellular and molecular basis of depression

Beyond the empirical evidence for altered stress signaling in depression, there are multiple hypotheses about the origins of depressive illness (e.g. depression). Some

focus on specific chemical imbalances while others focus on the interactions between psychological and biological factors. Depression is a complex disorder; to date, no single hypothesis has yielded complete answers to the molecular pathology, but each has provided additional information about how depression affects the brain. Given the limitations of the monoamine hypothesis, additional hypotheses of how depression occurs in the brain have emerged based on evidence from human postmortem brain studies and neuroimaging data.

Neurotrophic hypothesis of depression

One such hypothesis is the well-developed neurotrophic hypothesis, which states that depression results in part from dysregulation of neurotrophins and growth factors. These molecules typically act by binding to receptor tyrosine kinases and activating a range of intracellular signaling cascades. Multiple lines of evidence indicate that alteration of brain-derived neurotrophic factor (BDNF) signaling occurs in depression (reviewed in (Duman, 2004; Dwivedi, 2009)). BDNF protein levels are decreased in the blood serum and platelets in patients with depression relative to non-depressed control subjects (Karege et al., 2002). Postmortem studies of BDNF in the brains of patients with depression have been limited, but patients with depression that were treated with antidepressants had higher hippocampal expression of BDNF protein compared with patients not treated with antidepressants (Chen et al., 2001). Animal models of depression display decreases in BDNF mRNA levels that can be normalized by antidepressant treatment (Angelucci et al., 2005), and infusion of BDNF protein itself into the brain has demonstrated antidepressant properties (Siuciak et al., 1997). BDNF

is thought to act in part by influencing neurogenesis in the hippocampus; decreased neurogenesis is observed in animal models of depression, and treatment with antidepressants can normalize neurogenesis (Malberg et al., 2000), making neurogenesis and cell proliferation an active area of current depression research. However, BDNF synthesis is also required for the rapid antidepressant action of ketamine (Autry et al., 2011), indicating that its antidepressant properties also involve acute actions in addition to influencing neurogenesis. Other growth factors that have also been implicated in mechanisms relevant to depression, including Igf-I (Hoshaw et al., 2005), VEGF (Warner-Schmidt and Duman, 2007), and FGF2 (Turner et al., 2008)); all of these molecules have been shown to regulate neurogenesis, demonstrate antidepressant properties in animal models of depression, and be responsive to antidepressant treatment. Multiple aspects of FGF signaling (ligands and receptors) have also been found to be disregulated in depression based upon gene expression studies using human postmortem brain samples (Evans et al., 2004).

Glutamate hypothesis of depression

Advances in clinical technology have given rise to intriguing hypotheses involving imbalances of neuronal circuits and glutamate neurotransmission in depression. Neuroimaging studies have identified specific brain regions that exhibit increased (e.g. subgenual cingulated cortex, amygdala) or decreased (e.g. dorsal lateral prefrontal cortex, striatal regions) metabolism in subjects with depression (Krishnan and Nestler, 2008). Subsequent clinical studies found that deep brain stimulation (phasic increases in neuronal firing) in regions associated with decreased activity in depression

produces antidepressant-like behavioral responses (Mayberg et al., 2005). Recent studies have extended this human work concerning the neural circuitry linked to depression to animal models that exploit optogenetic tools to modulate specific neurons (Covington et al., 2010). Expanding upon these animal studies, alterations of excitatory and inhibitory circuits in the prefrontal cortex of rodents has been found to modulate depressive-like behaviors, suggesting that the balance of neurotransmitter systems may be a circuit-level factor in depression. Since glutamate is the predominant neurotransmitter in the brain (estimated 80% of neurons in neocortex comprising 85% of synapses) and is often the functional output of monoamine circuits, glutamatergic signaling likely plays an important role in depression. Indeed, evidence for a glutamate hypothesis of depression (also termed neuroplasticity hypothesis (Pittenger and Duman, 2007)) has been further supported by recent studies revealing antidepressant effects by indirectly or directly targeting glutamatergic signaling (e.g. antidepressant action of riluzole that modulates glutamate release and facilitates glutamate uptake (Banasr et al., 2008), rapid antidepressant action of NMDA-antagonist ketamine (Berman et al., 2000)). Clinical studies have also reported alterations in glutamate metabolites in the brains of individuals with depression (Auer et al., 2000; Hasler et al., 2007). Based on these data, pathways that modulate glutamate neurotransmission could thus be involved in the biology of depression.

Associations between astrocytes and depression

Astrocyte pathology and depression; human postmortem studies

In terms of classification as a disease condition, depression was originally viewed as a neuron-based disorder. This "neuron-centric" view of depression is at least partially supported by the known actions of commonly used antidepressants that modulate brain levels of the neurotransmitters (e.g. SSRIs and serotonin). However, antidepressants target proteins expressed by multiple brain cell types (e.g. astrocytes express NMDA receptors (Conti et al., 1998) and serotonin receptors (Inazu et al., 2001)), and specific mechanisms and cell types involved in antidepressant action remain largely unknown. A series of studies in the late 1990s and early 2000s made unexpected associations between depression and changes in cell density of specific cell types in the brain. Rajkowska and colleagues used cell counting techniques to investigate how depression impacted cell density and morphology across various brain regions in mood disorders. Based on previous models, they suspected that depression would lead to cell death and decreased cell density in brain regions associated with depression. They found that there were indeed decreases in cell density in areas such as the prefrontal cortex (Rajkowska et al., 1999) and hippocampus (Stockmeier et al., 2004), but their findings were surprising in terms of the cell type; the decreases in cell density were associated with morphologies consistent with both neurons and glial cells (Figure 1.9A). These findings inspired biochemical experiments that revealed specific depression-linked decreases in astrocyte density based on decreased protein

expression of the astrocyte marker Gfap (Miguel-Hidalgo et al., 2000) (Figure 1.9B). In addition, recent microarray gene expression studies using human brain samples have also associated decreased astrocyte gene expression with depression in the locus coeruleus, including markers associated with astrocytes (i.e. S100B, Gfap), gap junctions (i.e. Gja1, Gjb6), and membrane channels (i.e. Aqp4) (Bernard et al., 2010). Together, these findings suggest that astrocyte pathology is associated with depression; however, whether these changes were causative of or a response to depression could not be determined from postmortem studies.

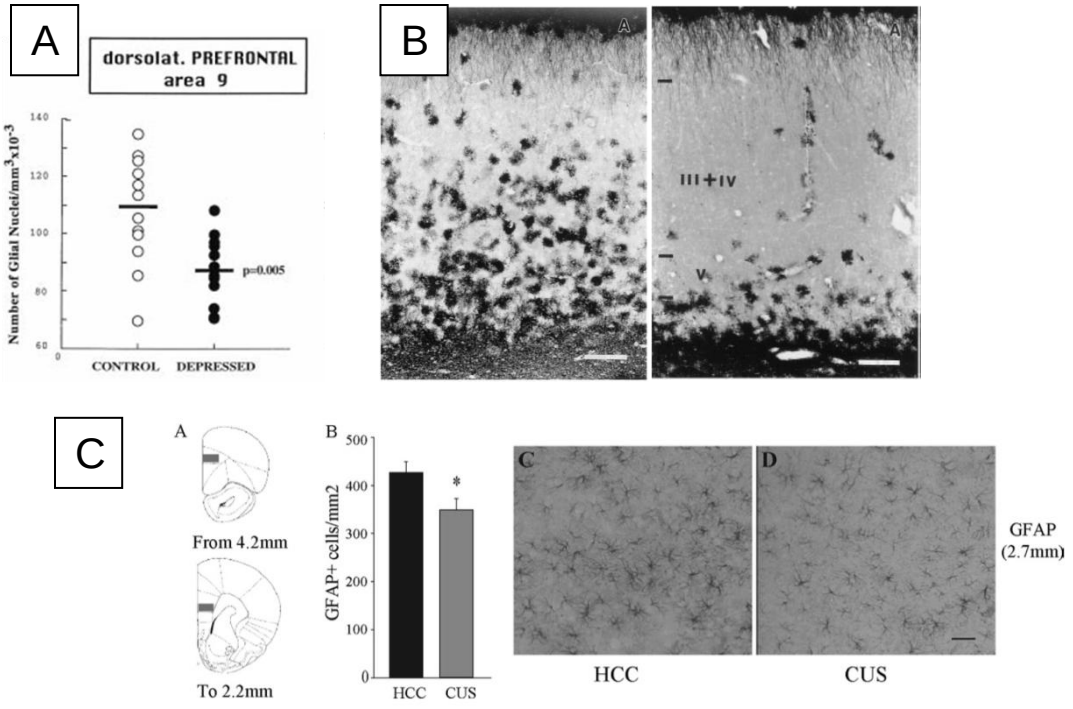

Figure 1.9: Astrocyte density changes associated with depression
(A) Representative Nissl stain cell counting data showing decrease in cell morphology associated with glia in prefrontal cortex (Brodman area 9, with permission from (Rajkowska et al., 1999)).
(B) Sample Gfap immunocytochemistry results in dorsolateral prefrontal cortex between non-depressed control (left panel) and MDD (right panel) in adults. Note significant decrease in the number of Gfap-positive astrocytes in MDD sample (Derived and used with permission from (Miguel-Hidalgo et al., 2000)).
(C) Decreases in Gfap-positive astrocytes in prefrontal cortex of mice associated with chronic unpredictable stress paradigm (CUS), an animal model of depression, compared with home cage control (HCC) (With permission from (Banasr and Duman, 2008)).

Investigating astrocytes in animal models of depression

Recent studies have begun to assess astrocyte pathology associated with depression using animal models of depression, and the initial evidence appears to suggest that astrocyte alteration can indeed be causative or at least a contributing factor to depression biology. A study by Banasr and Duman found that rats put through a chronic unpredictable stress paradigm showed decreases in astrocyte cell marker expression (GFAP) in the prefrontal cortex (Banasr and Duman, 2008) (Figure 1.9C), results parallel to the human findings. In a separate group of naïve rats, injection of an astrocyte toxin into the prefrontal cortex resulted in an increase in depressive-like behaviors; this effect was selective to astrocyte alteration in that injection of a neurotoxin did not result in any behavioral change (Banasr and Duman, 2008). Other studies have also implicated astrocyte alterations as causative components of depressive-like behaviors. For example, injection of a gap junction blocker into the prefrontal cortex of rats resulted in increased depressive-like behaviors; since astrocytes are the primary cells involved in gap junction communication in the brain, the results were attributed to astrocyte dysfunction (Sun et al., 2011). Taken together, these studies implicate astrocytes as important players in the initiation of depressive-like behaviors and imply further studies of astrocytes and depression biology may be critical to understanding depression.

Relationship between stress hormones and astrocytes; connections

Given the independent association of stress signaling and astrocytes with depression,

the interaction of glucocorticoids and astrocytes may be relevant to understanding depression biology. More generally, astrocytes may also play a role in modulating stress signaling via their response to glucocorticoids. A limited literature on glucocorticoid regulation in astrocytes indicates stress hormones do have specific effects on this cell type.

Astrocytes are known to express both GR and MR (Bohn et al., 1991) and thus are sensitive to glucocorticoid regulation. In terms of transcriptional regulation, glucocorticoids have been shown to regulate mRNAs in astrocytes on a gene by gene basis. Multiple mRNAs associated with astrocytes (e.g. GFAP, Glul, Slc1a2) have been shown to be regulated by glucocorticoids in cultured astrocytes (Rozovsky et al., 1995; Zschocke et al., 2005) and in the intact brain (Patel et al., 1983; O'Callaghan et al., 1989). A number of other mRNAs have been shown to be glucocorticoid-sensitive in cultured astrocytes, usually as an extension of studies of glucocorticoid regulation in neuronal cell cultures or other cell types (specified in Chapter II). How alteration of these mRNAs in astrocytes impacts brain physiology is currently not well characterized. In terms of functional impact, altering the expression of these genes would predictably alter cytoskeletal structure (Gfap) and glutamate-glutamine metabolism (Glul). Indeed, GFAP hypertrophy has been associated with glucocorticoid action (O'Callaghan et al., 1989). Glucocorticoids have also been shown to decrease glutamate uptake in cultured astrocytes (Virgin et al., 2006). Most of the functional impact of glucocorticoids on astrocytes has been demonstrated *in vitro*; the modulation of astrocytes *in vivo* remains to be investigated.

What we do know is that (1) glucocorticoids regulate specific mRNAs in astrocytes and (2) glucocorticoids alter specific astrocyte functions. What we do not know is the total range of glucocorticoid impact on the astrocyte transcriptome and subsequent functional consequences, both *in vitro* and especially *in vivo*.

Section 4: Hypothesis and experimental design

Given the established link between the HPA axis and depression and the emerging evidence for astrocyte pathology and depression, further understanding glucocorticoid action in astrocytes would inform our understanding of HPA axis signaling in the brain by cell type and may inform our understanding of depression biology. The goal of this thesis is to test the following hypothesis:

Glucocorticoids regulate specific mRNAs in astrocytes, including certain mRNAs regulated by glucocorticoids in other cellular contexts. A portion of mRNAs regulated by glucocorticoids in astrocytes are uniquely regulated in astrocytes compared to other cell types in the brain.

To test these hypotheses, we have employed the following strategies:

*Establish global impact of glucocorticoid regulation on astrocyte transcriptome *in vitro* using primary astrocyte cultures

*Test physiological significance of *in vitro* findings and neuroanatomical questions in 2 mouse models of elevated glucocorticoid exposure (acute and chronic).

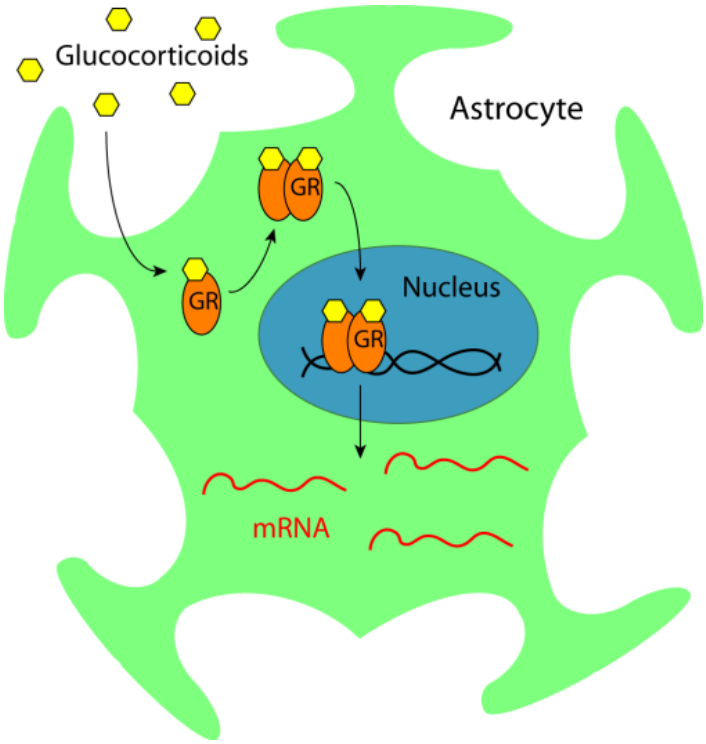

Figure 1.10: Visual representation of thesis hypothesis
Glucocorticoids regulate specific mRNAs in astrocytes, including certain mRNAs regulated by glucocorticoids in other cellular contexts. A portion of mRNAs regulated by glucocorticoids in astrocytes are uniquely regulated in astrocytes compared to other cell types in the brain.

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Chapter II

Glucocorticoid treatment of astrocytes results in temporally dynamic transcriptome regulation and astrocyte-enriched mRNA changes *in vitro*

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ABSTRACT

While general effects of glucocorticoids are well-established, the specific cellular mechanisms by which these hormones exert tissue-dependent effects continue to be elaborated. Diseases that demonstrate altered glucocorticoid signaling have been associated with alterations in astrocytes, yet relatively little is known about the effects of glucocorticoids upon this cell type. We have analyzed mRNA expression patterns following glucocorticoid treatment of mouse primary astrocyte cultures. Microarray

analysis of cortical astrocyte cultures treated with dexamethasone over an 8-point, 24-hour time course identified 854 unique genes with ≥two-fold change in mRNA expression at one or more time points. Clustering analysis associated subsets of these mRNA expression changes with gene ontology categories known to be impacted by glucocorticoids. Numerous mRNAs regulated by dexamethasone were also regulated by the natural ligand corticosterone; all of the mRNAs regulated ≥two-fold by corticosterone were substantially attenuated by co-treatment with the glucocorticoid receptor antagonist RU486. Of the mRNAs demonstrating ≥two-fold expression change in response to both glucocorticoids, 33 mRNAs were previously associated with glucocorticoid regulation, and 36 mRNAs were novel glucocorticoid targets. All genes tested by qPCR for glucocorticoid regulation in cortical astrocyte cultures were also regulated by glucocorticoids in hippocampal astrocyte cultures (18/18). Interestingly, a portion of glucocorticoid-regulated genes were astrocyte-enriched; the percentage of astrocyte-enriched genes per total number of regulated genes was highest for the early time points and steadily decreased over the time course. These findings suggest that astrocytes *in vitro* may initially deploy cell-type-specific patterns of mRNA regulatory responses to glucocorticoids and subsequently activate additional cell-type-independent responses.

INTRODUCTION

The brain is composed of a number of different cell types, resulting in complex molecular processes that underlie both normal brain function and disease pathology. While studies have historically attributed numerous functions to neurons, glial cells (e.g. astrocytes, oligodendrocytes, microglia) are increasingly identified as active participants in a range of brain functions (e.g. (Henneberger et al., 2010; Di Castro et al., 2011)). For example, astrocytes are now known to be intricate contributors to many fundamental brain processes, including energy metabolism (Brown and Ransom, 2007), metabolite transport (Gandhi et al., 2009), the blood brain barrier (Abbott et al., 2006), synapse regulation (Eroglu and Barres, 2010), intercellular communication and gliotransmission (Parpura and Zorec, 2010), and injury response (Buffo et al., 2010). In an effort to understand the transcriptional underpinnings of these intercellular distinctions, recent efforts in brain cell-specific transcriptome analyses have identified subsets of mRNAs that are enriched in a given cell type, including astrocytes (Cahoy et al., 2008). These results reinforce the notion that cell type context may be an important factor in understanding cellular mechanisms that contribute to defined neural circuits and related brain functions. How cell types defined by these differences in basal gene expression transcriptionally adapt to specific stimuli may inform our understanding of both normal and disease conditions.

The molecular distinctions between neurons and glial cell types are gaining interest as additional disease states are associated with glial pathology. Some single-gene diseases have been associated with specific glial alterations (e.g. Alexander's disease

with astrocytes (Messing et al., 2012), myelination disorders with oligodendrocytes (Franklin, 2008)), but recent results implicate a glial component in many complex disorders as well (e.g. psychiatric disorders (reviewed in (Cotter et al., 2001b); schizophrenia (Webster et al., 2005; Steffek et al., 2008), bipolar disorder (Rajkowska et al., 2001), major depression (Cotter et al., 2001a)). Changes in astrocyte density and size in distinct brain regions of the postmortem brains of patients with depression imply that distinct subpopulations of astrocytes are disrupted in the disorder (Rajkowska et al., 1999; Miguel-Hidalgo et al., 2000). Postmortem brain transcriptional profiling studies have also identified astrocyte-associated mRNAs as being altered in specific brain regions in depression (Barley et al., 2009; Bernard et al., 2010), further suggesting the relevance of transcriptional control by cell type in this disorder.

Even with growing interest in glial biology in the face of increasing evidence of disease alteration, relatively little is known about effects of common transcriptional regulators in astrocytes. Given the differences in baseline gene expression between cell types, the effects of transcriptional regulators may also vary as a function of cell type. If cell-specific transcriptional alterations contribute to disease states associated with astrocytes, then discerning how known pathological alterations influence gene transcription in a cell-type-dependent fashion may enhance our understanding of disease mechanisms. For example, in the case of major depression, a well-established physiological alteration is the clinical presentation of hypercortisolemia (i.e. elevated levels of cortisol/glucocorticoids in the blood) (e.g. (Halbreich et al., 1985), reviewed in (Murphy, 1997; Gillespie and Nemeroff, 2005)).

Glucocorticoids are a class of steroid hormones that mediate the effects of the hypothalamic-pituitary-adrenal (HPA) axis. This system is commonly associated with stress signaling and the "fight-or-flight" response (reviewed in (De Kloet et al., 2005; Chrousos, 2009)). Glucocorticoids act as powerful transcriptional regulators that signal through two types of receptors, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). MR is a high affinity receptor associated with mechanisms involving lower glucocorticoid concentrations (e.g. circadian rhythmicity of cortisol). GR is a modestly lower affinity receptor that responds to higher concentrations of glucocorticoids, such as levels associated with stressful experiences. Hypercortisolemia is thought to particularly impact GR-mediated regulation. Alterations in glucocorticoid signaling are thought to contribute to some disorders (e.g. major depression (McEwen, 2005), Alzheimer's disease (Green et al., 2006)) and be causative for others (e.g. Cushing's syndrome (Newell-Price et al., 2006)). How stress hormones influence astrocyte gene expression at the transcriptome level and how these steroid-mediated transcription patterns change across time remains largely uncharacterized.

A small number of mRNAs have been identified individually as glucocorticoid-sensitive in astrocytes (e.g. glutamine synthetase (Khelil et al., 1990) , GFAP (Rozovsky et al., 1995)), suggesting unique impact of glucocorticoids by cell type. These mRNAs have been identified using two experimental strategies: (1) *in vitro* studies that use astrocyte cultures to examine glucocorticoid regulation of individual mRNAs (predominant

strategy, e.g. (Khelil et al., 1990)) and (2) *in vivo* studies that manipulate stress steroid levels and identify known glial-enriched mRNAs as steroid-regulated (e.g. (Nichols et al., 1990)). However, neither of these approaches has addressed the temporal patterning of mRNA expression changes at the transcriptome level. These approaches were thus not able to investigate associations between multiple mRNAs regulated by glucocorticoids or previously uncharacterized mRNA targets of glucocorticoid regulation in astrocytes.

Here we have characterized global mRNA expression changes induced by glucocorticoid treatment in astrocytes *in vitro*. Primary mouse astrocyte cell cultures were treated with glucocorticoids in a series of experiments designed to determine GR-mediated mRNA regulation over a 24-hour time course. We used the synthetic GR-selective agonist dexamethasone to identify temporally regulated mRNAs. Similarly, we demonstrate that all of the mRNAs regulated ≥two-fold by the natural ligand corticosterone were attenuated with co-treatment of the GR-selective antagonist RU486. Understanding glucocorticoid transcriptional regulation in astrocytes may provide additional insights into cell-specific effects of glucocorticoids in the brain.

MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle's media (DMEM, 11960-044), fetal bovine serum (FBS, Cat. #16000-044), antibiotic-antimycotic (Cat. #15240-062), charcoal-stripped FBS (CS-FBS, Cat. #12676-029), trypsin (Cat. #15090-046), and Hank's balanced salt solution (HBSS,

Cat. #14170-112) were obtained from Invitrogen (Gibco). Dextrose (Glucose, Cat. #BP350-1) and Trypan Blue (Cat. #SV3008401) were obtained from Fisher Scientific. Dexamethasone (Dex, Cat. #D-2915), corticosterone (Cort, Cat. #C2505), and mifepristone (RU486/RU, Cat. #M8046) were obtained from Sigma Aldrich. Standard cell culture media was 10% FBS in DMEM, 1:100 dilution of antibiotic-antimycotic, and 33mM glucose.

Astrocyte cell culture

Adult C57B/6 mice were obtained from Charles River. All animal procedures at the University of Michigan were approved by UCUCA (University Committee on Use and Care of Animals) and monitored by ULAM (Unit for Laboratory Animal Medicine). Mice were bred to obtain postnatal brain tissue. Primary astrocyte cell culture was obtained using a protocol derived from previous studies (Ruzicka et al., 1995). Brains were extracted from P0-P2 mice (average 6 mice/litter, meninges removed, placed in cold HBSS), and cortex and hippocampus were dissected using a dissecting microscope. Tissue dissections were pooled across animals and manually triturated. The tissue was then treated with a 0.05% Trypsin solution in HBSS for ~15 minutes to chemically release the cells from connective tissues. The trypsin was neutralized using 10% FBS/DMEM media, and cells were pelleted via centrifugation. The supernatant was removed, and the cells were washed using 10% FBS/DMEM media and centrifuged again. The resulting supernatant was then removed and the cells were resuspended in 10% FBS/DMEM. Sample cell density was determined using a hemocytometer and Trypan blue dye. Cells were then plated on uncoated plastic 6-well dishes (Costar

3516) at 250,000-500,000 cells/well in 2mL 10% FBS/DMEM media. Cells were grown in an incubator at 37C/5% CO2. All media was removed 24 hours after plating, and fresh media was then applied. For subsequent media replacement, 50% of media was changed every 2-3 days. Cells were grown for 9-11 days *in vitro* until near confluency and then used for experiments. Cell culture derived using this protocol yielded 97% GFAP-positive cells (data not shown), implicating the majority of cells as GFAP+ astrocytes.

RNA sample generation; Glucocorticoid and antagonist *in vitro* treatments

On the day prior to glucocorticoid treatment, all media was removed and replaced with 10% CS-FBS/DMEM to limit residual serum steroid effects. For experiments, cells were treated with 50% fresh media to a final concentration of (1) 100nM glucocorticoids (Corticosterone or Dexamethasone), (2) 100nM glucocorticoids + 500nM antagonist (Corticosterone+RU486), (3) 500nM antagonist (RU486), or (4) vehicle (solution containing identical components to treatment group in a given experiment except for steroid/antagonist). For dexamethasone experiments, cells were exposed to a specific treatment for 1 of 8 different lengths of time (0.25-hour, 1-hour, 2-hour, 4-hour, 6-hour, 12-hour, 18-hour, 24-hour). For corticosterone/RU486 experiments, cells were exposed to a specific treatment for 2 hours, 4 hours, or 6 hours. For all treatment conditions examined, samples for the 2-hour, 4-hour, and 6-hour treatments were generated as triplicate samples per group (i.e. 3 glucocorticoid-treated, 3 vehicle-treated); samples at other time points were generated as duplicate samples (i.e. 2 glucocorticoid-treated, 2 vehicle-treated). Total RNA samples were collected using Trizol reagent per

manufacturers protocol (Invitrogen). RNA concentrations were obtained using a Nanodrop spectrophotometer (Thermo Scientific).

Microarray studies

RNA samples were prepared using Illumina TotalPrep RNA Amplification kit (Ambion AMIL1791, 500 ng/sample). Biotinylated RNA samples (1.5 ug/sample) were then applied to MouseWG-6 v2.0 Expression Beadchips (Illumina BD-201-0202) and processed per manufacturers recommendations. Microarrays were scanned on a Illumina BeadArray Reader. Microarray data were analyzed using Illumina GenomeStudio software (version 1.1.1). GenomeStudio analyses were performed using quantile normalization with an Illumina custom error model and referenced to the corresponding vehicle group. Multiple testing corrections were applied using a Benjamini and Hochberg False Discovery Rate (FDR) with differential expression defined as $p<0.05$; p-values were obtained through the GenomeStudio software. Subsequent calculations (e.g. fold-changes) were derived using Microsoft Excel. For the corticosterone studies, mRNAs were considered to be regulated by corticosterone and attenuated by RU486 by meeting one of the following statistical criteria: (1) Cort treatment $p<0.05$ and Cort+RU co-treatment $p>0.05$ or (2) Cort treatment $p<0.05$ and Cort-RU co-treatment $p<0.05$ but Cort-RU co-treatment resulted in ≥50% reduction in the magnitude of fold-change compared to Cort treatment. Regulated Illumina probes were manually analyzed using the NCBI Gene database to update annotations and identify single genes associated with multiple regulated probes (http://www.ncbi.nlm.nih.gov/gene); for probes that targeted the same gene, the probe

containing the highest magnitude of changes was used for downstream analyses. For Figure 1, if a cell type marker had multiple probes, the probe with the highest average signal across all samples was used and then averaged per group per time point for analysis (16 total values). All microarray data is deposited on the NCBI GEO database (accession numbers: GSE39270 (corticosterone), GSE39272 (dexamethasone)).

Gene Ontology analysis

Gene ontology (GO) analyses were conducted using the DAVID software database (Da Wei Huang and Lempicki, 2008; Sherman and Lempicki, 2009) using Illumina probe ID notations for specified sets of mRNAs. In an effort to identify more specific GO terms (vs. broad), analyses were performed using default settings for terms of the GO Biological Processes (BP_FAT) and GO Molecular Function categories (MF_FAT).

Hierarchical clustering analysis

Hierarchical clustering was performed using k-means clustering in R. Clustering analysis was performed on the set of all probes regulated ≥two-fold at any time point by dexamethasone. Cluster number (8 clusters) was chosen based on analyzing the lowest number of clusters that produced a sum of squares statistically indifferent from immediately higher numbers of clusters.

qPCR validation studies

The same RNA samples used for the microarray studies were also used for qPCR analyses. RNA samples (1 ug) were converted to cDNA using Superscript II via random

hexamer priming (Invitrogen). ~50% of each cDNA reaction was used for Applied Biosystems (ABI) Taqman mRNA qPCR assays in custom Low-Density Array (TLDA) format (ABI #4346799) with Taqman reagents (ABI #4440048). The Taqman arrays were processed on an Applied Biosystems Viia7 instrument. Genes measured were selected based on a series of factors (highest magnitude regulation, known/unknown glucocorticoid regulation, astrocyte enrichment, house-keeping gene expression controls). For validation experiments, genes were measured in technical triplicate per sample. Differential expression analysis was performed using the delta-delta-Ct method ((Livak and Schmittgen, 2001), β-actin as control reference) using Statminer software (Integromics). An average of Ct-values from technical replicates was taken as the Ct-value for each gene measurement; individual Ct-values identified as outliers via the Grubbs' outlier test were excluded from downstream analyses. Specific Taqman mRNA assays used in this study are for the following targets: Actb (Mm01205647_g1), Adora2b (Mm00839292_m1), Aldh1l1 (Mm00550947_m1), Atp6v1b2 (Mm00431987_m1), Ch25h (Mm00515486_s1), Egr2 (Mm00456650_m1), Fkbp5 (Mm00487401_m1), Folh1 (Mm00489655_m1), Foxo1 (Mm00490672_m1), Gap43 (Mm00500404_m1), Gfap (Mm01253033_m1), Gja1 (Mm00439105_m1), Gjb6 (Mm01317508_m1), Glul (Mm00725701_s1), Klf9 (Mm00495172_m1), Mapk4 (Mm00554001_m1), Mertk (Mm00434920_m1), Pdk4 (Mm01166879_m1), Per1 (Mm00501813_m1), Phlda1 (Mm00456345_g1), Sgk1 (Mm00441380_m1), Slc1a2 (Mm00441457_m1), Slc1a3 (Mm00600697_m1), Sult1a1 (Mm01132072_m1), Syn2 (Mm00449780_m1), Txnip (Mm00452393_m1), Wnt7a (Mm00437354_m1).

Glucocorticoid regulation literature searching and astrocyte mRNA enrichment analyses

Previously reported glucocorticoid mRNA regulation was assigned based on analysis of the literature for each gene (using PubMed and Google Scholar search tools, terms used: gene symbol and/or probe ID + "glucocorticoids, corticosteroids, corticosterone, dexamethasone, prednisone"). Astrocyte mRNA enrichment analyses were performed using *in vivo* astrocyte enrichment gene expression data from "Supplemental Table 4" from Cahoy and colleagues (Cahoy et al., 2008).

RESULTS

Astrocyte cell cultures robustly express astrocyte markers and do not express markers for other brain cell types

Previous studies have utilized these cell culture protocols for studying astrocytes *in vitro* (Ruzicka et al., 1995; Ruzicka et al., 1996). To confirm that our cell culture protocol successfully selected for astrocytes, we examined the mRNA expression of cell-type markers associated with different brain cell types (Figure 2.1). Across all groups of the dexamethasone time course, the cell culture model expressed a number of astrocyte-specific cell markers (e.g. glial fibrillary acidic protein (Gfap), connexin-43 (Gja1), GLAST (Slc1a3), aquaporin-4 (Aqp4), aldehyde dehydrogenase 1L1(Aldh1l1)) while exhibiting limited or no expression above background of cell markers associated with other brain cell types (i.e. connexin-47 (Cx47), myelin-associated glycoprotein (Mag), myelin oligodendrocyte glycoprotein (Mog), myelin basic protein (Mbp) [oligodendrocytes]; neurofilament (Nef3), neuronal K-Cl co-transporter (KCC2/Slc12a5), synaptosomal-associated protein 25 (Snap25), neuron-specific β-tubulin (Tuj1/Tubb3)

[neurons]; Cd11b/Itgam, Cd45/Ptprc [microglia]). These results suggest that the vast majority of cells in this culture system are indeed astrocytes. In addition, nearly identical gene expression patterns of these cell-type mRNA markers were detected in individual groups of samples (i.e. vehicle-treated or glucocorticoid-treated; data not shown), suggesting that glucocorticoid treatment did not impact the cell-type character of the culture system.

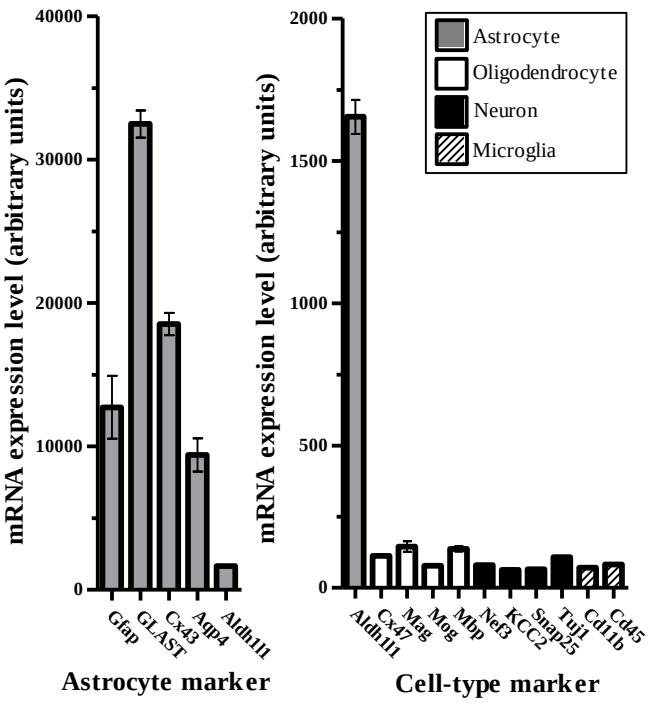


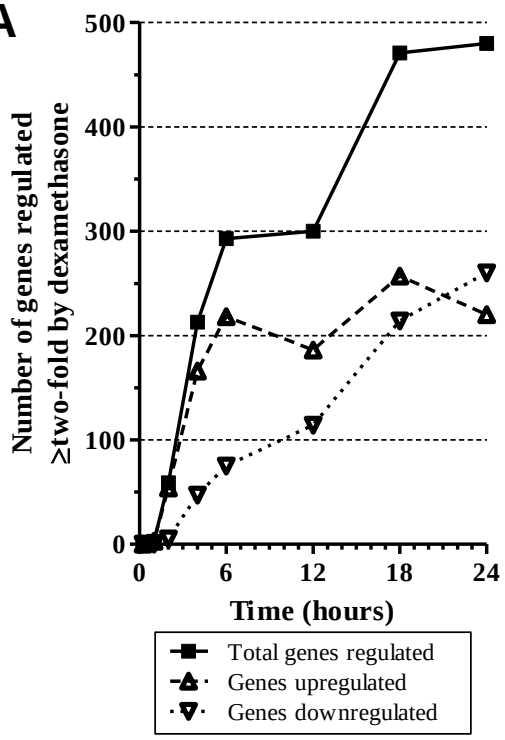
Figure 2.1: Cell culture system specifically expresses astrocyte mRNA cell markers. (A) Plot of average mRNA expression for subset of astrocyte cell markers among sample groups from 8 time points in dexamethasone time course. (B) Plot of average mRNA expression for subset of cell markers associated with additional brain cell types (neurons, oligodendrocytes, microglia). Bars for each mRNA are shaded according to the cell type association. Error bars display S.E.M.

Dexamethasone dynamically regulates mRNAs in astrocytes over time in vitro

To establish the transcriptome profile of primary glucocorticoid receptor regulation in astrocytes *in vitro*, primary astrocyte cell cultures were treated with the GR-selective synthetic agonist dexamethasone (100 nM) at 8 different time intervals spanning a 24-hour time course (i.e. 0.25, 1, 2, 4, 6, 12, 18, 24 hours). Microarray analyses were then performed on the resulting RNA samples to identify glucocorticoid-sensitive mRNAs in

this model system. Treatment of primary astrocyte cell cultures with dexamethasone resulted in dynamic gene expression changes over a 24-hour period of exposure (total 886 probes for 854 unique genes regulated ≥two-fold, Figure 2.2A).

A



B

Dexamethasone-sensitive mRNAs						
Time	**2HR**		**4HR**		**6HR**	
Gene	**Array**	**qPCR**	**Array**	**qPCR**	**Array**	**qPCR**
Gjb6	**8.93**	**31.85**	**20.20**	**13.99**	**20.38**	**82.06**
Fkbp5	**9.10**	**15.53**	**20.78**	**11.32**	**23.44**	**50.98**
Adora2b	**3.30**	**8.45**	**3.77**	**4.80**	**2.77**	**12.12**
Pdk4	**5.11**	**7.45**	**8.78**	**5.09**	**10.50**	**14.39**
Klf9	**4.74**	**6.58**	**6.27**	**4.10**	**6.91**	**8.54**
Mertk	**4.31**	**5.56**	**6.33**	**3.88**	**4.64**	**6.44**
Sgk1	**3.84**	**5.31**	**6.32**	**4.16**	**5.12**	**10.98**
Per1	**4.60**	**5.29**	**5.00**	**3.33**	**6.10**	**6.30**
Glul	**2.22**	**3.93**	*3.98*	**2.88**	**4.95**	**8.30**
Sult1a1	**2.56**	**3.72**	**6.05**	*3.62*	**9.20**	**12.08**
Mapk4	*2.32*	**3.59**	**6.01**	**4.58**	**5.06**	**12.96**
Txnip	**2.54**	**3.08**	**2.47**	**2.09**	**3.03**	**3.60**
Egr2	*1.78*	**2.79**	**3.87**	**2.95**	**3.52**	**4.96**
Folh1	**2.99**	**2.51**	**5.65**	*3.08*	**6.25**	**10.24**
Prodh	*1.86*	**2.36**	**6.62**	**3.99**	**9.99**	**23.90**
Wnt7a	*-2.21*	**-2.32**	**-5.13**	*-2.41*	**-3.67**	**-4.40**
Ch25h	**-3.47**	**-3.62**	**-4.41**	*-4.80*	**-5.00**	**-4.92**
Phlda1	**-4.13**	**-4.73**	**-3.95**	**-2.98**	**-4.33**	**-3.44**
Common astrocyte-associated mRNAs						
Aldh1l1	*-1.11*	*1.11*	*1.13*	*1.12*	*-1.35*	**1.22**
Gfap	*-1.02*	*1.11*	*1.18*	*1.05*	*-1.00*	*1.05*
Gja1	*1.03*	*1.09*	*1.08*	*1.06*	*-1.14*	*1.02*
Slc1a2	*1.15*	*1.09*	*1.15*	*1.16*	*1.21*	**1.18**
Slc1a3	*-1.03*	*1.11*	*1.07*	*1.04*	*-1.01*	*1.06*

Figure 2.2: Dexamethasone dynamically regulates specific mRNAs in astrocytes *in vitro* over 24-hour time course. (A) Plot of number of genes that show more than two-fold regulation by dexamethasone over time ($p<0.05$). (B) Comparison of linear fold-change mRNA expression values of microarray data with qPCR data for select mRNAs regulated by dexamethasone treatment (vs. vehicle). Data ordered based on magnitude of mRNA expression change in two-hour qPCR data. (HR = hours, **Bold** = $p<0.05$, *Italic* = $p>0.05$).

The total number of mRNAs whose expression level changed ≥two-fold increased with the duration of steroid exposure. In addition, the overall directionality of dexamethasone-mediated changes varied across the time course (e.g. majority of genes whose expression changed at early time points were upregulated by

dexamethasone). These findings were also highly reproducible in a replicate microarray study of triplicate samples from the 2-hour, 4-hour, and 6-hour time points based on the same statistical criteria for differential expression at similar magnitudes of regulation (≥97% overlap of mRNAs regulated ≥two-fold in replicate data with mRNAs regulated ≥1.5-fold in time course data, data not shown). qPCR studies confirmed the observed microarray findings for all mRNAs tested for glucocorticoid regulation (18/18 mRNAs, Figure 2B). The expression level of GR mRNA was similar in all data, suggesting that GR levels were consistent across astrocyte cultures (data not shown). 5 astrocyte-associated genes (Slc1a3, Slc1a2, Gfap, Gja1, Aldh1l1) were not altered due to glucocorticoid treatment, indicating that observed mRNA changes were not generic to all astrocyte mRNAs (Figure 2B).

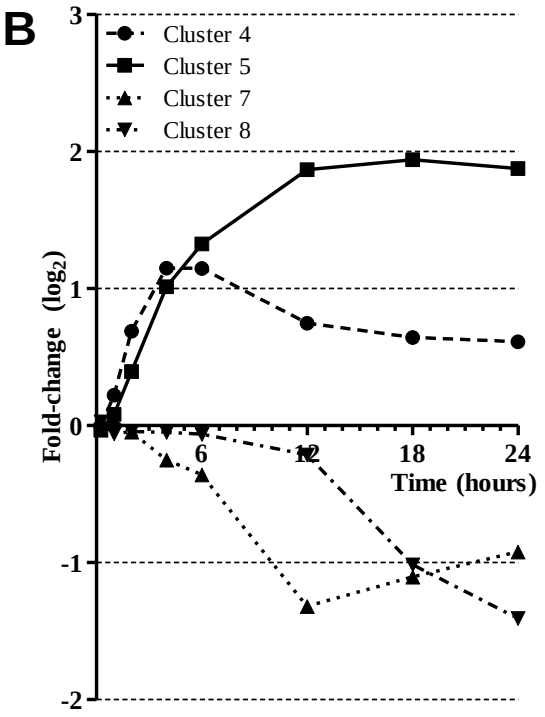
Hierarchical clustering analysis reveals subsets of dexamethasone-regulated genes associated with specific cellular functions

To determine temporal patterns of gene expression changes in astrocytes due to glucocorticoid treatment, a clustering analysis was performed on the set of all mRNAs regulated ≥two-fold by dexamethasone using the k-means method (Figure 2.3). Based on sum of squares analysis, the k-value that best explained the temporal variation using the least amount of clusters was an 8-cluster analysis. Of the 8 clusters, 5 of the cluster averages/centers were upregulated (Clusters 1, 2, 3, 4, 5) while 3 were downregulated (Clusters 6, 7, 8) by the 24-hour time point (Figure 2.3A). Some clusters exhibited average mRNA changes that peaked at earlier time points (e.g. 4 hours, 6 hours) and tailed off at later time points (Clusters 3, 4, 6).

A

Clustering analysis: centers (log_2 data)								
Cluster	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
# genes	15	175	48	130	69	90	167	191
0.25HR	-0.05	-0.01	-0.03	-0.01	-0.03	0.02	-0.03	0.03
1HR	0.56	0.02	0.65	0.22	0.08	-0.23	0.04	-0.06
2HR	2.12	0.09	1.37	0.69	0.39	-0.51	-0.05	-0.05
4HR	3.27	0.32	1.96	1.15	1.01	-0.96	-0.25	-0.05
6HR	3.54	0.44	2.05	1.15	1.33	-1.12	-0.36	-0.06
12HR	3.73	0.97	1.77	0.75	1.87	-0.67	-1.32	-0.21
18HR	3.66	0.98	1.56	0.64	1.94	-0.57	-1.10	-1.02
24HR	3.52	0.91	1.50	0.61	1.88	-0.40	-0.92	-1.41

B



C

Gene ontology associations of select clusters of dexamethasone-regulated mRNAs in astrocyte cultures		
Cluster	**Most statistically significant GO terms**	**p-value**
Cluster #4 Genes: 130 GO term category: Biological Process	Negative regulation, myeloid cell differentiation	3.10E-04
	Regulation of myeloid cell differentiation	3.11E-04
	Regulation of inflammatory response	4.78E-04
	Regulation of apoptosis	7.79E-04
	Negative regulation of apoptosis	8.44E-04
	Regulation of programmed cell death	8.63E-04
	Regulation of cell death	9.02E-04
	Negative regulation of programmed cell death	9.53E-04
	Negative regulation of cell death	9.76E-04
	Regulation of response to external stimulus	4.28E-03
	Response to abiotic stimulus	5.43E-03
Cluster #5 Genes: 69 GO term category: Molecular Function	Pyridoxal phosphate binding	1.44E-02
	Vitamin B6 binding	1.44E-02
	Cofactor binding	4.08E-02
	Transition metal ion binding	4.51E-02
	Peptidase activity (L-amino acid peptides)	5.19E-02
	Peptidase activity	6.02E-02
	Vitamin binding	6.32E-02
	Metal ion binding	7.38E-02
	Transaminase activity	7.83E-02
	Cation binding	7.96E-02
	Ion binding	8.83E-02
Cluster #7 Genes: 167 GO term category: Biological Process	DNA metabolic process	2.32E-08
	DNA replication	8.32E-08
	Cell cycle	7.14E-07
	DNA replication initiation	3.60E-05
	DNA-dependent DNA replication	8.15E-05
	Cell cycle process	1.46E-03
	DNA recombination	1.72E-03
	Cellular response to stress	1.76E-03
	Cell cycle phase	1.82E-03
	M phase	3.25E-03
	Response to DNA damage stimulus	3.51E-03
Cluster #8 Genes: 191 GO term category: Biological Process	Cell cycle	1.78E-53
	M phase	8.14E-43
	Cell cycle phase	1.28E-42
	Cell cycle process	3.53E-40
	Cell division	4.47E-40
	Mitotic cell cycle	7.08E-40
	M phase of mitotic cell cycle	1.49E-39
	Mitosis	7.21E-37
	Nuclear division	7.21E-37
	Organelle fission	2.88E-36

Figure 2.3: Clustering analysis of dexamethasone gene expression time course data yields distinct sets of genes that contain similar gene ontology categories. (A) Table of number of genes per cluster and cluster centers (average log_2 value) over the time course. (B) Graph of four GO term-associated cluster centers from k-means cluster analysis. (C) Table of graphed clusters and their corresponding most significant GO terms.

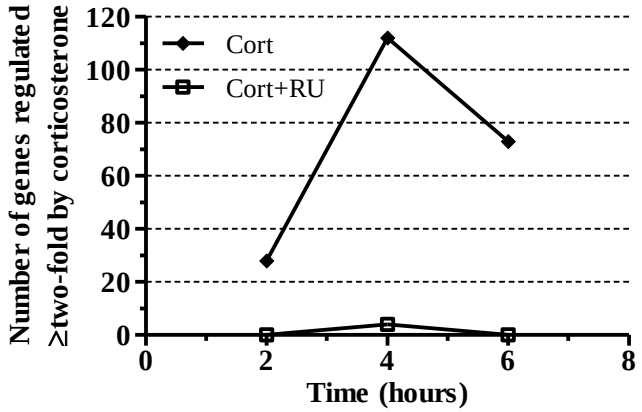
Other clusters exhibited more delayed glucocorticoid-mediated changes (e.g. not changed until ≥12 hours) (Clusters 2, 7, 8). Gene ontology analyses were then

performed on the genes in each cluster using the DAVID software. GO analysis of these clusters revealed distinct cellular targets of glucocorticoid regulation based on the duration of steroid exposure. Of the 8 clusters examined, the most significantly associated biological processes in 4 of them were highly enriched for specific cellular functions; the other 4 clusters were not highly correlated with any one type of biological process. The 4 enriched clusters were associated with (1) apoptosis (Cluster 4, increase of negative regulation), (2) cofactor and ion binding (Cluster 5, positive correlation), (3) DNA replication (Cluster 7, negative correlation), and (4) cell cycle (Cluster 8, negative correlation) (Figure 2.3B, 2.3C).

Dexamethasone-sensitive mRNAs also regulated by natural ligand corticosterone via the glucocorticoid receptor

To determine if dexamethasone-regulated mRNAs were similarly regulated by the natural ligand of the glucocorticoid receptor, primary astrocyte cultures were treated with corticosterone (100 nM) for an abbreviated time course targeting the time points of initial regulation in the dexamethasone time course (i.e. 2 hours, 4 hours, 6 hours). Corticosterone treatment altered the expression level of numerous genes at these 3 time points (2 hours: 28 mRNAs, 4 hours: 112 mRNAs, 6 hours: 73 mRNAs) (Figure 2.4A). To determine if the observed mRNA expression differences were mediated through the glucocorticoid receptor, parallel cultures were treated with both corticosterone and RU486 (500 nM), a GR-selective antagonist.

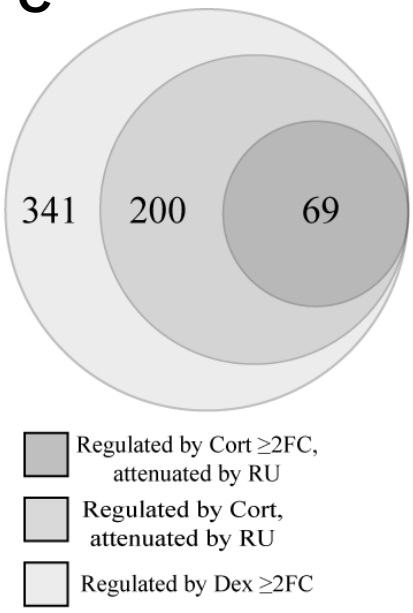
A



B

Corticosterone-sensitive/RU486-attenuated genes						
Time	**2HR**		**4HR**		**6HR**	
Gene	**Cort**	**Cort+RU**	**Cort**	**Cort+RU**	**Cort**	**Cort+RU**
Fkbp5	**4.60**	**1.18**	**15.51**	**1.30**	**16.36**	**1.43**
Sgk1	**4.35**	**1.39**	**5.71**	**1.30**	**5.76**	**1.43**
Pdk4	**3.74**	**1.26**	**6.64**	**1.21**	**7.96**	**1.32**
Per1	**3.32**	*1.20*	**4.26**	**1.50**	**5.74**	**1.48**
Folh1	**3.22**	*1.23*	**6.61**	**1.42**	**5.17**	**2.04**
Gjb6	**3.18**	*1.16*	**9.82**	*1.06*	**13.13**	**2.07**
Klf9	**3.11**	*1.10*	**4.19**	*1.10*	**4.60**	*1.06*
Sult1a1	**2.95**	**1.31**	**7.78**	**1.44**	**5.42**	**1.94**
Glul	**2.31**	**1.67**	**2.54**	*1.13*	**2.69**	**1.38**
Prodh	**2.02**	**1.37**	**7.55**	*1.04*	**5.54**	**1.47**
Adora2b	**1.74**	*1.23*	**2.22**	*-1.30*	**3.58**	**1.64**
Mertk	**1.66**	*1.01*	**3.62**	**1.19**	**3.20**	*1.15*
Egr2	**1.64**	*1.06*	**1.29**	*-1.03*	**2.23**	*1.21*
Txnip	**1.51**	*1.15*	**1.90**	*-1.05*	**2.03**	*1.17*
Mapk4	**1.44**	*-1.05*	**7.41**	**1.41**	**7.93**	**1.60**
Phlda1	**-1.49**	**1.32**	**-3.28**	*-1.18*	**-2.75**	*-1.15*
Ch25h	*-1.50*	**1.99**	**-2.97**	*-1.22*	*-1.20*	*1.23*
Wnt7a	**-1.76**	*1.05*	**-1.78**	*-1.18*	**-1.61**	*-1.09*
Common astrocyte-associated genes						
Aldh1l1	*-1.17*	*1.02*	*-1.10*	*-1.07*	*1.14*	*-1.01*
Gfap	*-1.12*	*1.06*	*-1.15*	*-1.06*	*-1.01*	*1.02*
Gja1	*-1.19*	*-1.05*	*-1.06*	*1.01*	*-1.03*	*1.03*
Slc1a2	*-1.28*	*-1.13*	*-1.10*	*-1.10*	*1.01*	*-1.03*
Slc1a3	*-1.21*	*-1.03*	*-1.06*	*-1.05*	*-1.01*	*-1.06*

C



D

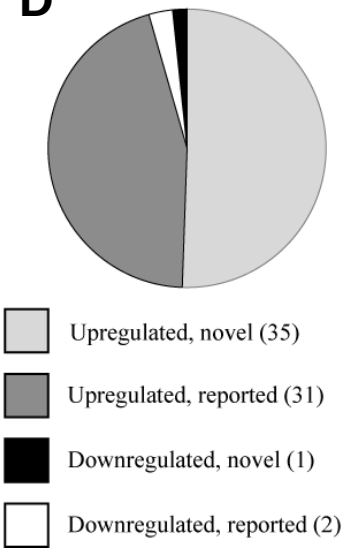


Figure 2.4: mRNAs regulated by synthetic glucocorticoid in primary astrocyte cultures are also regulated by natural ligand and the regulation is attenuated by GR antagonist. (A) Plot of number of genes that show more than two-fold mRNA expression change due to corticosterone (Cort) versus corticosterone and RU486 co-treatment (Cort+RU) ($p<0.05$). (B) Table of qPCR validation data on select corticosterone-sensitive mRNAs. Genes ordered by magnitude of fold-change due to 2-hour corticosterone exposure. (C) Proportional Venn diagram of overlap of genes regulated more than two-fold by dexamethasone at 2 hours, 4 hours, or 6 hours versus genes regulated by corticosterone ($p<0.05$) and attenuated by RU486 ($p>0.05$) at those same time points. (C) Graph characterizing genes regulated by both dexamethasone and corticosterone ≥two-fold and also attenuated by RU486 in terms of previous literature reporting and direction of regulation. (Regulated by Cort = $p<0.05$, attenuated by RU = $p>0.05$, HR = hours, FC = fold-change, **Bold** = $p<0.05$, *Italic* = $p>0.05$).

All 141 unique mRNAs with ≥two-fold expression changes induced by corticosterone were attenuated by co-treatment with RU486 at one or more of the time points (Figure 2.4A). qPCR data of a subset of Cort-regulated/RU486-attenuated mRNAs matched the directionality and magnitude of the microarray data (18/18 mRNAs, Figure 2.4B). Comparison of Cort-regulated/RU486-attenuated mRNAs demonstrating ≥two-fold expression change with mRNAs regulated ≥two-fold by dexamethasone at similar time points revealed substantial but incomplete overlap (Figure 2.4C), identifying more genes regulated ≥two-fold by dexamethasone than corticosterone. The set of Cort-regulated/RU486-attenuated mRNAs as defined by statistical criterion but with the two-fold change criterion removed (i.e. Cort $p<0.05$, Cort+RU $p>0.05$) contained a greater number of mRNAs regulated ≥two-fold by dexamethasone (Figure 2.4C). Based on this latter comparison, ~60% of mRNAs regulated ≥two-fold by dexamethasone were also regulated by corticosterone and attenuated by RU486. mRNAs that were regulated ≥two-fold by corticosterone and dexamethasone and whose corticosterone regulation was attenuated by RU486 were primarily upregulated by glucocorticoid treatment (Figure 2.4D). Of these 69 mRNAs regulated by both glucocorticoids, 33 have been previously associated with glucocorticoid regulation (Table 1) whereas 36 demonstrated novel glucocorticoid regulation (Table 2).

mRNAs regulated by glucocorticoids in cortical astrocyte cultures are also regulated in hippocampal astrocyte cultures.

To determine if observed glucocorticoid-mediated mRNA changes were specific to astrocytes derived from cortical brain regions, additional qPCR experiments were conducted on RNA samples isolated from primary astrocyte cell cultures derived from hippocampus and treated with dexamethasone. All mRNAs tested for dexamethasone regulation by qPCR in cortical astrocyte cultures were also regulated by dexamethasone in hippocampal astrocyte cultures (18/18 mRNAs, $p<0.05$) (Figure 2.5).

Figure 2.5: Portion of mRNAs regulated by glucocorticoids in cortical astrocyte cultures are also regulated by glucocorticoids in hippocampal astrocyte cultures. qPCR data of select mRNAs regulated by dexamethasone treatment in cortical astrocyte cultures that are also hippocampal astrocyte cultures. Genes ordered based on 2-hour fold-change values. (HR = hours, **Bold** = $p<0.05$, *Italic* = $p>0.05$).

Dexamethasone regulation of mRNAs in hippocampal astrocyte cultures

Gene	2HR	4HR	6HR
Gjb6	**24.35**	**92.70**	**51.38**
Fkbp5	**12.31**	**43.42**	**39.92**
Adora2b	**5.94**	**8.32**	**6.62**
Per1	**4.46**	**5.11**	**6.46**
Mertk	**4.00**	**3.90**	**5.58**
Sgk1	**3.74**	**5.02**	**4.90**
Pdk4	**3.41**	**11.82**	**10.39**
Klf9	**3.40**	**4.65**	**5.70**
Mapk4	**2.75**	**10.20**	**10.20**
Sult1a1	**2.37**	**9.13**	**8.20**
Glul	**2.22**	**8.26**	**4.73**
Folh1	**2.13**	**6.00**	**4.82**
Foxo1	**1.91**	**1.50**	**1.83**
Txnip	**1.55**	**2.32**	**2.28**
Prodh	*1.24*	**10.64**	**6.77**
Wnt7a	**-2.27**	**-5.37**	**-4.89**
Phlda1	**-3.80**	**-3.78**	**-3.53**
Ch25h	**-5.02**	**-9.30**	**-10.13**

Glucocorticoid-regulated mRNAs include sets of astrocyte-enriched genes

A portion of glucocorticoid-regulated mRNAs regulated by glucocorticoids in these data were known to be specifically expressed in astrocytes (e.g. Glul, Gjb6; Figure 2.6A). In an effort to determine how glucocorticoids might modulate unique astrocyte cell functions, mRNAs regulated by glucocorticoids were compared to mRNAs reported to be enriched in astrocytes, thus described as "astrocyte-enriched" (Cahoy et al., 2008). On average, the astrocyte cultures expressed ≥60-70% of astrocyte-enriched mRNAs

(≥1.5-fold enrichment) that represented 13%-14.5% of the detected mRNAs across samples (data not shown). Many genes identified as glucocorticoid-sensitive in these data are reported to be astrocyte-enriched (overall percentage 23.4%, Figure 2.6B).

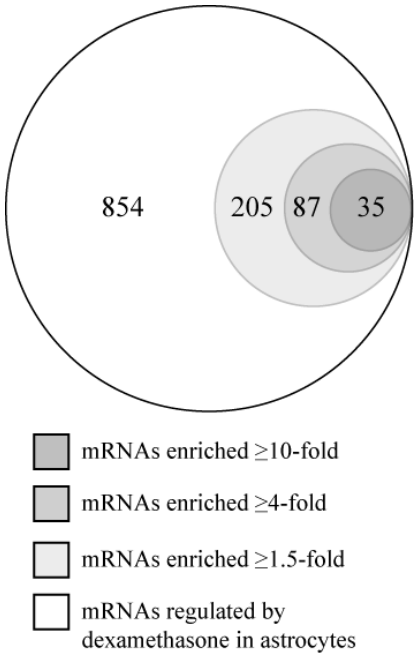
A

qPCR-validated, glucocorticoid-regulated mRNAs in astrocytes	
mRNA	**Astrocyte enrichment***
Mertk	33.0
Gjb6	32.7
Prodh	29.4
Pdk4	20.8
Adora2b	17.4
Sult1a1	12.8
Folh1	11.4
Glul	8.5
Mapk4	5.6
Txnip	5.3
Klf9	3.1
Per1	1.9

C

Gene ontology associations with glucocorticoid-regulated astrocyte-enriched mRNAs		
Astrocyte enrichment	**Most significant GO biological pathways**	**p-value**
≥1.5-fold	Cell cycle	2.21E-07
	Cell cycle process	4.20E-06
	Cell cycle phase	3.57E-05
≥4-fold	Sulfur metabolic process	1.25E-03
	Nitrogen compound biosynthetic process	1.75E-02
	Oxidation reduction	1.85E-02
≥10-fold	Sulfur metabolic process	7.43E-04
	Oxidation reduction	1.41E-03
	Embryonic organ development	1.06E-02
	Nitrogen compound biosynthetic process	1.94E-02

B



D

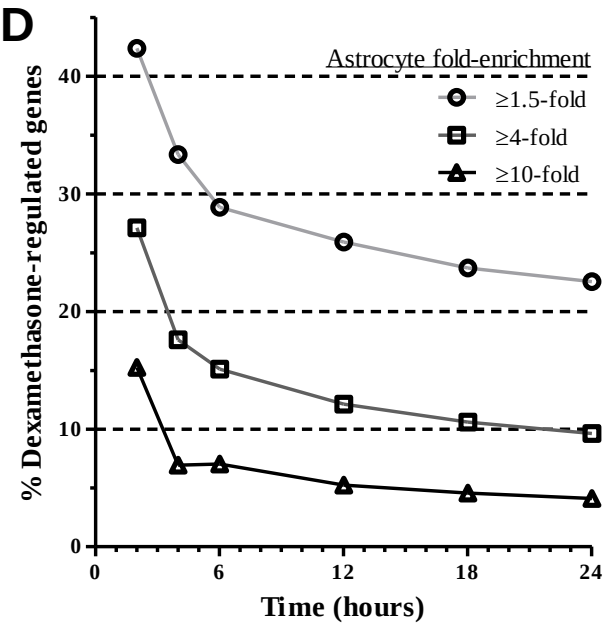


Figure 2.6: Certain mRNAs regulated by dexamethasone are astrocytes-enriched.
(A) Table of genes found to be regulated ≥two-fold by dexamethasone and corticosterone in both microarray and qPCR experiments and their reported enrichment in astrocytes relative to other neural cell types. Genes arranged by magnitude of enrichment. *=Cahoy, et. al., J. Neurosci. (2008). (B) Proportional Venn diagram of overlap of total number of unique dexamethasone-regulated mRNAs with 3 subsets of astrocyte-enriched mRNAs. (C) Table containing most statistically significant gene ontology category associated with 3 astrocyte-enriched mRNA subsets of dexamethasone-regulated mRNAs in (B). (D) Plot of percentage of astrocyte-enriched mRNAs as a function of total mRNAs regulated ≥two-fold by dexamethasone over time.

To investigate the functional implications of these findings, glucocorticoid-regulated, astrocyte-enriched genes were analyzed for gene ontology term association (Figure 2.6C). The most significant “biological processes” term associated with the broadest enrichment category (≥1.5-fold enrichment) was related to cell cycle. The “biological processes” terms most associated with greater astrocyte-enrichment levels (≥4-fold, ≥10-fold) included (1) sulfur metabolic processes (genes upregulated), (2) nitrogen compound biosynthesis (genes upregulated), and (3) oxidation reduction (genes upregulated) (Table 3). Among glucocorticoid-sensitive mRNAs, the percentage of astrocyte-enriched mRNAs as a function of all glucocorticoid-regulated mRNAs was higher for shorter durations of glucocorticoid exposure compared with longer durations over the time course (e.g. 2-hour=42.4%, 24-hour=22.5%); this percentage consistently trended downward over the entire time course, reaching a value at the 24-hour time point that was slightly below the overall percentage for the entire time course (Figure 2.6D).

DISCUSSION

Cell-specific mechanisms in the brain have long been of interest in an effort to understand normal neural processes and disease pathology. The goal of these studies was to characterize glucocorticoid transcriptional regulation in astrocytes *in vitro*. Previous studies had identified genes regulated by glucocorticoids in astrocytes, but (1) mRNA regulation was observed on an individual gene basis and (2) few published reports have looked extensively at patterns of mRNA changes due to glucocorticoid exposure at time points less than 24 hours. To extend current knowledge of

glucocorticoid receptor-mediated transcriptional regulation to shorter durations of glucocorticoid exposure, we treated cortical astrocyte cultures with the synthetic GR agonist dexamethasone over 8 time points from 15 minutes to 24 hours. We chose a glucocorticoid concentration relevant to stress mechanisms (100 nM) to parallel (1) concentrations commonly used in other *in vitro* gene expression studies and (2) the range of corticosterone concentrations observed in the brain as measured by microdialysis following acute stress in rodents (e.g. (Reul et al., 2000; Thoeringer et al., 2007; Droste et al., 2009)).

Over the 24-hour time course, we identified 854 unique genes that were regulated more than two-fold at one or more time points by dexamethasone compared to vehicle control treatment. In an effort to validate the observed mRNA regulation, qPCR experiments were performed on samples at the earliest time points that contained large numbers of regulated mRNAs (i.e. 2 hours, 4 hours, 6 hours). All of the genes tested for glucocorticoid sensitivity by qPCR showed significant differences similar in magnitude and direction to the microarray data (18/18 mRNAs). We also observed that mRNAs previously reported to be glucocorticoid-regulated in astrocytes were regulated in manner consistent with the literature (e.g. Glul (Kumar et al., 1986), Glt-1/Slc1a2 (Zschocke et al., 2005), MAO-B (Carlo et al., 1996), Ndrg2 (Takahashi et al., 2012), GDH (Hardin-Pouzet et al., 1996)), with some exceptions (e.g. Fgf2 (Meisinger et al., 1996), GFAP (Rozovsky et al., 1995)). The exceptions may be the result of a number of factors (e.g. methods of cell preparation, species differences (e.g. mouse vs. rat)), including primary vs. secondary effects of glucocorticoids based on length of

glucocorticoid exposure in culture (e.g. GFAP glucocorticoid regulation sensitive to time of exposure (Rozovsky et al., 1995)). The overall consistency of our results with previous findings supports the validity of this data in terms of characterizing glucocorticoid regulation in astrocytes *in vitro*.

The dynamic temporal gene expression regulation by glucocorticoids observed over 24 hours raises the possibility of coordinated pathway regulation. To examine relationships between the regulated mRNAs, we performed (1) a clustering analysis using 8 clusters to group mRNAs according to glucocorticoid-mediated expression changes over time and (2) gene ontology (GO) analyses on the mRNAs in each cluster. The clustering analysis identified novel temporal patterns of regulation among the glucocorticoid-regulated mRNAs. Clusters of mRNAs varied by the magnitude and rate of glucocorticoid-induced activation or repression (Figure 3). Our results are similar to spatial patterns observed in previous reports characterizing time-dependent mRNA expression changes mediated by glucocorticoids in other cell culture systems (John et al., 2009; Reddy et al., 2009). From the GO analysis, the most statistically-enriched biological processes changed between time points over the 24-hour time course; different pathways were impacted at earlier time points compared to later time points. Among the 8 clusters, specific clusters associated with distinguishable sets of similar GO terms (Cluster 4 = increased gene expression related to negative regulation of apoptosis, Cluster 5 = increased gene expression related to cofactor binding and ion binding, Cluster 7 = decreased gene expression related to DNA replication, Cluster 8 = decreased gene expression related to cell cycle regulation). Negative regulation of

apoptosis, DNA replication, and cell cycle pathways have often been associated with glucocorticoid regulation; glucocorticoids are known to inhibit proliferation in many cell types, including astrocytes (Crossin et al., 1997). Cofactors and ions associated with mRNAs represented in the binding-related cluster included zinc (Bcl6b, Dpep1, Trp63, SceI, Cpm, Gm22, Lims2, Zhx3), pyridoxal phosphate (Cbs, Got1l1, Agxt2l1), and divalent cations (calcium: Dlk1, S100a7a, Arsj, Galntl2; magnesium: Pgm5, Acsl3, Ppm1k; iron: Cdo1, Cyp2d22). Together, these findings document that specific pathways in astrocytes are targeted by glucocorticoids across time *in vitro*.

To extend the observed synthetic glucocorticoid regulation to the endogenous ligand and further verify the GR-based mechanism of regulation on target mRNAs in astrocytes, we conducted microarray experiments on cortical astrocyte cell culture samples treated with (1) the endogenous ligand corticosterone and (2) corticosterone co-administered with RU486 (mifepristone), a GR-selective antagonist. Co-treatment of corticosterone and RU486 attenuated the regulation of almost all of the mRNAs regulated ≥two-fold by corticosterone alone, suggesting they were regulated via GR. RU486 acted as an antagonist to corticosterone action for these robustly glucocorticoid-regulated mRNAs, consistent with reports involving glucocorticoid-antiglucocorticoid co-administration (e.g. (Baulieu, 1991; Cadepond et al., 1997; De Kloet, 2004)). Although RU486 has been reported to have agonistic properties under select circumstances (e.g. (Cairns et al., 1993; Oakley et al., 1996)), we did not observe significant agonist activity by RU486 in these experiments (i.e. RU486 treatment alone). RU486 agonist activity has been suggested to be cell-type dependent (Meyer et al., 1990). Although RU486

action has not been explicitly studied in astrocytes, the combination of GR concentration (Zhang et al., 2007), corepressor expression (Jackson et al., 1997; Meijer et al., 2006), and other cellular factors in astrocytes may not permit substantial RU486 agonist activity in this context.

Because both dexamethasone and corticosterone signal through the glucocorticoid receptor, we predicted the two steroids would regulate mRNAs in our astrocyte culture system in a similar manner. Previous comparative *in vitro* studies have found similar regulation between the two steroids for select mRNA targets (e.g. (Slieker et al., 1996)). Compared with dexamethasone treatment, corticosterone treatment regulated a smaller number of mRNAs ≥two-fold at 2 hours, 4 hours, and 6 hours of steroid exposure (absolute number of regulated genes: 341 (Dex) vs. 141 (Cort)). While a portion of the discrepancy may be due to experimental variation and astrocyte heterogeneity, this difference in the number of mRNAs regulated ≥two-fold by these two glucocorticoids may be due in part to the higher GR affinity of dexamethasone vs. corticosterone (Funder et al., 1973; Reul and De Kloet, 1985; De Kloet, 2004). Differential responses to time-limited treatments of dexamethasone vs. corticosterone has been documented for other mRNAs (e.g. (Eberwine and Roberts, 1984; Logsdon et al., 1985; Ihara and Nakanishi, 1990)). In our experiments, the set of genes statistically regulated by corticosterone ($p<0.05$) without the fold-change criteria included additional genes regulated ≥two-fold by dexamethasone (133 additional genes, Figure 4). These results are consistent with the interpretation that dexamethasone is a more robust regulator of initial mRNA expression changes than corticosterone at specific time points within our

time course. The equivalence of dexamethasone and corticosterone regulation observed in previous studies may be due to longer duration of glucocorticoid exposures (e.g. 24+ hours) that may overcome temporal pharmacological distinctions between these molecules. Based on our findings, future temporal studies comparing regulation by dexamethasone and corticosterone may yield more precise comparisons of regulated mRNAs by matching physiological criteria other than absolute steroid concentration (e.g. specified IC_{50} or EC_{50} to glucocorticoid receptor).

We then compared the sets of (1) mRNAs regulated by dexamethasone with (2) mRNAs regulated by corticosterone and attenuated by RU486 co-treatment at any of the 2-hour, 4-hour, or 6-hour time points. We found 69 mRNAs regulated more than two-fold by both glucocorticoids that meet this criteria. Among these mRNAs, 33 had previously been associated with glucocorticoid regulation (31 upregulated, 2 downregulated; Table 1) and 36 displayed novel glucocorticoid regulation (35 up, 1 down; Table 2). Our findings extend the observed glucocorticoid regulation in other systems to astrocytes; among the mRNAs known to be regulated by glucocorticoids, all of the mRNA changes in this study were in the same direction as previously reported (data not shown). Both previously reported and the unreported glucocorticoid-regulated mRNAs may have unique actions in astrocytes; their functional roles in astrocytes remain to be investigated.

Comparisons of astrocyte gene expression between brain regions are infrequent despite the growing evidence for astrocyte heterogeneity and brain region-dependent

astrocyte differences associated with both transcriptional regulation and specific diseases (Zschocke et al., 2005; Zhang and Barres, 2010). Most astrocyte cell culture studies have derived cells in a brain region-independent manner (e.g. whole brain or hemispheres), yet astrocytes derived from different brain regions have been reported to have different gene expression profiles (Yeh et al., 2009). To determine if the observed gene expression changes were specific to cortical astrocyte cultures, we measured the glucocorticoid sensitivity of select mRNAs in hippocampal astrocyte cultures. All of the genes measured were regulated by glucocorticoids in both cortical cultures and hippocampal cultures, indicating that these glucocorticoid effects occur in astrocytes derived from multiple brain regions (Figure 2.5). This observed regulation could result from common mechanisms for robustly regulated mRNAs (i.e. directly mediated by glucocorticoid receptor), although we cannot exclude the possibility that astrocytes *in vitro* undergo cumulative cellular changes that disrupt or remove brain-region dependent differences. Whether the glucocorticoid regulation of mRNAs in astrocytes characterized in our experiments occurs in all astrocytes regardless of brain region source remains an open question. Additional experiments will be needed for comprehensive transcriptional comparisons of glucocorticoid regulation between astrocyte cell cultures derived from different brain regions (e.g. basal gene expression concordance for all mRNAs by brain region source, examining lower fold-change expression changes).

Some mRNAs regulated by glucocorticoids in our experiments have been previously reported to have enriched expression in astrocytes compared to other brain cell types

(i.e. neurons, oligodendrocytes) (Cahoy et al., 2008) (Figure 2.6A, 2.6B). Transcriptional regulation of these astrocyte-enriched mRNAs may have particular functional significance for astrocytes. Many mRNAs previously associated with glucocorticoid regulation as well as mRNAs displaying novel regulation are also astrocyte-enriched, representing 205 of the 854 unique mRNAs regulated by glucocorticoids (24.0%). In addition, multiple mRNAs (Atp6v1b2, Gap43, Syn2) reported to be both (1) enriched in neurons (Cahoy et al., 2008) and (2) regulated by glucocorticoids (Federoff et al., 1988; Skynner et al., 2006) were not regulated in our astrocyte culture system (data not shown), further suggesting that glucocorticoids act in a cell-specific manner among brain cell types.

What are the functional consequences of regulating these astrocyte-enriched mRNAs? GO analyses of mRNAs from the entire time course data set that were enriched in astrocytes ≥four-fold and ≥ten-fold both identified the terms “sulfur metabolism” (Papss2, Mamdc2, Cbs, Mgst1, Gstk1), “nitrogen compound biosynthetic process” (Atp1a2, Cbs, Prodh, Slc7a2, Rora, Glul), and “oxidation reduction” (Adhfe1, Aldh1a1, Cyp2d22, Cyp4v3, Dio2, Fmo1, Prodh, Aass, Aldh6a1) as significantly enriched processes (Figure 6C, Table 3). The regulated mRNAs associated with nitrogen biosynthetic processes and oxidation reduction encode for proteins associated with a range of cellular processes, including nitric oxide metabolism (e.g. Slc7a2, Rora) and amino acid metabolism and transport (e.g. Cbs, Prodh, Slc7a2, Glul, Aass, Aldh6a1). These pathways are associated with the cellular stress response, a reaction to

glucocorticoid stimulation that is known to increase reactive oxygen species and cellular metabolic load (De Kloet et al., 2005).

We also observed striking basal and temporal trends in the percentage of glucocorticoid-regulated mRNAs that were astrocyte-enriched. First, the percentage of glucocorticoid-regulated mRNAs that are astrocyte-enriched was significantly higher for all lengths of glucocorticoid exposure (23% of dexamethasone-regulated mRNAs) than the average percentage for all detected mRNAs (~14% of expressed mRNAs). All astrocyte-enriched percentages for glucocorticoid-regulated mRNAs were >1.5x greater and up to 3x greater than the astrocyte-enriched percentages for all expressed mRNAs. The difference in percentages implies that astrocyte-enriched mRNAs are on average more likely to be regulated by glucocorticoids compared with all mRNAs expressed in our astrocyte cultures. Our data is one of the first to empirically demonstrate a bias in transcriptional regulation in astrocytes toward astrocyte-enriched mRNAs at the level of the transcriptome *in vitro*. Second, we noticed that astrocyte-enriched mRNAs represented a greater percentage of glucocorticoid-regulated genes at earlier time points compared to later time points in the time course. This percentage steadily declined over the entire time course (e.g. for 1.5-fold enriched mRNAs, 42.4% at 2 hours → 22.5% at 24 hours). This temporal trend suggests an additional bias of glucocorticoid regulation toward astrocyte-enriched mRNAs in initial astrocyte responses to steroid exposure.

Generalizability of these findings to other brain cell types and additional transcriptional regulators is an intriguing potential extension of these findings. Glucocorticoids are thought to act in a cell-type dependent fashion based upon a number of factors, including (1) basal gene expression patterns (e.g. genes expressed only in astrocytes based on chromatin structure and gene availability/regulation) and (2) the compliment of transcriptional regulators present in the cell (e.g. cell-dependent transcription factor expression and interactions with GR in astrocytes) (Vardimon et al., 1999; Le et al., 2005; So et al., 2007). We have added empirical evidence suggesting that astrocyte-enriched mRNAs are regulated by the initial responses of astrocytes to glucocorticoids. Based on our findings, understanding the response of a specific cell type to a given signaling cascade may be enhanced by (1) knowing which mRNAs are enriched in that cell type relative to other cells in a given tissue and (2) investigating the regulation of cell-type-enriched mRNAs. We currently do not know how the observed glucocorticoid regulation in astrocytes compares with glucocorticoid regulation in other brain cell types (e.g. neurons, oligodendrocytes), although there is data available on glucocorticoid mRNA regulation in hippocampal slices and a neuronal cell line (Morsink et al., 2006a; Morsink et al., 2006b). As additional cell-type-enriched expression data becomes available, comparing and contrasting our findings with glucocorticoid regulation in other cell types, both in the brain and in other tissues, may increase our understanding of the mechanisms of cell-type specific glucocorticoid regulation.

There are caveats to consider in interpreting these results. First, we used stringent criteria to define differential expression. mRNAs were considered differentially

expressed in the microarray experiments when they were changed more than two-fold by both dexamethasone and corticosterone vs. vehicle treatment. While this conservative criterion increases the likelihood of identifying authentic glucocorticoid regulation by limiting the identification of false positives, it also excludes mRNAs that are regulated at a lower magnitude by dexamethasone, corticosterone, or RU486. In addition, some genes were statistically regulated by one glucocorticoid and not the other; such genes may be authentic glucocorticoid targets but would not be identified as such based on these criteria (e.g. qPCR validation of Adora2b and Wnt7a). Second, all of these gene expression experiments were conducted on astrocytes *in vitro*. This approach isolated the glucocorticoid response in astrocytes from other cell types, but it does not assess the effects *in vivo*. As with many primary culture systems, cultured astrocytes are reported to have different gene expression profiles compared to astrocytes *in vivo* (Cahoy et al., 2008; Foo et al., 2011). Additional experiments will be needed to determine how the glucocorticoid regulation in astrocytes *in vitro* translates to the *in vivo* environment and corresponding physiological influence. Third, astrocytes in the brain are regulated in the context of intercellular interactions; how glucocorticoids impact astrocytes and these mRNAs in their interactions with neurons and other glial cell types in the intact brain remains to be investigated. In addition, although a subset of regulated mRNAs are reportedly astrocyte-enriched, these mRNAs may be expressed in other cell types. We are interested in knowing if the glucocorticoid regulation of these mRNAs observed in astrocytes also occurs in a similar manner in other cell types. Fourth, while the time course of regulation is consistent with primary transcriptional regulation, we have not demonstrated that GR directly modulates the

transcription of these genes but rather that mRNA levels change. Future studies can be designed to assess whether GR binds the promoter domains near these the genomic origins of these glucocorticoid-regulated mRNAs. Finally, the associations mentioned in terms of astrocyte enrichment and gene ontology analyses are derived from transcriptome-level data sets. Individual gene connections and pathway enrichment must be further investigated to confirm the neurological relevance of these statistical connections.

In summary, we have characterized the responsiveness of gene expression in astrocytes to glucocorticoids *in vitro*. To our knowledge, our study is the most extensive reported investigation of the temporal patterning of glucocorticoid-mediated mRNA regulation at the transcriptome level in a specific glial cell type. We find that (1) glucocorticoids regulate a specific set of mRNAs in astrocytes *in vitro*, (2) the observed dynamic gene expression regulation contains associations with specific biological pathways, (3) glucocorticoids regulate specific mRNAs in astrocyte cultures derived from multiple brain regions, and (4) a subset of glucocorticoid-regulated mRNAs are enriched in astrocytes. Together, these data add to the knowledge of glucocorticoid-mediated gene expression regulation and further enhance our understanding of glucocorticoid signaling and stress biology in the brain.

NOTE: Additional supplemental data from original publication are available online:

- Table S1 - Microarray data with fold-change + differential p-values for all probes measured in 24-hour dexamethasone time course

- Table S2 - Microarray data for mRNAs regulated by dexamethasone treatment with ≥two-fold change ($p<0.05$)
- Table S3 - Individual log_2 data for genes in clusters with similar GO terms
- Table S4 - Microarray data for unique genes (1) regulated by corticosterone ≥two-fold ($p<0.05$) and (2) attenuated by RU486 ($p>0.05$) or abs(Cort/C+R)≥2 at 2HR, 4HR, or 6HR treatment

Additional text; predicted functional consequences of glucocorticoid mRNA regulation in astrocytes *in vitro*

The following section is additional functional information on mRNAs of interest identified as glucocorticoid-regulated in astrocytes in vitro as well as an additional point regarding cell-enriched mRNA regulation patterns; this component of this chapter was not part of the initial publication.

Genes previously associated with glucocorticoid regulation but not in astrocytes

A portion of genes regulated by dexamethasone in astrocytes have been previously reported as glucocorticoid-sensitive in other systems (33 genes, Table 1). Our findings extend the observed glucocorticoid regulation in other systems to astrocytes; almost all of the mRNA changes in this study were in the same direction as the previously reported glucocorticoid regulation (exception: Lpin3, reported glucocorticoid insensitivity in hepatocytes (Manmontri et al., 2008)).

Among the mRNAs whose glucocorticoid regulation was validated by qPCR, we find mRNAs associated with brain function but not specifically with astrocyte mechanisms,

including transcription factors (Klf9, Per1, Txnip, Egr2, Foxo1), kinases (Pdk4, Mertk, Sgk1), and other proteins (Fkbp5, Sult1a1, Ch25h, Phlda1, Prodh). Although the influence of these glucocorticoid-regulated genes on astrocytes are not yet characterized, we can infer potential functional consequences of their glucocorticoid regulation in astrocytes based on known roles of the gene products in other contexts. For example, Klf9 is an immediate early gene and transcription factor associated with neuronal development and maturation (Bonett et al., 2009). Per1 is well-characterized as part of circadian gene expression circuitry and has been shown to contain an active glucocorticoid response element (Yamamoto et al., 2005). Txnip inhibits reduction of protein sulfide bonds, and glucocorticoid regulation of Txnip has been linked to apoptosis mechanisms (Wang et al., 2005). Sgk1 is a kinase known to indirectly upregulate the activity of EAAT1 (GLT-1) (Boehmer et al., 2003), a glutamate transporter enriched in astrocytes. Mertk is a receptor tyrosine kinase that has displayed glucocorticoid sensitivity in macrophages and immune cells; in this context, induction of Mertk increased phagocytosis capacity and is associated with anti-inflammatory effects (Zahuczky et al., 2011). Sult1a1 is a sulfur transferase associated with thyroid hormone metabolism (Duanmu et al., 2001). Interpreting the effects of glucocorticoid-mediated regulation of mRNAs whose roles in astrocytes have not been previously investigated will require additional studies to understand their function under basal and glucocorticoid-stimulated conditions in this cellular context.

Pdk4 is a pyruvate dehydrogenase kinase isoform that is involved in energy metabolism. Pdk4 regulates the pyruvate dehydrogenase complex (PDC) and the

subsequent oxidative metabolism of pyruvate into acetyl-CoA for the Krebs cycle, increasing available cellular energy via ATP production. Pdk4 has also been shown to be glucocorticoid-sensitive in kidney (Huang et al., 2002). Recent studies have reported that Pdk4 mRNA and protein expression in the brain are highly enriched in astrocytes relative to neurons (Halim et al., 2010). A glucocorticoid-mediated increase in Pdk4 levels would hypothetically inhibit PDC activity and result in higher glycolytic metabolism and lower oxidative metabolism in astrocytes, increasing the available energetic compounds for neurons (e.g. pyruvate into lactate (Boumezbeur et al., 2010)). This shift in metabolism may be functionally important for the neural stress response to elevated glucocorticoids.

Proline dehydrogenase (Prodh) converts the amino acid proline to delta-1-pyrroline-5-carboxylate (P5C) at the mitochondrial membrane, resulting in ATP production via cytochrome c and connecting proline catabolism with neurotransmitter metabolism (Tanner, 2008). P5C is a precursor for both glutamate and GABA. Proline itself has been shown to decrease glutamate uptake in cortex and hippocampus (Delwing et al., 2007). While hyperprolinemia is considered detrimental to neuronal function (Delwing et al., 2003), a glucocorticoid-mediated increase in Prodh levels and activity would theoretically decrease proline levels and (1) be neuroprotective against oxidative stress and reactive oxygen species generation, (2) maintain or increase mitochondrial ATP production, and (3) increase glutamate uptake. In addition to hyperprolinemia, Prodh mutations have been highly associated with schizophrenia due to its genomic location in the chromosome 22q11 locus (Liu et al., 2002); a deletion of this locus is the most

frequent interstitial deletion known and has been extensively correlated as a risk allele for schizophrenia (Jacquet et al., 2002). With evidence established for astrocyte morphological alterations and hypothesized effects of hypercortisolemia associated with schizophrenia (Tandon et al., 1996; Gispen-de Wied, 2000), the functional regulation of Prodh in astrocytes may inform our understanding of processes involved in schizophrenia.

Genes exhibiting novel glucocorticoid regulation

We also identified novel glucocorticoid regulation for a subset of mRNAs not previously reported (36 genes, Table 2). These previously unreported glucocorticoid-regulated mRNAs may have unique actions in astrocytes. Among genes demonstrating novel glucocorticoid regulation that were validated by qPCR, we identified a number of mRNAs associated with signaling pathways that may be specifically relevant to astrocyte physiology (Gjb6, Adora2b, Mapk4, Folh1, Wnt7a).

Gjb6 encodes for the gap junction protein connexin-30. Astrocyte gap junctions are known to mediate intercellular communication between astrocytes. In addition to a number of reports on Connexin-30/Connexin-43 double knockout phenotypes (e.g. weakened blood-brain barrier (Ezan et al., 2012), demyelinating phenotype (Lutz et al., 2009), disrupted excitatory synaptic transmission (Rouach et al., 2008)), connexin-30 has been independently associated with inherited deafness (Grifa et al., 1999) and hippocampal interastrocytic communication (Gosejacob et al., 2011). Changing gap junction levels may alter the passive diffusion potential between cells, indirectly

regulating cell size, ionic flux, and cellular uptake based on resulting changes in osmolarity (for review on glial gap junctions, see (Scemes and Spray, 2009)). Recent studies have suggested GR regulation of astrocyte gap junctions may contribute to the increases in depressive-like behavior, exemplified by downregulation of the primary astrocyte gap junction protein Cx43 in a chronic unpredictable stress animal model of depression (Sun et al., 2011). Although Cx43 mRNA does not appear to be glucocorticoid sensitive in our study, Cx30 mRNA expression appears to be strongly regulated by glucocorticoids (Figures 2.1, 2.3). The functional distinctions between the two astrocyte connexins are not well characterized. Cx43 and Cx30 are thought to be partially functionally redundant but also have some unique roles and interactions. Both connexins can act as homomeric complexes that couple astrocytes together and are also know to act as hemichannels. As a hemichannel, Cx30 is known to pass ATP (Essenfelder et al., 2004). These connexins can also form isoform-specific associations with oligodendrocyte connexins; Cx30 associates with Cx32, and Cx43 associates with Cx47 (Nagy et al., 2003). In terms of permeability, Cx43 gap junctions and Cx30 gap junctions exhibit different gating properties (Banach and Weingart, 1996; Valiunas et al., 1999), implying that these gap junctions passage molecules differently based on various properties such as charge or size (e.g. most frequently reported difference is permeability of common dye Lucifer yellow (Cx43 permeable, Cx30 not permeable)). Cx30 has also been associated with metabolite diffusion (e.g. glucose (Chang et al., 2008)), suggesting additional non-ionic transport may be an important role of this connexin. Given the short half-life of connexin proteins (e.g. 1.5-4 hours (Berthoud et al., 2004)), cells expressing connexins (e.g. astrocytes) may have developed gene-

dependent transcriptional mechanisms to proportionally regulate specific connexins in response to certain physiological or pathological stimuli (Oyamada et al., 2005). With regard to transcriptional regulation, Cx30 has only been associated with EGF sensitivity (Essenfelder et al., 2005). The EGF-mediated induction of Cx30 was also cell-line specific (i.e. regulated in keratinocyte cell line vs. not regulated in kidney cell line), indicating that cell-specific factors influence Gjb6 transcriptional regulation. How altering astrocyte networks and connexin ratios contributes to the action of glucocorticoids and stress axis mechanisms is yet to be determined.

Folate hydrolase (Folh1, glutamate carboxypeptidase II) converts N-acetylaspartylglutamate (NAAG) to glutamate and N-acetylapartate (NAA). NAAG acts as an mGluR3 agonist and indirectly decreases presynaptic glutamate release. Folh1 influences synaptic transmission via regulating NAAG levels (Wroblewska, 2006). As a membrane protein on the external surface of astrocytes, Folh1 can generate additional glutamate in the extracellular space that can be excitotoxic (Thomas et al., 2000). Positive regulation of Folh1 by glucocorticoids predicts a decrease in NAAG concentration and subsequent increase in free glutamate. NAAG action on astrocytes has been associated with increased cAMP and cGMP levels; increased Folh1 activity would attenuate these responses. Most therapeutic applications have investigated Folh1 inhibition (e.g. neuropathy (Zhang et al., 2006), ischemia (Slusher et al., 1999), schizophrenia (Zhou et al., 2005)); the impact of increased Folh1 activity is not well characterized.

Wnt7a is a secreted signaling molecule associated with synaptic plasticity. Wnt7a can activate adenylyl cyclase and induce CREB phosphorylation. Wnt7a acts through the Frizzled-5 (Fz5) receptor and the Lrp6 receptor, mediating excitatory synapse formation in an activity-dependent manner through local activation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (Sahores et al., 2010; Ciani et al., 2011). Wnt7a has been functionally characterized as a transforming factor that acts on the beta-catenin pathway (Caricasole et al., 2003). Little is known about the impact of Wnt7a on astrocytes, although it was recently identified as a trophic factor that promotes survival of astrocytes in culture (Foo et al., 2011). Glucocorticoid-mediated decrease of Wnt7a could result in decreased CaMKII activation and reduced synaptogenesis. Negative regulation of Wnt7a has not been reported previously; the functional impact of dynamic regulation of this molecule and its role in astrocytes are not yet known.

Although adenosine signaling is of particular interest for astrocyte biology (Fields and Burnstock, 2006), the functionality of the GPCR adenosine receptor 2B (Adora2b) remains largely uncharacterized in the brain. Adora2b has a lower affinity for adenosine than Adora1 and Adora2a; because of this difference in sensitivity, Adora2b signaling is thought to be particularly active in pathophysiological conditions where there are high levels of adenosine present (e.g. low oxygen, high metabolic demands) (Feoktistov and Biaggioni, 1997; Aherne et al., 2011). Both Adora2a and Adora2b act through the G_s protein and adenylyl cyclase to increase cAMP levels and protein kinase A (PKA) activation. Unlike Adora2a but like Adora1 (A1) and Adora3, Adora2b also acts through the G_q protein and phospholipase C (PLC) to increase protein kinase C (PKC) activity

and intracellular calcium levels. In the nervous system, A1 receptors have generally been associated with inhibitory effects on neurotransmission while A2 receptors (A2a/A2b) have generally been associated with excitatory effects. Most studies have focused on Adora2a because of selective pharmacological agents available for that receptor and not Adora2b; physiological roles are attributed to Adora2b based on a lack of response to Adora2a agonists. A previous study found indirect evidence for glucocorticoid-mediated downregulation of Adora2b in Jurkat cells (Svenningsson and Fredholm, 1997). Given that glucocorticoids reliably increased Adora2b mRNA expression in our study, our results implicate the importance of cellular context in adenosine receptor gene expression regulation by glucocorticoids. With the roles of astrocyte-associated adenosine signaling implicated in multiple physiological processes (e.g. sleep (Halassa et al., 2009), coordinating neuronal activity (Panatier et al., 2011)), understanding the selective regulation of Adora2b by glucocorticoids in astrocytes may reveal important links between adenosine signaling and glucocorticoid action.

Additional point regarding astrocyte-enriched mRNAs; comparison with glucocorticoid regulation of neuron-enriched mRNAs and oligodendrocyte mRNAs in astrocyte cultures

I described in this chapter the unexpected observations regarding apparent selective regulation of astrocyte-enriched mRNAs in astrocytes by glucocorticoids. This notion of selectivity is further emphasized when compared to the impact of glucocorticoid regulation on neuron-enriched mRNAs and oligodendrocyte-enriched mRNAs in astrocyte cultures (Cahoy 2008). Although the relative expression of cell-type-enriched

genes in the astrocyte cultures is similar (i.e. approximately 50% of neuron-enriched (~1000 mRNAs), 60% of oligodendrocyte-enriched (~1300 mRNAs), and 66% of astrocyte-enriched mRNAs expressed in cultures (~1700 mRNAs)), the percentage of dexamethasone-regulated mRNAs that are astrocyte-enriched is higher than expected compared to either the percentage of dexamethasone-regulated mRNAs that are neuron-enriched or oligodendrocyte-enriched in a manner (Figure 2.7A). Further, these other two populations do not demonstrate the temporal pattern observed among glucocorticoid-regulated mRNAs as a function of percent astrocyte-enrichment (Figure 2.7B). Together, these data support the notion that glucocorticoid regulation in astrocytes preferentially target genes whose expression is known to be enriched in astrocytes versus genes whose expression is known to be enriched in other brain cell types.

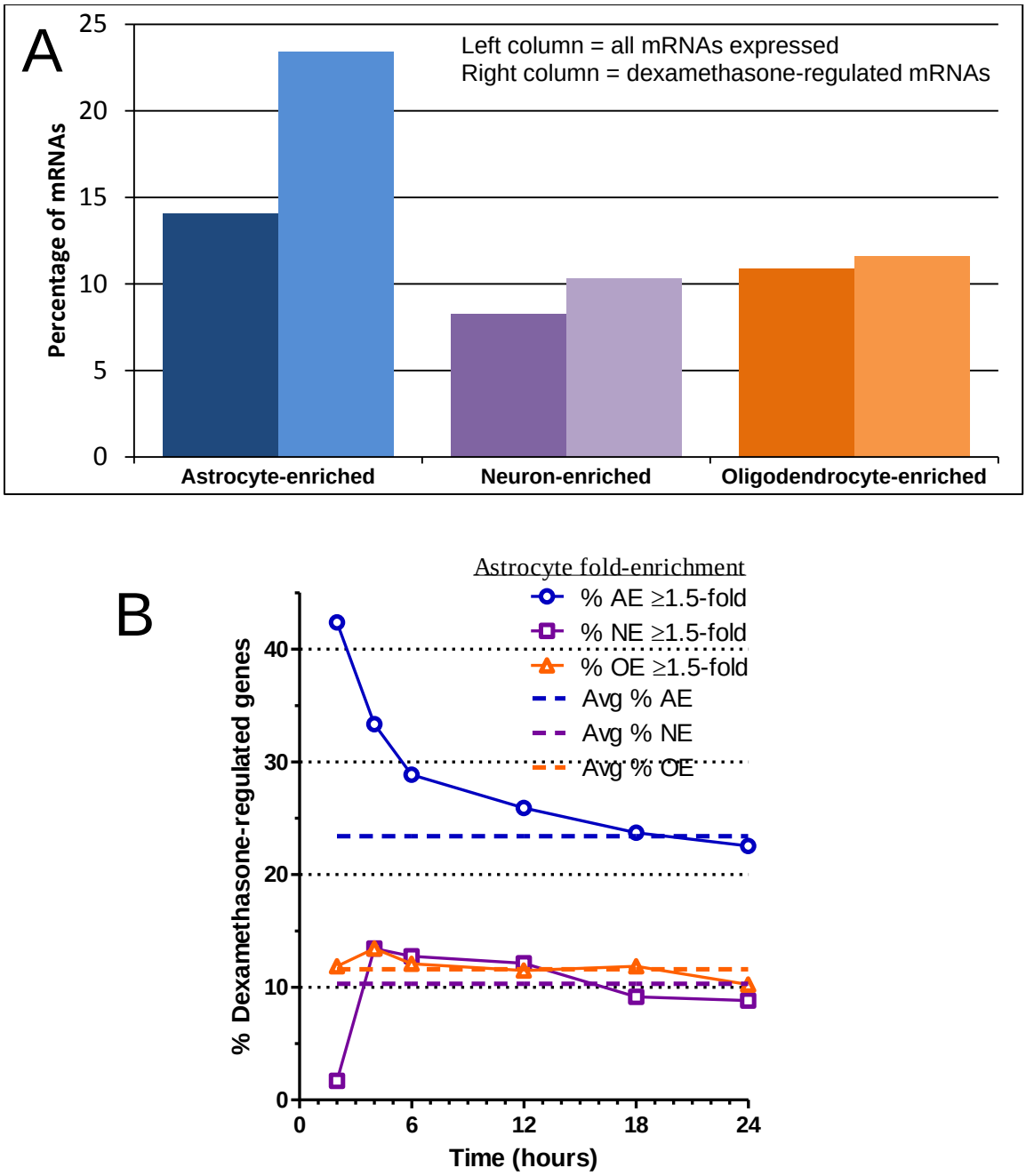


Figure 2.7: Comparison of percentage of glucocorticoid-regulated mRNAs as a function of astrocyte enrichment, neuron enrichment, and oligodendrocyte enrichment (1.5-fold enrichment). (A) Average percentages of all mRNAs expressed in astrocytes *in vitro* (left bars) and mRNAs regulated by dexamethasone in astrocytes *in* vitro (right bars) as a function of cell-enriched mRNAs of astrocytes (blue), neurons (purple), and oligodendrocytes (orange). (B) Plot of percentage of cell-type enriched mRNAs among dexamethasone-regulated mRNAs over time. Dashed lines indicate % enrichment from all time points; solid lines indicate % enrichment per time point. Note that astrocyte-enriched mRNAs represent a much larger percentage of dexamethasone-regulated mRNAs in astrocyte cultures compare to neuron-enriched mRNAs or oligodendrocyte-enriched mRNAs.

Chapter II references

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TABLE 1: Glucocorticoid-sensitive genes with reported GR-mediated regulation			
Gene Symbol	**Definition**	**Direction**	**Neural reported?**
Ada	Adenosine deaminase	Upregulated	
Bcl6	B-cell leukemia/lymphoma 6	Upregulated	
Cbs	Cystathionine beta-synthase	Upregulated	+
Ccnd2	Cyclin D2	Downregulated	
Errfi1	ERBB receptor feedback inhibitor 1	Upregulated	+
Fam107a	Family with sequence similarity 107 member A	Upregulated	+
Fkbp5	FK506 binding protein 5	Upregulated	+
Gadd45g	Growth arrest and DNA-damage-inducible 45 gamma	Upregulated	+
Hdc	Histidine decarboxylase	Upregulated	+
Hspa1a	Heat shock protein 1A	Upregulated	
Ipas/Hif3a	Inhibitory PAS domain AKA Hypoxia-inducible factor 3	Upregulated	+
Klf9	Kruppel-like factor 9	Upregulated	+
Lcn2	Lipocalin 2	Upregulated	+
Lims2	LIM and senescent cell antigen like domains 2	Upregulated	
Map3k6	Mitogen-activated protein kinase kinase kinase 6	Upregulated	
Mapk4	Mitogen-activated protein kinase 4	Upregulated	
Mertk	c-mer proto-oncogene tyrosine kinase	Upregulated	+
Mgll	Monoglyceride lipase	Upregulated	
Pdk4	Pyruvate dehydrogenase kinase, isozyme 4	Upregulated	+
Per1	Period homolog 1	Upregulated	+
Phlda1	Pleckstrin homology-like domain family A member 1	Downregulated	
Pim3	Proviral integration site 3	Upregulated	
Prodh	Proline dehydrogenase	Upregulated	
Rhou	Ras homolog gene family member U	Upregulated	
S3-12	Plasma membrane associated protein S3-12	Upregulated	
Sesn1	Sestrin 1	Upregulated	
Sgk1	Serum/glucocorticoid regulated kinase 1	Upregulated	+
Sphk1	Sphingosine kinase 1	Upregulated	
Sult1a1	Sulfotransferase family 1A phenol-preferring member 1	Upregulated	+
Tsc22d3	TSC22 domain family member 3	Upregulated	+
Txnip	Thioredoxin interacting protein	Upregulated	
Zbtb16	Zinc finger and BTB domain containing 16	Upregulated	

TABLE 2: Glucocorticoid-sensitive genes demonstrating novel GR-mediated regulation		
Gene Symbol	**Definition**	**Direction**
1810011O10Rik	RIKEN cDNA 1810011O10 gene	Upregulated
9930004G02Rik	RIKEN cDNA 9930004G02 gene	Upregulated
Accn1	Amiloride-sensitive cation channel 1 neuronal (degenerin)	Upregulated
Acsl3	Acyl-CoA synthetase long-chain family member 3	Upregulated
Axud1	AXIN1 up-regulated 1	Upregulated
Bcat1	Branched chain aminotransferase 1 cytosolic	Upregulated
C030009J22Rik	RIKEN cDNA C030009J22 gene	Upregulated
Chk	Choline kinase alpha	Upregulated
Cldn2	Claudin 2	Upregulated
E230024B12Rik	RIKEN cDNA E230024B12 gene	Upregulated
Fam46b	family with sequence similarity 46, member B (Fam46b)	Upregulated
Folh1	Folate hydrolase 1	Upregulated*
Galntl4	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase-like 4	Upregulated
Gjb6	Gap junction protein beta 6	Upregulated
Gm22	Gene model 22	Upregulated
Got1l1	Glutamic-oxaloacetic transaminase 1-like 1	Upregulated
Hspb1	Heat shock protein 1	Upregulated
Kcnt1	Potassium channel subfamily T member 1	Upregulated
LOC385279	LOC385279	Upregulated
Lpin3	Lipin3	Upregulated*
Lrrc8	Leucine rich repeat containing 8A	Upregulated
Mical2	Microtubule associated monoxygenase, calponin and LIM domain containing 2	Upregulated
Papss2	3'-phosphoadenosine 5'-phosphosulfate synthase 2	Upregulated*
Pex11a	Peroxisomal biogenesis factor 11a	Upregulated
Pgm5	Phosphoglucomutase 5	Upregulated
Rassf4	Ras association (RalGDS/AF-6) domain family member 4	Upregulated
Rbpms	RNA binding protein gene with multiple splicing	Upregulated
Scel	Sciellin	Upregulated
Slc10a6	Solute carrier family 10 (sodium/bile acid cotransporter family) member 6	Upregulated
Slc24a4	Solute carrier family 24 (sodium/potassium/calcium exchanger) member 4	Upregulated
Slc25a33	Solute carrier family 25 (pyrimidine nucleotide carrier) member 33	Upregulated
Smox	Spermine oxidase	Upregulated
Spsb1	SplA/ryanodine receptor domain and SOCS box containing 1	Upregulated
Tcfcp2l1	Transcription factor CP2-like 1	Upregulated
Tilz3c	TSC22-related-inducible leucine zipper 3c	Upregulated
Tmem200a	Transmembrane protein 200A	Downregulated

*Previously reported as glucocorticoid- insensitive

TABLE 3: Gene ontology associations of glucocorticoid-sensitive astrocyte-enriched genes				
Illumina ID	**Symbol**	**Gene Name**	**Astrocyte enrichment***	**Direction**
GO term: sulfur metabolism				
ILMN_1253588	Papss2	3'-phosphoadenosine 5'-phosphosulfate synthase 2	32.7	Upregulated
ILMN_2750558	Mamdc2	MAM domain containing 2	16.2	Downregulated
ILMN_1230318	Cbs	cystathionine beta-synthase	23.4	Upregulated
ILMN_2669416	Mgst1	microsomal glutathione S-transferase 1	19.2	Upregulated
ILMN_2776008	Gstk1	Glutathione S-transferase kappa 1	4.7	Upregulated
GO term: nitrogen biosynthetic process				
ILMN_2722716	Atp1a2	ATPase, Na+/K+ transporting, alpha 2 polypeptide	29.8	Upregulated
ILMN_1230318	Cbs	cystathionine beta-synthase	23.4	Upregulated
ILMN_2636666	Prodh	proline dehydrogenase	29.4	Upregulated
ILMN_1219442	Slc7a2	solute carrier family 7 (cationic amino acid transporter, y+ system), member 2	21.2	Upregulated
ILMN_1243741	Rora	RAR-related orphan receptor alpha	4.8	Upregulated
ILMN_2644496	Glul	Glutamate-ammonia ligase (glutamine synthetase)	8.5	Upregulated
GO term: oxidation reduction				
ILMN_1222765	Adhfe1	alcohol dehydrogenase, iron containing, 1	22.6	Upregulated
ILMN_1222734	Aldh1a1	aldehyde dehydrogenase family 1, subfamily A1	11.7	Upregulated
ILMN_1214498	Cyp2d22	cytochrome P450, family 2, subfamily d, polypeptide 22	16.8	Upregulated
ILMN_1251504	Cyp4v3	cytochrome P450, family 4, subfamily v, polypeptide 3	13.6	Upregulated
ILMN_2766037	Dio2	deiodinase, iodothyronine, type II	56	Upregulated
ILMN_1250917	Fmo1	flavin containing monooxygenase 1	19.7	Upregulated
ILMN_2636666	Prodh	proline dehydrogenase	29.4	Upregulated
ILMN_2644092	Aass	Aminoadipate-semialdehyde synthase	6.1	Upregulated
ILMN_1258158	Aldh6a1	Aldehyde dehydrogenase family 6, subfamily A1	4.4	Upregulated

*Cahoy, et. al., *J Neurosci.* (2008)

Chapter III

Acute and chronic glucocorticoid treatments regulate astrocyte-enriched mRNAs *in vivo*

ABSTRACT

Alterations in glucocorticoid signaling have been implicated in a range of diseases, including pathologies of the stress axis and mental health disorders. Previous studies have primarily interpreted gene expression regulation by glucocorticoids in terms of impact on neurons; however, little is known about the corresponding impact of glucocorticoids on glia and specifically astrocytes *in vivo*. Previous microarray experiments identified glucocorticoid-sensitive mRNA transcripts in primary astrocyte cell culture. Here, we have tested whether elevated glucocorticoids similarly regulated a subset of these mRNAs *in vivo* following acute and chronic corticosterone exposure in mice. Acute corticosterone exposure was achieved by a single injection of 10 mg/kg corticosterone, and tissue samples were harvested at two hours post-injection. Chronic corticosterone exposure was achieved by administering 10 mg/mL corticosterone via drinking water for two weeks. Gene expression was then assessed by qPCR in two brain regions (prefrontal cortex and hippocampus) and by *in situ* hybridization. A majority of mRNAs regulated by glucocorticoids in astrocyte *in vitro* were similarly

regulated by acute and/or chronic glucocorticoid exposure *in vivo*. Select mRNAs were associated with astrocyte localization based on published astrocyte-enriched transcriptome data (Cahoy 2008) and/or in situ hybridization expression patterns similar to the pan-astrocyte marker Aldh1l1. Our findings suggest that glucocorticoids regulate gene expression in the brain in a cell type-dependent fashion *in vivo*. Future research on the impact of stress and glucocorticoids in the brain would profit by a more thorough understanding of these cell type-dependent actions.

INTRODUCTION

At the level of the organism, stress occurs via environmental or psychological stimuli (stressors) that disrupt homeostasis. The classic stress response involves the integration of signals within the brain that are ultimately communicated to the rest of the body through the hypothalamic-pituitary-adrenal (HPA) axis. Glucocorticoids are the major output hormones following activation of the HPA axis that mobilize resources and energy, enabling the organism to immediately adapt to the stressor (Lupien et al., 2009). The stress response is critical for appropriate responsiveness to external stimuli. Disruption of appropriate stress signaling (e.g. altered HPA axis signaling, chronic stress) can have drastic impact on well-being and has been linked to numerous disease states, including Cushing's disease (HPA hyperactivity (Newell-Price et al., 2006)), Addison's disease (HPA hypoactivity (Ten et al., 2001)), and multiple psychiatric disorders, including depression and bipolar disorder (McEwen, 2005). Given the importance of the stress response to health and disease, there is great interest in understanding the impact of glucocorticoids on the brain, both in terms of acute

exposure (e.g. stress action) and chronic exposure (e.g. disease states).

At the cellular level, glucocorticoids are known to act as transcriptional regulators through interaction with their receptors, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR) (Strachan and Read, 1999). GR is associated with actions at higher levels of corticosteroids (e.g. stress response). Glucocorticoids readily pass through the cell membrane and bind their inactive receptors that are complexed with chaperone proteins in the cytoplasm. Ligand-bound receptors dimerize to an active form that translocates to the nucleus (mechanisms reviewed in (Vandevyver et al., 2012)). The activated receptor complex binds to DNA sequences known as glucocorticoid response elements (GREs) and upregulates or downregulates target gene expression. Glucocorticoids are known to regulate many mRNAs in the brain. While numerous studies have reported *in vitro* findings of glucocorticoid regulation on a gene by gene basis, fewer studies have looked at glucocorticoid regulation in the brain at the level of the transcriptome *in vivo*. Gene expression profiling studies of hippocampal slices indicate that numerous mRNAs are also regulated by glucocorticoids in brain tissue (Datson et al., 2001) and that the observed regulation varies by time of exposure (Morsink et al., 2006).

The brain consists of many diverse cell types (e.g. neurons, astrocytes, oligodendrocytes, microglia) that vary significantly in function. All of these cell types are known to express glucocorticoid receptors and are thus responsive to glucocorticoid signaling (McEwen et al., 1968; Vielkind et al., 1990; Sierra et al., 2008). Interpretations

of previous studies of glucocorticoid action in the brain focused on how glucocorticoid mRNA regulation affects neurons; how these stress hormones alter gene expression in cell types other than neurons remains largely unknown. We recently characterized glucocorticoid regulation of mRNAs in astrocytes *in vitro*, defining both cell-common and astrocyte-specific glucocorticoid regulated mRNA targets (Carter et al., 2012). However, the physiological relevance of these findings in the intact brain is currently unknown. Here we have investigated whether a subset of mRNAs regulated by glucocorticoids in astrocytes *in vitro* are responsive to glucocorticoids *in vivo* following acute and chronic glucocorticoid exposure, including mRNAs reported to be enriched in astrocytes (Cahoy et al., 2008).

MATERIALS AND METHODS

Animal treatments and brain tissue collection

Adult C57B/6 male mice were obtained from Charles River. All animal procedures at the University of Michigan were approved by UCUCA (University Committee on Use and Care of Animals) and monitored by ULAM (Unit for Laboratory Animal Medicine). For all experiments, mice were group housed (n = 3 or 5 per cage). Mice were housed for one week under basal conditions (14:10 light dark cycle) before experiments. To collect tissue samples, mice were euthanized using cervical dislocation and decapitation. For both the acute and chronic glucocorticoid exposure protocols, upon euthanasia, brains were removed and bisected along the mid-sagittal plane. One brain hemisphere was frozen in chilled isopentane (-35C) and stored at -80C prior to tissue sectioning and in situ hybridization analyses. The remaining brain

hemisphere was further dissected on wet ice to collect hippocampi and prefrontal cortex tissues. These tissues were frozen immediately on dry ice and stored at -80C prior to RNA extraction. Corticosterone (Cort, Cat. #C2505) was obtained from Sigma Aldrich.

Acute Glucocorticoid Mouse model

Mice were injected (s.c.) with 10 mg/kg corticosterone or vehicle (1% ethanol solution) one hour after lights on. Mice were returned to their home cages for specified time durations prior to euthanasia and sample collection.

Chronic Glucocorticoid Mouse model

Chronic corticosterone exposure was produced based on previously published protocols (Karatsoreos et al., 2010). Mice were group housed and given access to drinking water containing 10 mg/mL corticosterone or vehicle for 2 weeks (n=5/group). Drinking solutions were replaced 3 times over course of 2 weeks (every 4-5 days). On day 14, all mice were weighted on an analytic balance. Mice were sacrificed and trunk blood was collected. Adrenal glands, spleen and thymus tissues were dissected on ice and weighed on an analytic balance.

Plasma Corticosterone assay

Immediately following decapitation, trunk blood was collected and mixed with 0.5M EDTA (pH 8.0, final concentration with blood ~10uM) and stored immediately on wet ice. Blood samples were subsequently spun in a table-top centrifuge (3500xg for 10 minutes at 4C). Blood plasma was transferred to a fresh 1.5mL tube and stored at -

80C. Plasma corticosterone concentrations were measured using a corticosterone double antibody radioimmunoassay kit (MP Biomedicals, Cat. #07120102). Individual samples were measured in triplicate, including standard curve controls; the average value was used for subsequent calculations.

RNA isolation

Total RNA samples were isolated from tissues using Trizol reagent per manufacturer's protocol (Invitrogen), and RNA concentrations were obtained using a Nanodrop spectrophotometer (Thermo Scientific).

Real Time PCR analysis

RNA samples (500ng-1 ug) were converted to cDNA using Superscript II via random hexamer priming (Invitrogen). ~50% of each cDNA reaction was used for Applied Biosystems (ABI) Taqman mRNA qPCR assays in custom Low-Density Array (TLDA) format (ABI #4346799) with Taqman reagents (ABI #4440048). The Taqman arrays were processed on an Applied Biosystems Viia7 instrument according to manufacturer protocols. mRNAs measured were selected based on the following of factors as defined previously (Carter et al., 2012); (1) glucocorticoid sensitivity vs. insensitivity in primary astrocyte cultures, (2) magnitude of glucocorticoid regulation, (3) previous reporting of glucocorticoid sensitivity vs. novel regulation, and (4) previous association with astrocyte action or astrocyte-enrichment. mRNAs were measured in technical triplicate per sample. Differential expression analysis was performed using the delta-delta-Ct method ((Livak and Schmittgen, 2001), β-actin as control reference) using Statminer

software (Integromics). An average of Ct-values from technical replicates was taken as the Ct-value for each gene measurement; individual Ct-values identified as outliers via the Grubbs' outlier test (CIT) were excluded from downstream analyses. mRNA differential expression was defined statistically significant for p-value<0.05. Specific Taqman mRNA assays used in this analysis are: Actb (Mm01205647_g1), Adora2b (Mm00839292_m1), Aldh1l1 (Mm00550947_m1), Atp6v1b2 (Mm00431987_m1), Ch25h (Mm00515486_s1), Egr2 (Mm00456650_m1), Fgfr1 (Mm00438930_m1), Fgfr3 (Mm00433294_m1), Fkbp5 (Mm00487401_m1), Folh1 (Mm00489655_m1), Foxo1 (Mm00490672_m1), Gap43 (Mm00500404_m1), Gfap (Mm01253033_m1), Gja1 (Mm00439105_m1), Gjb6 (Mm01317508_m1), Glul (Mm00725701_s1), Hdac7 (Mm00469520_m1), Klf9 (Mm00495172_m1), Mapk4 (Mm00554001_m1), Mertk (Mm00434920_m1), Pdk4 (Mm01166879_m1), Per1 (Mm00501813_m1), Phlda1 (Mm00456345_g1), Prodh (Mm00448401_m1), Sgk1 (Mm00441380_m1), Slc1a2 (Mm00441457_m1), Slc1a3 (Mm00600697_m1), Sult1a1 (Mm01132072_m1), Syn2 (Mm00449780_m1), Txnip (Mm00452393_m1), Wnt7a (Mm00437354_m1).

In situ hybridization

Brain hemisections (10um thick) were cut on a cryostat, mounted on Superfrost microscope slides (2 sections per slide), and stored at –80C prior to ISH experiments. In situ hybridization probes were designed for several mRNAs in our study. Specific mRNA domains that displayed low levels of nucleotide homology were amplified by PCR (Native Taq DNA polymerase, Invitrogen) using primers designed with NCBI Primer Blast software (Supplementary Table 3.2). PCR amplicons were cloned into

pCR-II-TOPO Vector and grown in Top 10 cells (Invitrogen). The identity of all clones was verified by Sanger Sequencing (DNA sequencing core, University of Michigan). Linearized plasmids were used to create ^{35}S-RNA probes using T7 or SP6 RNA polymerases (varied by plasmid). Briefly, linearized plasmid (100ng-500ng) was combined with 1ul each of ATP, GTP, and CTP (10mM), 1 ul RNAse inhibitor, 1.66 ul DTT (100 uM), 4 ul 5x Transcription buffer (Promega) and 7.8 ul of ^{35}S-UTP (12.5 uCi/ul, Perkin Elmer). Probes were purified using BioRad P-6 columns, effluent counted and mixed with hybridization solution (~2 x 10^6 cpms of probe in 40 ul hybridization buffer per slide). All remaining tissue processing steps were performed according to published lab protocols (Carter et al., 2010). Hybridization specificity was determined in control experiments using sense probes which failed to yield autoradiographic signals above background (Supplemental Figure 3.1).

Densitometry analysis

Anatomical boundaries of the prefrontal cortex and hippocampus where defined based upon comparisons of cresyl violet stained tissue sections to the mouse brain atlas of Paxino and Franklin (Paxinos and Franklin, 2001). Autoradiograms from ISH were scanned with a ScanMaker 1000XL Pro Flatbed Scanner (Microtek, Carson, CA) using SilverFast Ai Imaging Software (LaserSoft Imaging, Sarasota, FL). The scanned images were analyzed based on optical density (OD) measurements using the ImageJ software (Version 1.45S, NIH). For each probe, a normalized OD value for each section image was determined by subtracting a background value from the OD value (Background= background mean + 3.5*standard deviation of background mean). Background

measurements were taken from a non-tissue area of each film. 4 section images were measured for each brain region per animal; an average normalized value of the 4 sections per probe was used for downstream analyses.

Statistical analysis

For comparisons between groups in terms of organ weight, body weight, corticosterone levels, and ISH analysis, measurements were compared using a two-tailed student's t-test; significance was defined as $p<0.05$.

RESULTS

Single corticosterone injection results in transient rise of corticosterone plasma levels

In order to determine the plasma concentrations of corticosterone *in vivo* following a single bolus injection of corticosterone, mice were injected with 10 mg/kg Corticosterone, and blood samples collected at 1 hour, 2 hours, and 4 hours. We found that a 10 mg/kg dose of corticosterone increased plasma corticosterone concentration to a supraphysiological level by one hour. The plasma corticosterone level remained significantly elevated at 2 hours at levels at a magnitude consistent with acute stressor exposure (Ma et al., 1997; McClennen et al., 1998) and then returned to baseline by 4 hours (Figure 3.1A). In order to assess the impact of elevated glucocorticoids over the greatest amount of time, we chose to analyze gene expression two hours post-injection in subsequent experiments; a replicate study yielded a similar corticosterone elevation (Figure 3.1B).

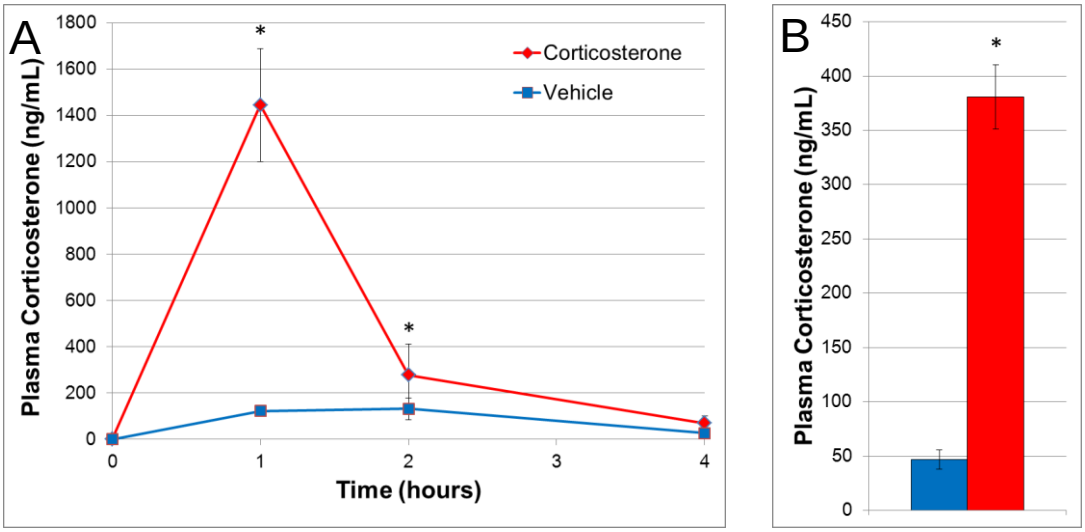


Figure 3.1: Single injection of 10mg/kg corticosterone results in transient rise in corticosterone levels in mice. (A) Time course of plasma corticosterone levels following injection (n=3/group) (B) Plasma corticosterone levels in replicate 2 hour exposure for use in qPCR experiments (n=8/group). Error bars = SEM. * = p<0.05.

Single injection of corticosterone results in acute regulation in vivo of mRNAs regulated by glucocorticoids in astrocytes in vitro

mRNA levels for 30 genes were measured in mRNA samples derived from prefrontal cortex and hippocampus collected 2 hours post-Corticosterone treatment by quantitative PCR using Taqman Low Density Arrays (TLDAs). 18 mRNAs were measured based on glucocorticoid-sensitivity in astrocytes *in vitro* (Figure 3.2). In total, 6 mRNAs were differentially expressed in both cortex and hippocampus following acute corticosterone treatment (upregulated; Fkbp5, Gjb6, Klf9, Pdk4, Sgk1, Sult1a1). In terms of differential regulation by acute corticosterone between brain regions, 2 mRNAs were regulated in cortex but not in hippocampus (upregulated: Txnip; downregulated: Phlda1), and 6 mRNAs were regulated in hippocampus but not cortex (upregulated: Adora2b, Egr2; downregulated: Hdac7, Prodh, Slc1a2, Wnt7a). 15 mRNAs that have been reported to be regulated by glucocorticoids *in vitro* (listed in (Carter et al., 2012)) were not

statistically regulated in either brain region 2 hours post-corticosterone treatment *in vivo* (previously reported upregulation: Ch25h, Folh1, Foxo1, Gap43, Glul, Mapk4, Mertk, Per1, Syn2; previously reported downregulation: Atp6v1b2, Gfap). 3 mRNAs were regulated by acute corticosterone treatment *in vivo* that were not regulated or regulated in the opposite direction by glucocorticoids in astrocytes *in vitro* (downregulated: Egr2, Hdac7, Slc1a2). The magnitude of regulation ranged from 3.15-fold upregulation (Sgk1 in hippocampus) to 1.55-fold downregulation (Egr2 in hippocampus). Complete numerical values of regulation by acute corticosterone exposure *in vivo* and corresponding statistical significances are listed in Supplemental Table 3.1.

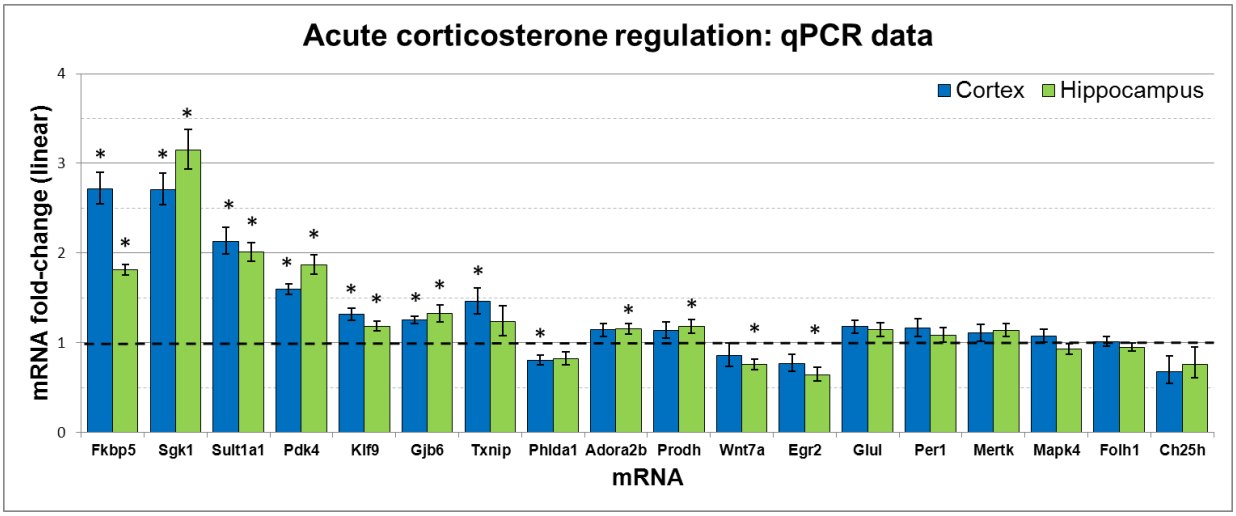


Figure 3.2: Acute corticosterone exposure regulates select mRNAs in hippocampus and cortex. Gene expression fold-changes for mRNAs regulated by glucocorticoids in astrocytes *in vitro*(CIT) in frontal cortex and hippocampus induced by 2 hour exposure following injection of 10 mg/kg corticosterone vs. saline control *in vivo* (n=8 per group). Dashed line indicates no change compared to vehicle. Error bars = SEM. * = $p<0.05$.

Mice given access to drinking water containing corticosterone for 2 weeks demonstrate hormonal and organ changes consistent with acute and chronic glucocorticoid elevation

Plasma corticosterone levels assessed on the morning after 14 days of treatment revealed statistically elevated corticosterone in the corticosterone-treated group relative

to the vehicle-treated group (Figure 3.3A). Chronic corticosterone-treated mice also had significantly reduced thymus, adrenal, and spleen organ weights compared to vehicle-treated mice (Figure 3.3B). There was no difference in the total body weight between treatment groups (Figure 3.3C).

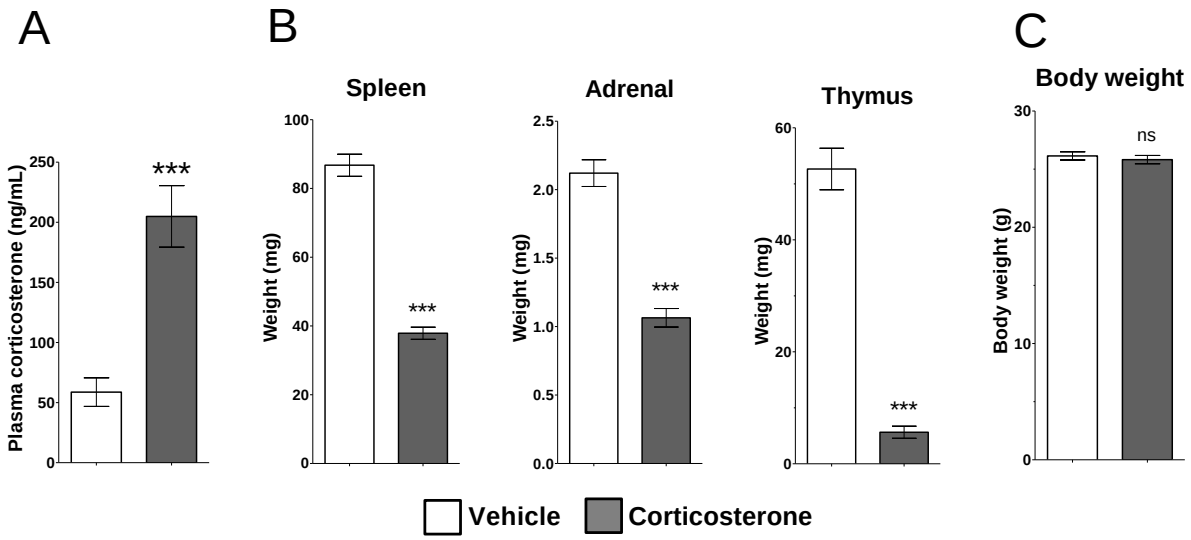


Figure 3.3: Chronic corticosterone administration via drinking water results in acute and chronic corticosterone elevations. (A) Plasma corticosterone levels after two weeks exposure to 10 mg/mL corticosterone vs. vehicle. (B) Weights of corticosterone-sensitive organs after chronic corticosterone administration. (C) Body weight of mice after chronic corticosterone administration. N=5 per group. Error bars = SEM. ***=p<0.0001. ns= not significant.

Chronic corticosterone exposure results in regulation in vivo of mRNAs regulated by glucocorticoids in astrocytes in vitro

mRNA levels were measured by quantitative PCR using Taqman Low Density Arrays in RNA samples derived from two brain regions (prefrontal cortex, hippocampus) of chronic corticosterone and vehicle treated mice (Figure 3.4). 15 mRNAs were regulated in at least one brain region in a manner consistent with previously reported glucocorticoid regulation *in vitro* (e.g. (Carter et al., 2012)) (upregulated: Adora2b,

Fkbp5, Folh1, Gap43, Gjb6, Glul, Mertk, Prodh, Sgk1, Sult1a1, Syn2; downregulated: Ch25h, Gfap, Gja1, Wnt7a) (Figure 3.4A).

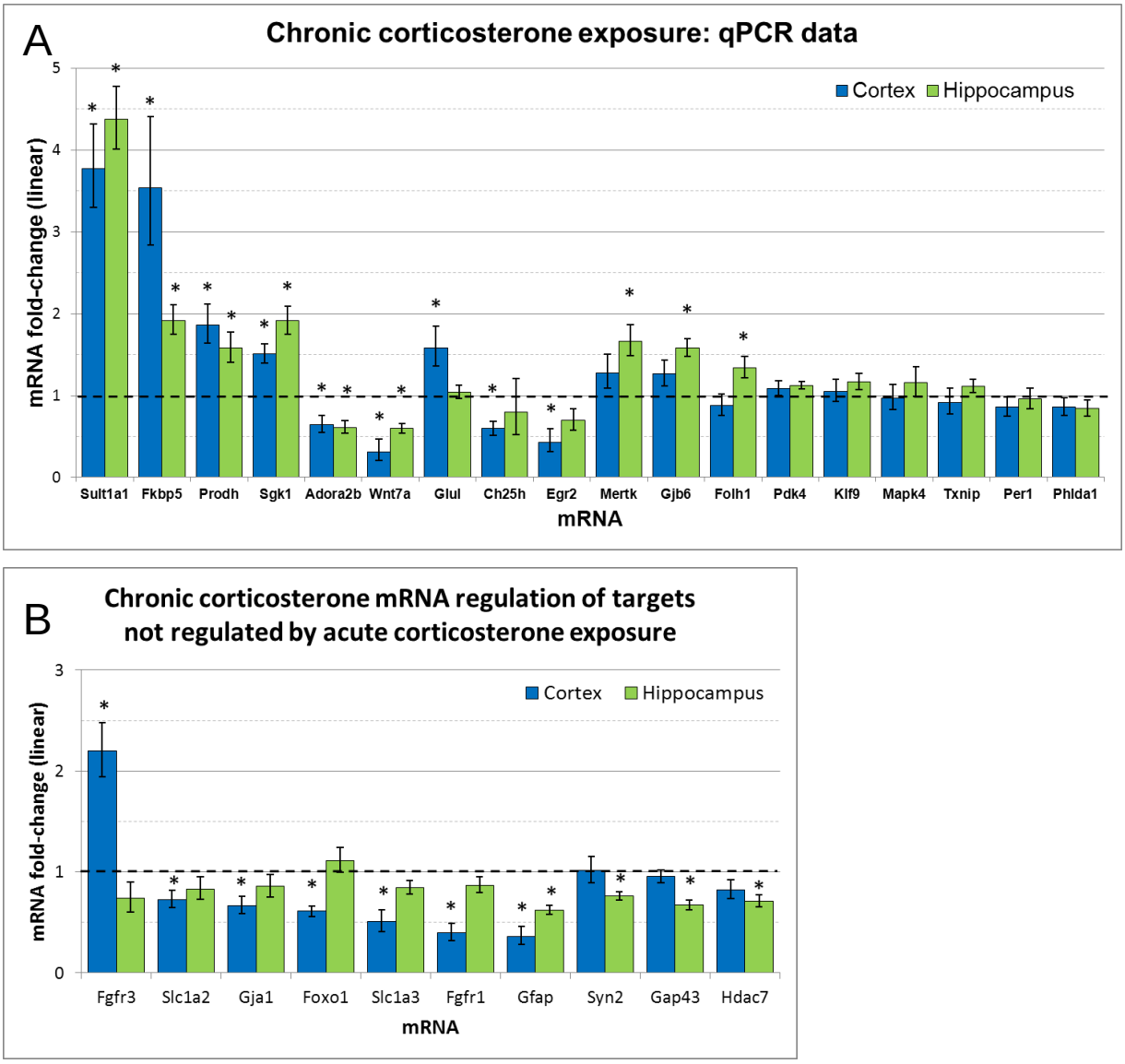


Figure 3.4: Chronic corticosterone exposure regulates select mRNAs in hippocampus and cortex. Linear gene expression fold-changes in frontal cortex and hippocampus induced by chronic corticosterone exposure for 2 weeks vs. vehicle control (n=8 per group). (A) Fold-changes of mRNAs regulated by glucocorticoids in astrocytes *in vitro*. (B) Fold-changes of mRNAs not regulated by glucocorticoids in astrocytes *in vitro*. Dashed line indicates no change compared to vehicle. Error bars = SEM. * = $p<0.05$.

7 mRNAs were regulated by chronic corticosterone exposure in both cortex and hippocampus (upregulated: Adora2b, Fkbp5, Prodh, Sgk1, Sult1a1; downregulated:

Gfap, Wnt7a). 9 mRNAs were regulated by chronic corticosterone exposure in cortex but not in hippocampus (upregulated: Ch25h, Egr2, Fgfr3, Foxo1, Glul; downregulated: Ch25h, Fgfr3, Gja1, Slc1a2, Slc1a3); 6 mRNAs were regulated by chronic corticosterone exposure in hippocampus but not cortex (upregulated: Folh1, Gap43, Gjb6, Mertk, Syn2; downregulated: Hdac7). 7 mRNAs that have been reported to be regulated *in vitro* were not regulated in either region by chronic corticosterone exposure *in vivo* (Atp6v1b2, Klf9, Mapk4, Per1, Pdk4, Phlda1, Txnip CITs). 7 mRNAs were regulated by chronic corticosterone treatment *in vivo* that were not regulated or regulated in the opposite direction by glucocorticoids in astrocytes *in vitro* (upregulated: Egr2, Fgfr3, Foxo1; downregulated: Fgfr1, Hdac7, Slc1a2, Slc1a3) (Figure 3.4B). Magnitude of regulation ranged from 4.38-fold upregulation (Sult1a1 in hippocampus) to 3.24-fold downregulation (Wnt7a in frontal cortex). Complete numerical values of regulation by chronic corticosterone exposure *in vivo* and corresponding statistical significance are listed in Supplemental Table 3.1.

Select mRNAs regulated by chronic corticosterone exposure have expression patterns consistent with astrocyte marker Aldh1l1

Differential regulation of select mRNAs was further assessed in the prefrontal cortex and hippocampus by semi-quantitative radioactive in situ hybridization (Figure 3.5). Of the 6 mRNAs examined, 3 mRNAs were statistically regulated in at least one brain region in the same direction as the qPCR data (upregulated: Fkbp5, Gfap, Gjb6). 2 mRNAs demonstrated non-significant trends in the same direction as the qPCR data (upregulated: Glul, downregulated Gja1), and 1 mRNA demonstrated differential

expression in at least one brain region that was not observed by qPCR (upregulated: Aldh1l1). In terms of hippocampal expression patterns between chronic corticosterone treatment and vehicle treatment, these mRNA showed consistent global expression patterns between groups (Figure 3.5C).

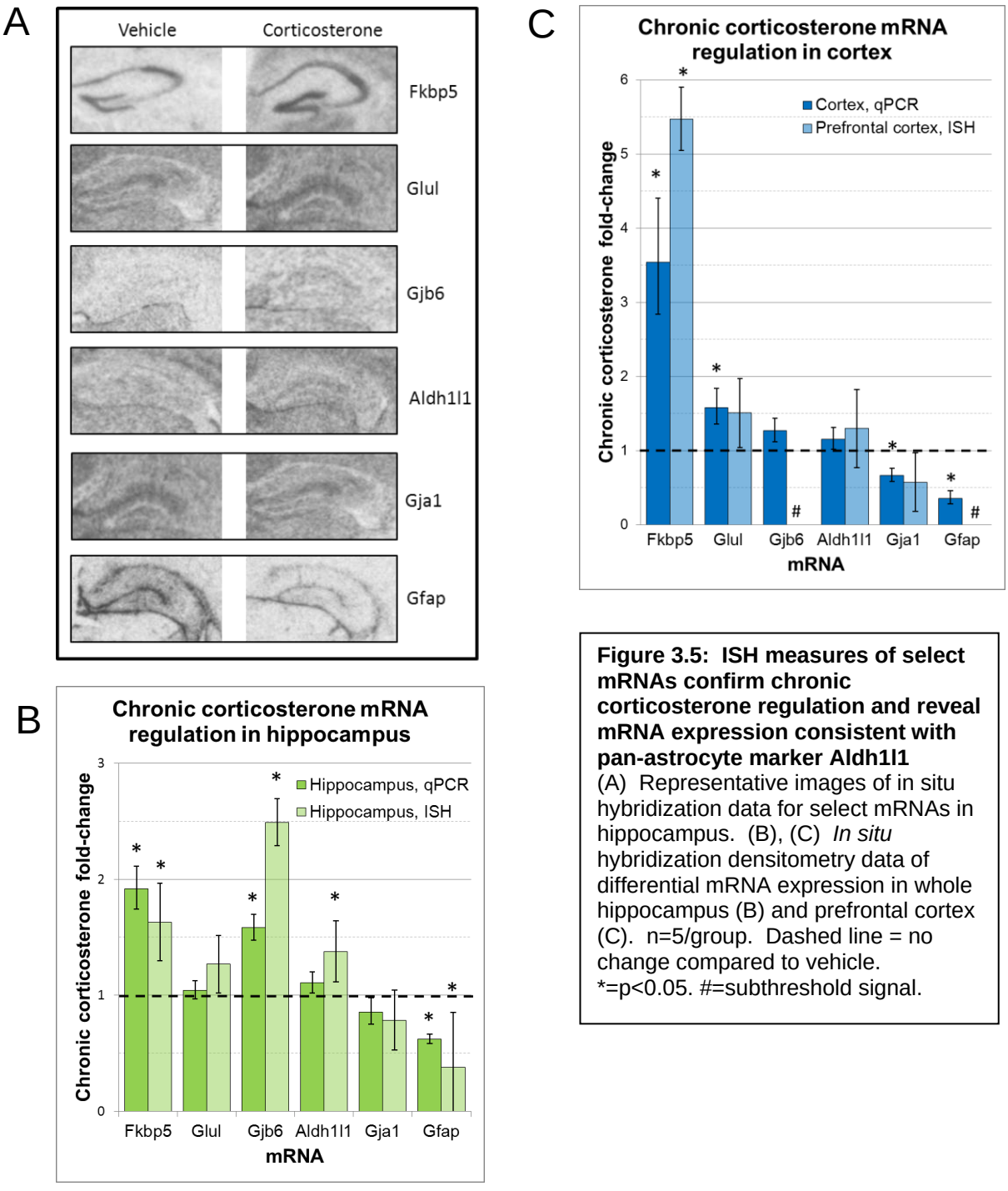


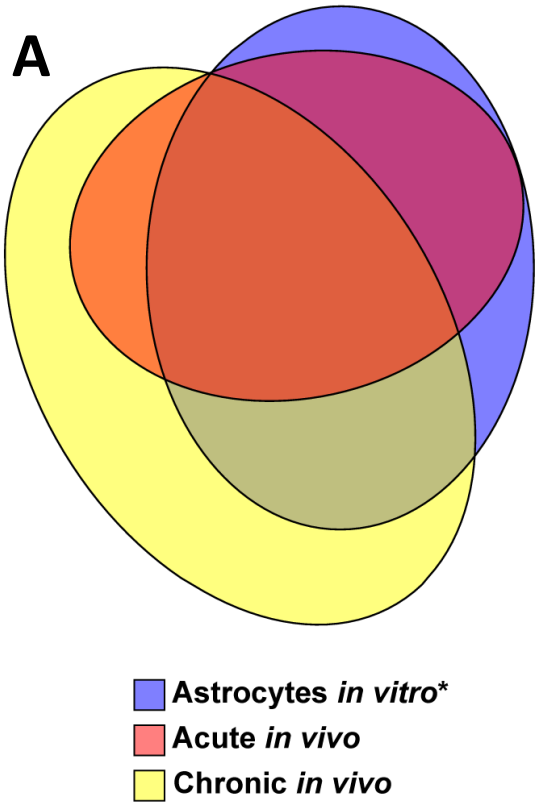
Figure 3.5: ISH measures of select mRNAs confirm chronic corticosterone regulation and reveal mRNA expression consistent with pan-astrocyte marker Aldh1l1
(A) Representative images of in situ hybridization data for select mRNAs in hippocampus. (B), (C) *In situ* hybridization densitometry data of differential mRNA expression in whole hippocampus (B) and prefrontal cortex (C). n=5/group. Dashed line = no change compared to vehicle. *=p<0.05. #=subthreshold signal.

Fkbp5 appeared predominantly expressed in pyramidal cell subfields, while expression of Gfap, Gja1, Gjb6, and Glul was noticeably lacking in the pyramidal cell regions but displayed diffuse levels of expression in molecular cell regions. This latter anatomical distribution was highly similar to the pan-astrocytic marker Aldh1l1.

Acute corticosterone exposure in vivo and chronic corticosterone exposure in vivo regulate distinct sets of mRNAs among mRNAs regulated by glucocorticoids in astrocytes in vitro

Comparison of select mRNAs measured by qPCR that were regulated by glucocorticoids in astrocytes *in vitro* (18 mRNAs, (Carter et al., 2012)), acute corticosterone exposure *in vivo* (14 mRNAs), or chronic corticosterone exposure *in vivo* (22 mRNAs) reveals significant overlap yet also distinct patterns between conditions (*in vitro* + acute overlap: 10 mRNAs, *in vitro* + chronic overlap: 12 mRNAs, acute + chronic overlap: 10 mRNAs, Figure 3.6). 8 mRNAs were regulated in all scenarios; all mRNAs regulated by acute corticosterone exposure *in vivo* were also regulated in one of the other paradigms (Figure 3.6A). For mRNAs regulated in multiple conditions, the directionality of glucocorticoid regulation was fairly consistent across conditions (Figure 3.6B).

A



KEY	
	Upregulated
	Downregulated
	Not regulated
C	Cortex
H	Hippocampus

B

mRNA	*In vitro**	*Acute*	*Chronic*
Fkbp5		C/H	C/H
Gjb6		C/H	H
Prodh		H	C/H
Sgk1		C/H	C/H
Sult1a1		C/H	C/H
Adora2b		H	C/H
Egr2		H	C
Klf9		C/H	
Pdk4		C/H	
Txnip		C	
Folh1			H
Glul			C
Mertk			H
Mapk4			
Per1			
Wnt7a		H	C/H
Phlda1		C	
Ch25h			C
Hdac7		H	H
Slc1a2		H	C
Fgfr3			C
Fgfr1			C
Foxo1			C
Gap43			H
Gfap			C/H
Gja1			C
Slc1a3			C
Syn2			H
Aldh1l1			
Atp6v1b2			

**In vitro* = regulated ≥two-fold in astrocyte cultures at 2-hour, 4-hour, or 6-hour time point

Figure 3.6: Acute corticosterone exposure and chronic corticosterone exposure regulate distinct sets of mRNAs regulated by glucocorticoids in astrocytes in vitro. (A) Proportional Venn diagram comparing glucocorticoid regulation of mRNAs measured by qPCR among results from astrocytes *in vitro*, acute exposure *in vivo*, and chronic exposure *in vivo*. (B) Heatmap summarizing directionality of glucocorticoid regulation of mRNAs across conditions. Key summarizes table terminology. Regulated = $p<0.05$.

DISCUSSION

Glucocorticoids are important mediators of the classic stress response. Previous reports have characterized extensive glucocorticoid-mediated regulation in the CNS via *in vitro* systems and brain slices, indicative of broad impact of stress hormones on the brain. Although many cell types in the brain express receptors for glucocorticoids (McEwen et al., 1968; Vielkind et al., 1990; Sierra et al., 2008) and are thus presumably responsive to glucocorticoid signaling, most studies have largely interpreted *in vivo* results in terms of glucocorticoid regulation in neurons; little is known about the impact of glucocorticoids on glia. We recently investigated glucocorticoid regulation in astrocytes *in vitro* and characterized extensive time-dependent gene expression changes, including regulation of astrocyte-enriched mRNAs (Cahoy et al., 2008). While this *in vitro* evidence was intriguing, physiological significance would hinge upon *in vivo* evidence of similar glucocorticoid regulation of astrocyte-enriched mRNA expression. Towards this end, we conducted a series of studies to examine the consequences of both acute and chronic glucocorticoid treatments upon a subset of mRNAs that were regulated by glucocorticoids in astrocytes *in vitro*.

We first determined if a subset of mRNAs regulated by short-term glucocorticoid treatment in astrocytes *in vitro* (Carter et al., 2012) were also regulated by acute glucocorticoid exposure *in vivo*. A majority of the measured mRNAs were regulated by acute corticosterone *in vivo* (12/18 regulated, Figure 3.2). A portion of the regulated mRNAs are known to be both enriched in astrocytes and involved in specific processes,

such as cellular metabolism (Pdk4, Prodh, Sult1a1), intercellular signaling (Adora2b, Gjb6, Slc1a2), and transcriptional regulation (Klf9, Txnip). While this qPCR data does not identify the cell types in which these glucocorticoid-regulated mRNA changes take place, these data confirm that these mRNAs are glucocorticoid-sensitive *in vivo*. An intriguing subsequent question will be to determine if other physiological challenges known to acutely increase glucocorticoids (e.g. stress) similarly regulate these same mRNAs. Additionally, a portion of mRNAs regulated by glucocorticoids in astrocytes *in vitro* were not regulated by acute corticosterone treatment *in vivo* (Ch25h, Folh1, Glul, Mapk4, Mertk). This result may indicate these particular mRNAs are not regulated by elevated glucocorticoids *in vivo* or that the cellular environment *in vivo* is sufficiently different than that *in vitro* that glucocorticoid regulation of these mRNAs is delayed and/or less robust.

In general, the absolute magnitude of the glucocorticoid-mediated changes observed *in vivo* were significantly lower than mRNA expression differences observed following similar steroid treatment times in astrocytes *in vitro*. One explanation for this difference may be due in part to the active clearance of glucocorticoids from the brain compared to cell culture. In cell culture, the glucocorticoid concentration remains high and relatively constant, and there are less intercellular interactions that may counteract or limit cell-type dependent glucocorticoid responses. In contrast, the cellular environment in the brain is constantly opposing alterations to homeostasis; in this case, the brain would engage systems to limit responsiveness to continued steroid exposure by activating opposing regulatory mechanisms to control the response to changes induced by

glucocorticoid signaling. Indeed, active clearance of cortisol was directly observed in the acute exposure paradigm, returning corticosterone concentrations to baseline within 4 hours (Figure 3.1A).

Clinical conditions involving alterations in glucocorticoid signaling are often chronic in nature. Given that our acute paradigm resulted in a temporary increase in glucocorticoid levels, we wanted to further investigate regulation of these mRNAs following chronically elevated glucocorticoid levels *in vivo*. We assessed the impact of chronic glucocorticoid exposure on mice using an established paradigm administering corticosterone via drinking water (Karatsoreos et al., 2010). Consistent with prior studies, 2 weeks of corticosterone treatment of mice in this study lead to elevated plasmid levels of corticosterone, indicative that all mice in the steroid treatment group had consumed water containing corticosterone at least in the short term. Physiological indices of chronic corticosterone treatment were observed in the form of hypotrophy of several glucocorticoid-sensitive tissues (i.e. adrenal glands, thymus and spleen) without changes in body weight.

Among the panel of mRNAs measured, the majority of mRNAs regulated by glucocorticoids in astrocytes *in vitro* or acute corticosterone *in vivo* were also statistically regulated in at least one brain region by chronic corticosterone exposure *in vivo* (12/18 mRNAs). mRNAs regulated by chronic corticosterone exposure in the same direction as the glucocorticoid regulation observed in astrocytes *in vitro* or acute corticosterone exposure *in vivo* (upregulated: Fkbp5, Folh1, Gjb6, Glul, Mertk, Prodh, Sgk1, Sult1a1;

downregulated: Ch25h, Hdac7, Slc1a2, Wnt7a) have consistent response to glucocorticoids independent of treatment duration. In contrast, several other mRNAs in the chronic corticosterone experiment were either not regulated (Klf9, Pdk4, Txnip, Mapk4, Per1, Phlda1) or regulated in the opposite direction (Adora2b, Egr2) when compared with glucocorticoid regulation in acute corticosterone exposure and astrocyte cultures. Chronic corticosterone exposure also regulated mRNAs that were not regulated in either of the previous two experiments (upregulated: Fgfr3; downregulated: Fgfr1, Foxo1, Gap43, Gfap, Gja1, Slc1a3, Syn2). These discrepancies may be due to indirect, compensatory or adaptive regulation to prolonged glucocorticoid exposure. A summary of the results comparing the three conditions are shown in Figure 3.6. Additionally, in response to acute or chronic corticosterone exposure, certain mRNAs were statistically regulated in one brain region but not the other (e.g. Hdac7 only in hippocampus). While there may indeed be differential glucocorticoid mRNA regulation by brain region, such results may be due to specific factors unique to these particular experimental paradigms (e.g. exposure times, criteria used to define differential expression). Additional experiments showing consistent brain-region-dependent regulation across multiple exposure times and conditions will be needed to address this possibility.

The high concordance of *in vivo* regulation by corticosterone with glucocorticoid regulation in astrocytes *in vitro* is important in part because most of these mRNAs have not been reported as glucocorticoid-sensitive *in vivo*. A subset of measured mRNAs have been reported to be regulated *in vivo* by glucocorticoids or stress paradigms, but a

number of the other regulated astrocyte-enriched mRNAs demonstrate novel *in vivo* regulation in the mouse brain (e.g. Gjb6, Prodh, Adora2b, Klf9, Pdk4, Txnip, Folh1, Mertk, Wnt7a). Of the mRNAs associated with glucocorticoid regulation *in vivo*, the directionality matches the previous reports, giving further confidence that our data is consistent with glucocorticoid regulation. The additional *in vivo* regulation observed in these experiments may not have been previously observed for multiple reasons; the most likely possibility is that the magnitude of changes are small enough that they may not have been measured with techniques less sensitive that qPCR.

Although real-time PCR measures substantiated the notion of glucocorticoid regulation *in vivo* of mRNAs regulated by glucocorticoids in astrocyte *in vitro*, these techniques cannot localize mRNA regulation to specific cell types in a multicellular brain tissue sample. In order to examine the neuroanatomical nature of these glucocorticoid-regulated mRNAs *in vivo*, we performed in situ hybridization studies in mice that were subject to chronic corticosterone or vehicle treatments. Using semi-quantitative densitometry, we were able to confirm the glucocorticoid regulation of a number of mRNAs in the prefrontal cortex and hippocampus (Figure 3.5). Although only a subset of differential expression as measured by qPCR were validated by ISH, ISH data was directionally consistent with the qPCR data in almost all cases; the discrepancy of statistical significance may be in part due to a more robust sensitivity of qPCR measurements. We then examined the neuroantomical expression patterns in an effort to associate the observed mRNA regulation with the location of specific cell types. The hippocampus has a well-recognized distinctive cytoarchitecture that can readily

associate cell types with specific locations in this brain region. Within the hippocampus, there are well known layers such as the pyramidal cell layer characterized by a higher density of neurons contained therein compared with other regions (e.g. astrocyte presence and lower neuron density in molecular layer). Among mRNAs measured, we detected ISH signal for Fkbp5 predominately in the pyramidal cell layer throughout multiple hippocampal subfields (Figure 3.5C). Following chronic glucocorticoids, the hybridization signal appears to rise equally across all pyramidal subfields without any noticeable increase in signal in the molecular layer. While additional high resolution images will be necessary to fully resolve in which cell types Fkbp5 is being regulated, this expression pattern seems mostly likely to be the result of mRNA regulation in pyramidal neuronal cell populations. In contrast, mRNAs regulated by chronic corticosterone but not expressed in pyramidal cell regions may be regulated in other cell types (e.g. astrocytes). Strikingly, all of the other corticosterone-regulated mRNAs analyzed by ISH in this study demonstrate an absence of expression in pyramidal neuron subfields (Gfap, Glul, Gja1, Gjb6). The gene expression patterns of these mRNAs appear much more homogeneous across all areas of the hippocampus, an anatomical distribution that is largely consistent with the known distribution of astrocytes (or other glial cells) in this tissue. Interestingly, these mRNAs also show similar expression patterns as that of Aldh1l1, a mRNA previously shown to be substantially enriched in astrocytes and described as a pan-astrocytic marker (Cahoy et al., 2008). Similarly diffuse expression of these mRNAs was observed by ISH in both vehicle and cort-treated prefrontal cortex samples. While further studies will be required to definitively identify the cell type of this differential glucocorticoid-sensitive gene

expression, the highly similar anatomical distribution of Gja1, Gbj6, and Glu1 with Aldh1l1 suggests that these mRNAs are regulated by glucocorticoids *in vivo* in astrocytes.

We also note a differential regulation of astrocyte cell markers by corticosterone treatment *in vivo* that may be important for future studies of glucocorticoid signaling involving astrocytes. Interestingly, GFAP, a marker historically associated with astrocytes in the brain, yields a different expression pattern compared with the pan-astrocytic marker Aldh1l1 and the other astrocyte-enriched mRNAs in the hippocampus (Figure 3.5C). Gfap mRNA is detected at reduced levels throughout the hippocampus but higher expression at the boundary of the tissue; this boundary expression is consistent with previously reported Gfap expression in ependymal cells (i.e. pial distribution) (Liu et al., 2006). Following chronic glucocorticoid-treatment, GFAP mRNA levels decrease across all areas (pial and non-pyramidal cell areas), consistent with our qPCR data. Recent studies on major depression have used Gfap in human postmortem experiments and animal models of depression to measure astrocyte cell number; multiple reports have reported decreased astrocyte cell density in these conditions (Rajkowska et al., 1999; Miguel-Hidalgo et al., 2000). However, this interpretation may be biased by glucocorticoid-induced downregulation of Gfap mRNA and corresponding protein in these conditions. If astrocyte cell numbers are changing, then all astrocyte markers would also be expected to change in parallel. We have measured Aldh1l1, a more recently identified pan-astrocyte marker, and found that Aldh1l1 is not downregulated by glucocorticoids under any measured condition (in contrast, ISH data

suggests possible upregulation by chronic corticosterone exposure). This finding suggests that astrocyte numbers may not be decreasing in conditions of hypercortisolemia but that the astrocyte cytoskeleton may be dynamically regulated by glucocorticoids. Future studies of stress signaling in astrocytes would benefit from using multiple cell markers or a marker known not to be affected by any component of the manipulation or disease of interest.

These data also have interesting parallels to clinical findings in postmortem studies of major depression, a condition associated with elevation of glucocorticoids. Multiple mRNAs regulated by chronic corticosterone treatment were changed in the same direction in depression (e.g. downregulation of Gja1, Slc1a2, and Slc1a3) (Bernard et al., 2010). While the data presented in these studies cannot be directly linked to depression biology, they do provide a potential mechanism (glucocorticoid regulation) by which these genes may be altered. Alterations of FGF receptors observed here (upregulation of Fgfr3, downregulation of Fgfr1) may also be relevant to depression biology given the findings of altered expression of FGF receptors and ligands in depression (Evans et al., 2004). We would be interested in determining if these same mRNAs are regulated in other animal models demonstrating elevated corticosterone levels, such as animal models of depression. Given the associations of increased depressive-like behavior and astrocyte dysfunction (Banasr and Duman, 2008), perhaps these mRNAs are relevant to the underlying mechanisms of these observations. A caveat of such extrapolations is that we have documented glucocorticoid-mediated mRNA changes but not changes in corresponding proteins; further studies are needed

to measure correlations between changes in mRNA expression and any changes in protein levels.

In summary, our data demonstrate that select mRNAs regulated by glucocorticoids in astrocytes *in vitro* are also regulated by acute and/or chronic corticosterone exposure *in vivo*. A number of these mRNAs have been reported as astrocyte-enriched (Cahoy et al., 2008), and ISH data reveal anatomical expression patterns within the hippocampus that are highly suggestive that such changes are occurring within regions containing astrocytes. Together, these data suggest that the observed regulation may be occurring in astrocytes. These data convey physiological significance to parallel gene expression findings in astrocyte cell culture and suggest that additional findings of glucocorticoid regulation in astrocyte cultures *in vitro* may extend to the brain *in vivo*. Based on these results, we would predict that differential responses in the brain to glucocorticoids by cell type contribute additional complexity to stress signaling. The functional contribution of glucocorticoid regulation in astrocytes to stress signaling remains to be investigated and may be integral to both the classical stress response and pathological conditions associated with elevations in glucocorticoid levels.

Additional text; functional consequences of corticosterone mRNA regulation *in vivo*

The following section describes known functional information on mRNAs regulated by either acute or chronic corticosterone exposure *in vivo* that were not regulated by glucocorticoids in astrocytes *in vitro*. This section is designed to complement the functional descriptions of mRNAs regulated by glucocorticoids in astrocytes *in vitro* that is found in Chapter II.

Genes regulated by acute and/or chronic corticosterone exposure in vivo but not in astrocytes in vitro

Genes regulated by glucocorticoids *in vivo* but not in astrocyte cultures may be regulated in neurons or regulated in concert with additional intercellular signals. Hdac7 was upregulated and Slc1a2 was downregulated by both acute and chronic corticosterone exposure *in vivo*. Hdac7 is a class II deacetylase involved in protein-protein interactions. Recent data has demonstrated that Hdac7 expression can be neuroprotective to neurons by an unknown mechanism that is independent of enzyme activity (Ma and D'Mello, 2011). Increased expression of Hdac7 by glucocorticoids *in vivo* may thus also be neuroprotective. Slc1a2 (i.e. EAAT2, GLT-1) and Slc1a3 (i.e. EAAT1, GLAST) are glial high affinity glutamate transporters expressed in astrocytes; these transporters remove the majority of glutamate from the synapse. Decreases in glutamate transporter expression and function would likely disrupt fidelity of excitatory neurotransmission and lead to elevations of glutamate at the synapse. Previous studies have reported upregulation of GLT-1 and no regulation of GLAST by glucocorticoids (Reagan et al., 2004), results that are opposite the data presented here, although these previous reports were primarily in cell culture (Zschocke et al., 2005). However, acute

glucocorticoid exposure is generally thought to increase glutamate levels in the hippocampus and prefrontal cortex (Popoli et al., 2012), while research in depression generally report reductions in glutamate in specific brain regions (Auer et al., 2000). Further clarifying the physiological regulation of glutamate transporters by glucocorticoids is important given the prominent functional role of these transporters in astrocytes.

A subset of genes was regulated by chronic corticosterone exposure that were not regulated by acute corticosterone exposure nor by glucocorticoids in astrocyte cultures. These genes may represent indirect, adaptive regulation to glucocorticoid signaling. Fgfr1 and Fgfr3 are fibroblast growth factor receptors that act as receptor tyrosine kinases and are widely expressed in the brain (Belluardo et al., 1998). Fgfr3 is associated with astrocyte localization (Pringle et al., 2003), and both receptors are functionally implicated in regulating proliferation (Stachowiak et al., 1997; Inglis-Broadgate et al., 2005). Although Fgfr1 and Fgfr3 are regulated in opposite directions in our data, determining their functional roles in the brain may be of particular interest in light of reports that Fgfr1 and Fgf ligands are downregulated in human depression (Evans 2004). Of the remaining three other genes downregulated by chronic corticosterone exposure *in vivo* that were not regulated in astrocytes *in vitro*, Foxo1 is a transcription factor associated with PI3K signaling (Belgardt et al., 2008), Gap43 is a membrane protein associated with neuronal growth (Vitković et al., 1988), and Gja1 is connexin-43, the predominant gap junction protein expressed in astrocytes. In contrast, Syn2 is a synapsin that is enriched in neurons and previously reported as glucocorticoid-sensitive (Revest et al., 2010). The functional consequence of

glucocorticoid-mediated downregulation of these mRNAs *in vivo* remains to be investigated.

Genes regulated by glucocorticoids in astrocyte in vitro but not both acute and chronic corticosterone treatment in vivo

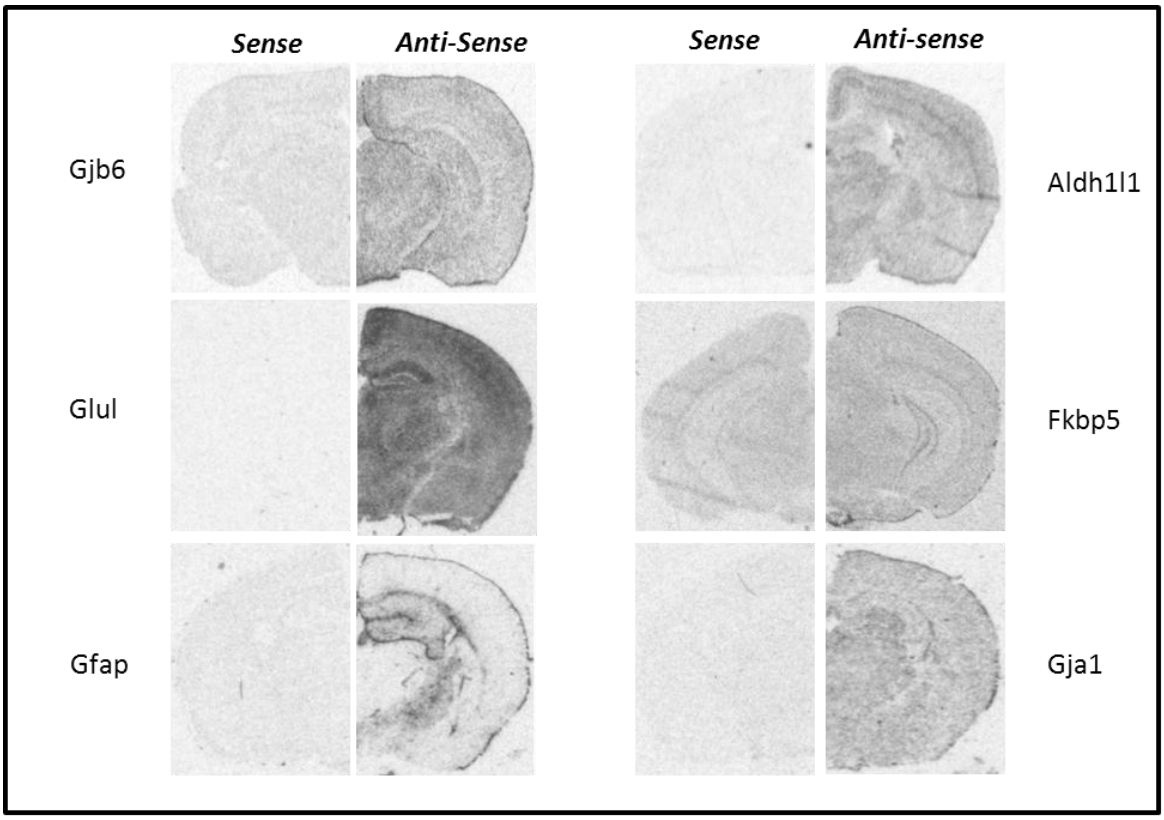
A subset of mRNAs regulated by glucocorticoids in astrocytes *in vitro* were regulated by acute corticosterone exposure *in vivo* but not under chronic corticosterone exposure *in vivo* (Klf9, Pdk4, Txnip). These genes may be tightly regulated or involved in homeostatic mechanisms but not long-term adaptive mechanisms. Other mRNAs regulated by glucocorticoids in astrocytes *in vitro* were regulated by chronic corticosterone exposure *in vivo* but not by acute corticosterone exposure *in vivo* (Folh1, Glul, Mertk). While these mRNAs may not be regulated by acute corticosterone exposure, one interpretation of these data is that the paradigm used for acute corticosterone exposure was not adequate to regulate these particular mRNAs (e.g. may be regulated by different acute corticosterone parameters, dose or duration or that neighboring cells might respond to glucocorticoids acutely that alter how surrounding cells respond to the same steroid). Finally, a subset of mRNAs regulated by glucocorticoids in astrocytes *in vitro* were not regulated in a consistent manner by either acute corticosterone exposure *in vivo* or chronic corticosterone exposure *in vivo* (not regulated: Mapk4, Per1; opposite directionality: Egr2). The mRNAs not regulated *in vivo* may not actually be physiologically regulated by glucocorticoids *in vivo*, but given that both Mapk4 and Per1 have been previously reported as glucocorticoid-sensitive, a more likely explanation is that the corticosterone exposure paradigms used did not induce these mRNAs.

Supplemental Table 3.1: Combined qPCR data for mRNAs measured for regulation by acute and chronic corticosterone exposure *in vivo*

mRNA	Acute Ctx FC	Acute Ctx p-value	Acute Hip FC	Acute Hip p-value	Chronic Ctx FC	Chronic Ctx p-value	Chronic Hip FC	Chronic Hip p-value
Adora2b	1.143	0.118	1.156	0.044	0.646	0.022	0.619	0.004
Aldh1l1	1.097	0.071	1.039	0.632	1.155	0.405	1.060	0.580
Atp6v1b2	0.985	0.825	0.980	0.750	1.022	0.897	0.984	0.874
Ch25h	0.681	0.147	0.762	0.295	0.595	0.008	1.490	0.580
Egr2	0.770	0.087	0.645	0.003	0.430	0.012	0.692	0.094
Fgfr1	1.155	0.121	1.013	0.853	0.395	0.001	0.862	0.157
Fgfr3	0.931	0.718	1.019	0.853	2.196	0.000	0.669	0.094
Fkbp5	2.716	0.000	1.812	0.000	3.540	0.000	1.861	0.000
Folh1	1.011	0.872	0.950	0.416	0.881	0.507	1.311	0.032
Foxo1	1.145	0.294	0.913	0.305	0.610	0.003	1.102	0.420
Gap43	1.120	0.079	1.071	0.493	0.955	0.774	0.655	0.001
Gfap	0.982	0.872	0.922	0.305	0.360	0.001	0.633	0.001
Gja1	1.077	0.294	1.011	0.866	0.669	0.021	0.847	0.234
Gjb6	1.254	0.000	1.324	0.003	1.269	0.169	1.543	0.001
Glul	1.179	0.055	1.143	0.109	1.585	0.017	0.978	0.851
Hdac7	0.886	0.121	0.861	0.047	0.825	0.229	0.673	0.004
Klf9	1.315	0.000	1.186	0.012	1.051	0.794	1.178	0.123
Mapk4	1.077	0.362	0.929	0.336	0.968	0.892	1.158	0.381
Mertk	1.107	0.324	1.138	0.109	1.279	0.225	1.656	0.002
Pdk4	1.596	0.000	1.871	0.000	1.087	0.589	1.153	0.103
Per1	1.161	0.147	1.087	0.336	0.860	0.405	0.955	0.741
Phlda1	0.807	0.021	0.823	0.051	0.859	0.401	0.841	0.157
Prodh	1.140	0.174	1.180	0.049	1.862	0.002	1.557	0.004
Sgk1	2.708	0.000	3.150	0.000	1.511	0.007	1.946	0.000
Slc1a2	0.997	0.960	0.876	0.044	0.727	0.050	0.848	0.234
Slc1a3	1.074	0.403	0.923	0.305	0.508	0.007	0.856	0.149
Sult1a1	2.130	0.000	2.012	0.000	3.774	0.000	4.317	0.000
Syn2	0.958	0.718	1.046	0.507	1.014	0.920	0.751	0.004
Txnip	1.464	0.003	1.235	0.170	0.916	0.704	1.199	0.149
Wnt7a	0.857	0.376	0.754	0.004	0.308	0.007	0.589	0.001

KEY: Ctx = cortex, Hip = hippocampus, pink = p<0.05

Supplemental Table 3.2: Primers for in situ hybridization probes		
mRNA	**Forward Primer**	**Reverse Primer**
Fkbp5	GGACCACGCTATGGTTTTGG	AACATGTTGGCGTACACCCT
Gja1	AGTGAAAGAGAGGTGCCCAG	TGCCGTGTTCTTCAATCCCA
Gjb6	AAGAACACAGGCGCAGAGAA	TTGTCCAGGTGACTCCAAGG
Glul	CCTGGACCCCAAGGCCCGTA	CGGTTGGCAACACCGGCAGA
Gfap	CTGGCCCAACAGCAGGTCCAC	TCCAGGCTGGTTTCTCGGATCTGG
Aldh1l1	GGGGACAGGAGGGTGCTAAGTC	TGTCATCCCCTGGAACTATCCC
*Primers listed 5'->3'		



Supplemental Figure 3.1: Specificity of mRNA in situ hybridization probes Sense (left) and antisense (right) comparisons of in situ hybridization probe signal. Sense probes demonstrate significantly lower signal compared to antisense probes for all mRNAs assessed.

Chapter III references

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Chapter IV

Thesis Summary and Conclusions

The goal of this dissertation research was to enhance our understanding of glucocorticoid action in the brain by characterizing glucocorticoid-mediated mRNA regulation in astrocytes. Overall, the data generated from both *in vitro* and *in vivo* experiments have implications not only for glucocorticoid mechanisms but potentially broader views on how a cell-type centric focus shapes our perspective of hormonal regulation of gene expression in the brain. This concluding chapter seeks to integrate the research findings, identify and discuss questions and future directions derived from these results, and specify the contributions of these studies to the field in terms of astrocytes, glucocorticoids, and stress signaling.

Integration of Major Findings of this Dissertation Research

Chapter I of this dissertation began with an introduction of stress and glucocorticoids, the primary mediator of the HPA axis and the classic stress response. In the brain, most cell types express receptors for glucocorticoids (e.g. neurons (McEwen et al., 1968), astrocytes (Vielkind et al., 1990), oligodendrocytes (Vielkind et al., 1990), microglia (Tanaka et al., 1997)) and are thus likely a part of the integrated response of the brain to stress and HPA axis stimulation; however, almost all previous work had

focused exclusively on glucocorticoid action in neurons. Altered glucocorticoid signaling has been implicated in numerous disease states, particularly in mental health disorders such as depression. Recent work has implicated cell-type specific alterations in the pathology of depression (e.g. astrocyte pathology (Rajkowska et al., 1999)). Despite these associations, there have been no comprehensive studies of how elevated levels of glucocorticoids alter gene expression at the transcriptome level and physiological functions of astrocytes. Prior to this thesis work, there were limited reports of individual mRNAs associated with astrocytes being regulated by glucocorticoids (e.g. (Kumar et al., 1986)). I wondered if glucocorticoids may have unique impact on astrocytes that would be relevant to understanding the stress response and alterations in HPA axis dysregulation, such as in depression. Given the gap in knowledge about glucocorticoid action in the brain by cell type, I set out to test the following hypothesis:

Glucocorticoids regulate specific mRNAs in astrocytes, including certain mRNAs regulated by glucocorticoids in other cellular contexts. A portion of mRNAs regulated by glucocorticoids in astrocytes are uniquely regulated in astrocytes compared to other cell types in the brain.

In order to isolate glucocorticoid regulation from other brain cell types, we measured mRNA changes in response to glucocorticoids in primary mouse astrocyte cultures (Chapter II). Since we were interested in glucocorticoid regulation under conditions of stress and/or elevated glucocorticoid levels, we used two strategies in separate experiments to target the receptor associated with action under these conditions (i.e.

glucocorticoid receptor, GR). GR was targeted using (1) the synthetic GR-selective agonist dexamethasone and (2) the natural ligand corticosterone alone or co-administered with the GR-selective antagonist RU486. In the first set of experiments using dexamethasone, we found 854 unique mRNAs regulated ≥two-fold compared to controls at one or more of 8 time points in a 24-hour time course. Given the extensive number of mRNAs regulated ≥two-fold by glucocorticoids, I used this criterion to define differential regulation across studies and limit false-positive identifications. There may be additional mRNAs authentically regulated at lower magnitudes; indeed, multiple mRNAs reportedly regulated by glucocorticoids in astrocytes were regulated less than two-fold (see Chapter II). In sum, these data represent a conservative estimate of regulation and establish the character of GR-mediated glucocorticoid regulation in astrocytes *in vitro*. These criteria indeed appeared robust for identifying glucocorticoid-regulated mRNAs; all differences measured by qPCR validated in direction and relative magnitude with the microarray data. Subsequent cluster and GO analyses found associations among the total set of regulated mRNAs with negative regulation of apoptosis (increased expression), binding to various ions and cofactors (increased expression), DNA replication (decreased expression), and cell cycle and cell division (decreased expression). Glucocorticoids have been associated with regulation of apoptosis, decreased DNA replication, and decreased cell division in other cellular contexts; we have identified subsets of mRNAs in this context that may be of interest for studies of these processes specifically in astrocytes.

In effort to further target primary natural glucocorticoid regulation, we also measured

mRNAs changes in response to the natural ligand corticosterone in an abbreviated time course (i.e. treatments for 2 hours, 4 hours, and 6 hours). Based on these experiments and comparisons with the dexamethasone data, we identified 69 mRNAs regulated more than two-fold by each glucocorticoid and whose regulation was dependent on GR activation; this list included 33 mRNAs reported to be regulated by glucocorticoids in other cellular contexts. Additional mRNAs previously reported to be glucocorticoid-sensitive were also identified in the data sets for either dexamethasone or corticosterone treatment (e.g. Fkbp5, Sgk1). Together, these data suggest that glucocorticoids also regulate these mRNAs in astrocytes, perhaps via mechanisms common across cell types. This finding also addresses or supports a component of our initial hypothesis. In addition, among the mRNAs regulated more than two-fold by each glucocorticoid, we identified 36 novel mRNA targets of glucocorticoid regulation. While we are confident of the glucocorticoid regulation of these mRNAs, whether they are regulated by glucocorticoids exclusively in astrocytes or also in additional cell types remains an open question.

Given the extensive microarray data of mRNAs regulated by glucocorticoids in astrocytes *in vitro*, we sought additional information to prioritize mRNAs to investigate in subsequent experiments. In a general sense, the specific cellular response to a stimulus such as glucocorticoids is presumed to rely upon the assortment of mRNAs expressed in that cell. Recent studies on mRNA expression in the brain have attempted to distinguish cell types based on their distinctive mRNA expression patterns (e.g. neurons vs. astrocytes vs. oligodendrocytes, (Cahoy et al., 2008)). In my examination

of this cell-type dependent mRNA analysis work, I noticed numerous examples of astrocyte-enriched mRNAs among our data sets of glucocorticoid-regulated mRNA. I hypothesized that glucocorticoid regulation of astrocyte-enriched mRNAs may have particular functional impact on astrocyte biology, separate to or in combination with the glucocorticoid regulation of mRNAs that were not astrocyte-enriched. Comparisons of astrocyte-enriched mRNAs with the set of mRNAs regulated by dexamethasone yielded a sizeable overlap. This result suggests that glucocorticoids regulate mRNAs specifically in astrocytes relative to other cell types, although we do not know at this point if these mRNAs are also regulated by glucocorticoids in other brain cell types. Given prior literature that identifies many of these glucocorticoid-regulated mRNAs as astrocyte-enriched, I speculate that these mRNAs are primarily responding to this steroid treatment in astrocytes. Future studies in other cell types and *in vivo* will be necessary to fully characterize the magnitude and time course of glucocorticoid regulation as it varies by cell type.

This comparison also yielded 2 striking observations in terms of the relationship between glucocorticoid regulation in astrocytes and astrocyte-enriched mRNAs. First, the percentage of astrocyte-enriched mRNAs among glucocorticoid-regulated mRNAs was substantially higher than the percentage among all mRNAs expressed in the culture system. Second, the percentage of astrocyte-enriched mRNAs among glucocorticoid-regulated mRNAs was highest at initial time points of regulation (i.e. 2 hours) and steadily decreased over the remainder of the time course. One interpretation of these observations is that (1) glucocorticoids via yet unknown

mechanisms selectively regulate astrocyte-enriched mRNAs in astrocytes and (2) glucocorticoid regulation in astrocytes initially regulate astrocyte-specific processes and subsequently activate responses common to other cell types. I will elaborate on my interpretation of these findings later in terms of research questions arising from this thesis work.

Having characterized glucocorticoid-mediated mRNA regulation in astrocytes *in vitro*, I wanted to test the physiological relevance of these findings based on both acute and chronic glucocorticoid exposure *in vivo* (i.e. acute corticosterone exposure: via single injection, 2-hour exposure; chronic corticosterone exposure: via drinking water, 2 week exposure). Chapter III describes two mouse studies in which the majority of mRNAs regulated by glucocorticoids in astrocytes *in vitro* as measured by qPCR were also regulated by corticosterone in mice *in vivo*, including changes in astrocyte-enriched mRNAs. Given the relatively brief corticosterone exposure in the acute paradigm (i.e. samples collected 2 hours post-glucocorticoid injection), one intriguing interpretation of these data is that these mRNAs that are regulated by glucocorticoids in astrocytes *in vitro* are also regulated by glucocorticoids in astrocytes *in vivo* and hence play a role in the brain's acute response to this stress hormone. One caveat of the acute corticosterone paradigm was that we observed significant but substantially reduced mRNA fold-change values in comparison with the *in vitro* astrocyte data. These differences could be due to a number of reasons, including intercellular interactions with other cell types and active clearance of glucocorticoids in the intact brain in response to a rapid increase in stress hormone concentration. Indeed, a time course experiment

investigating plasma corticosterone levels following steroid injection revealed an acute rise in corticosterone levels in the first hour post-injection that decreased rapidly over time, returning to baseline levels within 4 hours. Hence, the *in vivo* pharmacodynamics of elevated glucocorticoid levels did not replicate our well-controlled, static glucocorticoid treatments *in vitro*, making it difficult to precisely determine whether the reduced glucocorticoid response *in vivo* was due entirely to this variation in steroid levels *in vivo*. Beyond this, most diseases associated with HPA axis dysfunction result in chronic elevations of glucocorticoids; whether mRNAs regulated by acute corticosterone exposure were also regulated by chronic corticosterone exposure was not known. To address this question, I implemented a drinking water administration approach that provided a minimally invasive technique for producing chronic elevation of corticosterone levels. In addition to regulating many mRNAs regulated by glucocorticoids in astrocytes *in vitro*, chronic corticosterone exposure also regulated mRNAs that were not regulated by glucocorticoids in astrocytes *in vitro* or by acute corticosterone exposure *in vivo* (additional mRNAs included both astrocyte-enriched and non-enriched mRNAs). One interpretation of this finding is that chronic glucocorticoid exposure may result in adaptive changes in astrocyte physiology over time. I will expand on this possibility in the subsequent additional research questions section.

While the qPCR data can clearly establish differential regulation of mRNAs in response to corticosterone *in vivo*, additional data is needed to implicate such changes as occurring in astrocytes. In an effort to further validate the qPCR data and associate

mRNA changes with astrocytes, I selected a subset of astrocyte-enriched mRNAs for in situ hybridization analysis. ISH techniques allowed for more specific neuroanatomical analysis than qPCR methods as well as potential associations with astrocyte localization based on the known cytoarchitecture of the brain regions of interest. In terms of anatomical validation, the ISH data matched the directionality and relative magnitude of changes observed in the qPCR data, although some of these data did not reach statistical significance. I suspect this statistical disconnect speaks to the higher sensitivity of qPCR measures compared to the ISH measures combined with the lack of power in the ISH analysis based on the small number of animals in this study. In terms of astrocyte associations, the expression patterns of the selected mRNAs in the hippocampus suggest that they are expressed in astrocytes based on two observations: (1) the mRNAs demonstrated expression patterns similar to the pan-astrocytic marker Aldh1l1 and (2) the hybridization signals were notably absent from the pyramidal neurons of the hippocampal subfields, suggesting they are not expressed in these neurons and further that the observed signal is specific (i.e. hippocampal subfields are often a primary location for non-specific background signals). While these results do not definitively answer the question of whether these mRNAs are being regulated in astrocytes, they do add empirical correlative evidence to the discussion in addition to the literature-based associations.

Taken together, these data support physiological relevance of the glucocorticoid-mediated mRNA regulation in astrocytes *in vitro*. These *in vitro* and *in vivo* experiments

provide the first extensive empirical transcriptome-wide evidence of differential glucocorticoid regulation by cell type in the brain.

Research questions arising from this dissertation research and future directions

The results of this dissertation research add to our knowledge of glucocorticoid action in the brain. Given the novel focus on cell type differences, our findings have established principles relevant for understanding glucocorticoid regulation by cell type in the brain and have also raised a number of additional questions for consideration in future studies. A portion of these potential future directions are discussed in the following sections (diagrammed in Figure 4.1).

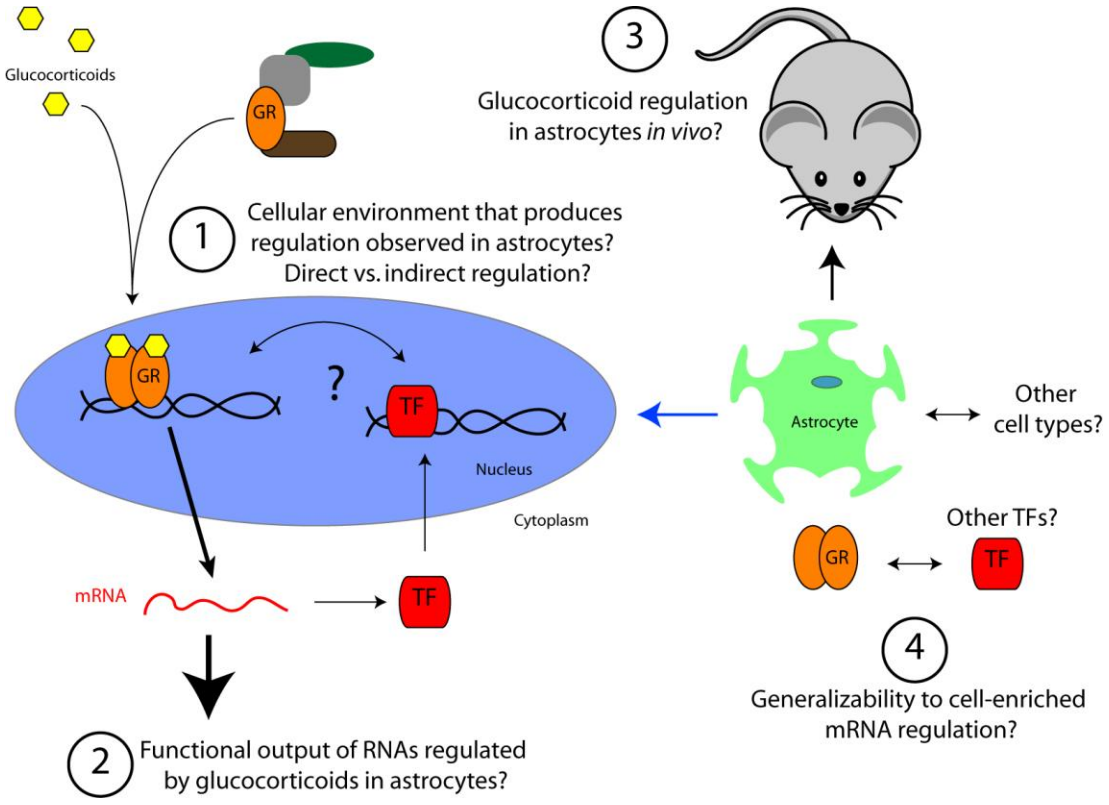

Figure 4.1: Graphical summary of research questions arising from this dissertation and future directions. TF = transcription factor

(1) How does the cellular environment of astrocytes produce specific patterns of mRNA regulation relative to other cell types?

Results discussed in Chapter II indicate that astrocytes exist in a state whereby their responses to glucocorticoids lead to selective regulation of mRNAs whose expression is enriched in astrocytes. I could picture a number of factors contributing to this phenomenon. The specific set of coregulators and other transcription factors that interact with glucocorticoid receptors in astrocytes would likely influence which genes would be targeted. Perhaps there are coregulators that are astrocyte-enriched and are particularly influential in this cell type compared to other cell types. Alternatively, the absolute expression of cofactors per se may not be the defining factor but rather their activity and/or the post-translational status of the receptor (e.g. GR phosphorylation modulates cofactor interactions (Chen et al., 2008)) that influences the nature or magnitude of glucocorticoid-mediated regulation. These factors or receptor modifications may also vary by cell type; if so, we may gain insight from further examining our microarray data sets for genes known to interact with GR. These questions could also be addressed by using GR pull-down experiments to determine which coregulators are bound to GR in astrocytes in the presence of glucocorticoids versus the absence of glucocorticoids. In addition, the chromatin structure in astrocytes defines which genes are available to be expressed and also regulated, and thus the epigenetic landscape may be an important factor in defining glucocorticoid regulation. Indeed, chromatin accessibility has been shown to be a predominant factor in determining glucocorticoid receptor binding (John et al., 2011), and the glucocorticoid receptor has been shown by chromatin immunoprecipitation (ChIP) assays to

preferentially bind genes in a lung cell line that are regulated in that cell line and not genes regulated in other cellular contexts (So et al., 2007). These data suggest that there are chromatin-modifying factors that vary by cell type and that such factors likely influence the basal state of chromatin in astrocytes that is permissive to additional levels of transcriptional regulation (e.g. via GR). In terms of testing the mechanisms of glucocorticoid regulation of specific genes in astrocytes, use of GR ChIP assays (GR-Chip or GR-Chip-Seq on astrocytes with and without steroid treatment) could identify which promoter elements bind GR in astrocytes. Given the apparent importance of cellular context for GR action, understanding the range of coregulators that interact with GR in astrocytes would also be another potential area of interest; ChIP-ChIP experiments could then be used to test which coregulators bind to GR for specific GR targets based on known interactions in other systems (e.g. coactivators: steroid receptor coactivators (SRCs), CBP/p300; corepressors: NCoR, SMRT; reviewed in (Meijer, 2002)).

(2) What are the functional consequences of glucocorticoid regulation in astrocytes?

The mRNAs regulated by glucocorticoids identified in these studies are individually associated with many different cellular processes; how does alteration of these mRNAs impact astrocyte physiology? In addition to the processes identified by the GO analyses (e.g. cell cycle, DNA replication, apoptosis, sulfur metabolism, nitrogen compound biosynthesis), one can first formulate hypotheses about the impact of specific genes that have known functions in astrocytes. For example, two functions that appear altered

by glucocorticoids in astrocytes are glutamate metabolism and transport as well as gap junction coupling. Glutamine synthetase is well-characterized as being upregulated by glucocorticoids, but we also found that the two glutamate transporters (Slc1a2, Slc1a3) were downregulated by chronic corticosterone treatment *in vivo*. If this change in mRNA levels is paralleled by changes in transporter protein, then there could be less transporter availability at the cell membrane and a corresponding decrease in the fidelity of glutamatergic neurotransmission (less clearance, increased glutamate in the synaptic cleft). This prediction could be tested initially by measuring protein products to see if there are parallel changes to the mRNA regulation; if there are correlated changes, subsequent measurements of glutamate levels could be made in the chronic corticosterone paradigm (e.g. microdialysis). Interestingly, this loss of glutamatergic fidelity due to decreases in astrocyte glutamate transporters is in conceptual agreement with recent results demonstrating rapid antidepressant effects of NMDA receptor antagonists in animal models of chronic stress (e.g. ketamine) (Li et al., 2011). In terms of gap junctions, we identified Gjb6 as a novel glucocorticoid target that was upregulated by glucocorticoid treatment (regulated in all paradigms). We also have found parallel increases in the protein product, connexin 30, in response to glucocorticoids in astrocytes *in vitro* (Targan and Carter, unpublished, Figure 4.2A).

An increase in gap junction expression may increase the connectivity between astrocytes and/or oligodendrocytes; perhaps increasing the passage of certain molecules through gap junctions or increasing the general permeability of an astrocyte

network as an adaptive response to acute stress. This concept seems plausible given that gap junctions are known to be transcriptionally regulated by a number of transcription factors, biological substances, and signal transduction pathways, including

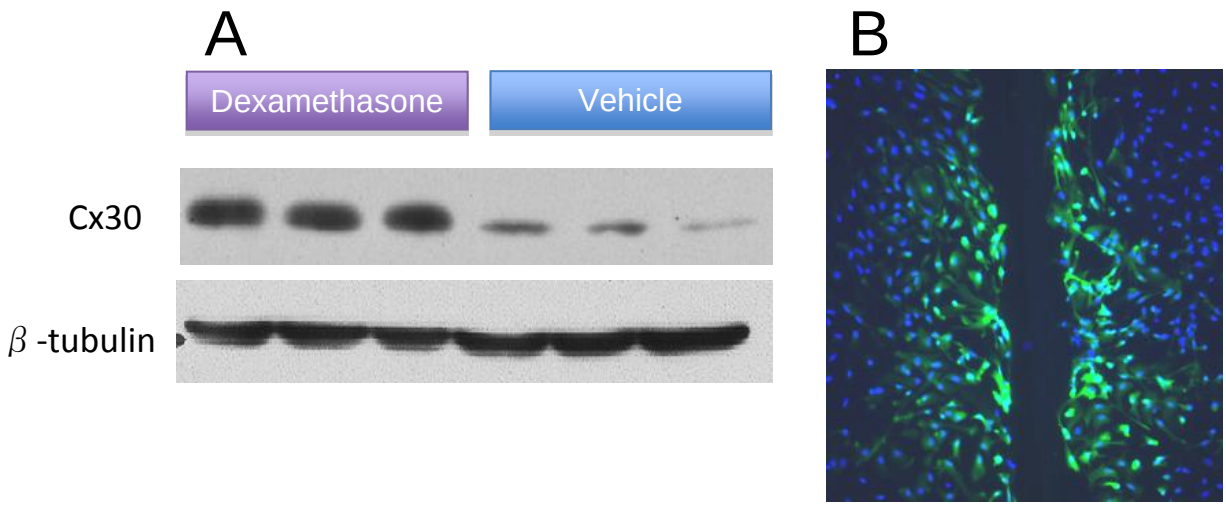

Figure 4.2: Glucocorticoid regulation of gap junction protein and function in astrocytes *in vitro* (A) Western blot of Cx30 protein from mouse cortical astrocyte cultures treated for 6 hours with 100nM dexamethasone (purple) or vehicle (blue) (beta-tubulin loading control). (B) Representative image of scrape loading dye coupling technique using astrocyte cell culture with neurobiotin dye and subsequent streptavidin-Alexafluor treatment. Green = dye, Blue = DAPI (nuclei). This experimental approach may be used in future studies to test changes in astrocyte connectivity.

some that act in a cell-type-specific fashion (reviewed in (Oyamada et al., 2005)). Conversely, we observed a downregulation of the other major astrocyte gap junction mRNAs (Gja1/Cx43) under chronic corticosterone exposure; reductions of gap junction expression would presumably decrease astrocyte coupling. One possible physiological output for opposite regulation of the two connexins is selective alteration of the permeability of specific molecules depending on what passes through each channel (e.g. increased permeability for molecules passing through Gjb6, decreased permeability for molecules passing through Gja1). In terms of decreasing Cx43, astrocytes may be limiting their connectivity in response to chronic glucocorticoid

elevation to isolate or otherwise reduce astrocyte signaling across the network (e.g. limit spread of toxic molecules/changes), a concept previously identified in the literature on astrocyte responses to inflammation and disease (reviewed in (Kielian, 2008)). These questions could be addressed by (1) understanding exactly what molecules and ions pass through different types of gap junctions in astrocytes (e.g. glucose, sodium, potassium, calcium) and (2) measuring astrocyte connectivity via dye transfer assays in glucocorticoid-treated astrocyte cultures and acute slices from mice following acute or chronic corticosterone exposure (e.g. hypothesize that increased gap junction expression = increased astrocyte coupling = faster and more expansive diffusion of dye through network in assay). While I was able to establish a dye transfer assay in astrocytes *in vitro* (Figure 4.2B), current limits of time and laboratory resources did not permit me to complete these functional studies. In a general sense, since connexins are thought to have relatively short half-lives, transcriptional regulation may provide a way to dynamically affect astrocyte coupling in response to stimuli. One could even imagine a "balance hypothesis" similar to glucocorticoid receptor function, where alterations of the ratio of connexins would yield an additional level of complexity to regulating gap junction coupling of astrocytes. Overexpression or knockdown of connexin proteins in culture may allow for understanding how changes in connexin ratios alter the coupling of astrocytes.

(3) How can mRNA expression changes be associated with astrocyte localization in vivo?

To associate glucocorticoid-mediated mRNA changes with astrocyte location, I have

relied on (1) previous studies that link genes to astrocytes or astrocyte functions and/or (2) their demonstrated mRNA expression patterns (based upon in situ hybridization) consistent with the pan-astrocyte marker Aldh1l1; while both of these approaches lend credence to the hypothesis that the observed mRNA changes in response to glucocorticoids are occurring in astrocytes, they do not provide explicit empirical evidence. Additional methods are needed to experimentally verify this claim.

We initially intended to associate glucocorticoid-mediated mRNA changes with astrocytes *in vivo* using dual in situ hybridization techniques but encountered technical challenges. We used a colorimetric nucleic acid probe targeting an astrocyte mRNA marker (e.g. Aldh1l1) with radiolabeled nucleic acid probes targeting mRNAs believed to be regulated by glucocorticoids in astrocytes and expected to colocalize changes in radioactive signal (silver grains) with labeled cells (blue cells). Similar strategies have been used successfully to localize mRNA changes with neuron localization (e.g. (Curran and Watson, 2004)). However, numerous attempts to accomplish this goal were unsuccessful because the radioactive probe signal (specific; not seen in sense control) tended to be diffuse and could not be uniquely associated with the astrocyte marker; this pattern was consistent across many astrocyte-enriched mRNAs. Given that the expression patterns of many of these mRNAs are well-associated with astrocytes, the most likely explanation is that the combination ISH approach is simply not sensitive enough in our hands to adequate measure these astrocyte-enriched mRNAs. Alternatively, the expansive cellular morphology of astrocytes may result in a diffuse distribution of mRNAs within this cell type as compared to the compartmentalized cell

body of the neuron. If this is true, then localizing mRNAs to astrocytes with dual in situ hybridization may be inherently challenging regardless of the experimental paradigm. An alternative method of inferring astrocyte localization is making correlation with the known cytoarchitecture of the hippocampus, comparing neuron-enriched regions (e.g. pyramidal cell layers) to less neuron-enriched regions (e.g. molecular cell layers); although less definitive and precise, this approach currently may be the best option for anatomically localizing mRNAs to astrocytes *in vivo*.

An additional approach to identify mRNA changes specifically in astrocytes that bypasses the neuroanatomical complexities would be to use genetic-based approaches in transgenic mice. One such approach involves a GFP-tagged L10 ribosomal protein that can be expressed in a cell-specific manner (i.e. Cre-recombinase-based method) (Doyle et al., 2008; Heiman et al., 2008). Expressing this protein under the control of an astrocyte-specific promoter (e.g. Aldh1l1, Gfap) would enable isolation of actively transcribed mRNAs selectively from astrocytes. Beyond this, there are additional approaches emerging that may prove to be equally valuable to selectively isolate all RNAs (not just actively translated RNAs) from distinct cell types using *in vivo* animal models (e.g. (Miller et al., 2009; Gay et al., 2013)). Repeating our *in vivo* corticosterone treatments (acute and chronic exposures) would enable us to collect mRNAs regulated in astrocytes; we are currently looking into collaborations to pursue this option. One challenge for this in vivo approach would be having a positive control of astrocyte regulation for comparison to ensure the mRNAs being isolated were coming from astrocytes; to address this issue, one parallel approach could be to measure mRNAs

isolated via ribosome tagging from astrocyte cell cultures treated with or without glucocorticoids and then compare these results to the glucocorticoid regulation observed in the transgenic mice. If the ribosome tagging technique is successfully isolating mRNAs from astrocytes *in vivo*, we may expect that this comparison would yield substantial overlap in regulated genes. Two caveats with this method are (1) the use of antibodies to collect mRNA samples and (2) specificity of transgene expression. Antibodies can have non-specific targeting and would select from a population of cells defined by transgene expression. The expression of Cre-recombinase will also be dependent on the promoter used, and ideally this promoter would drive expression exclusively in astrocytes. However, if this promoter drives any expression in non-astrocyte cells, then samples derived from this technique would not be exclusively from astrocytes (e.g. based on *in situ* experiments, Gfap promoter may drive expression in astrocytes as well as ependymal cells).

(4) In terms of cell-enriched mRNA regulation, how generalizable are the findings concerning glucocorticoid regulation of astrocyte-enriched mRNAs?

In attempt to investigate how glucocorticoids uniquely impact astrocytes, I looked specifically at astrocyte-enriched mRNAs that were regulated in our data for insight. I made the unexpected observation that glucocorticoids appear to selectively target astrocyte-enriched mRNAs relative to all mRNAs expressed in astrocytes *in vitro* and that this regulation varied in a time-dependent manner. Since our study is one of the most comprehensive gene expression studies of astrocytes, I wonder if these principles are a general feature of glucocorticoid regulation in the brain across cell types. For

example, if this finding is generalizable, then we might expect glucocorticoids to preferentially target neuron-enriched mRNAs in neurons and oligodendrocyte-enriched mRNAs in oligodendrocytes. To test this idea, one could imagine performing similar *in vitro* experiments on purified cultures of each cell type and making similar comparisons of regulated mRNAs with published cell-enriched mRNA data sets. To our knowledge, no such studies have been conducted to date. Another relevant question is if glucocorticoids are the only stimuli that result in the observed targeting bias toward astrocyte-enriched mRNAs in astrocytes. Do other transcription factorsshow a similar targeting bias toward astrocyte-enriched mRNAs in astrocytes? Does the glucocorticoid receptor demonstrate bias toward cell-enriched mRNAs in additional cell types? And if so, are certain cell types may be more (or less) dependent upon cell-type-specific responses to transcriptional regulation? The implications of these findings and the underlying mechanisms of cell-type dependent responses would be of interest across a range of questions both in the brain and other organs.

Glucocorticoids, astrocytes, and potential clinical relevance

The stress response is an adaptive mechanism that is critical for everyday life. Proper functioning of the HPA axis enables individuals to respond appropriately to stressors. How the stress response impacts the brain is still not fully known, and understanding the mechanisms of how glucocorticoids influence brain function is likely to inform our knowledge of stress in general. This thesis work has implicated astrocytes as active components of stress response and identified mRNAs in these cells altered by

stress signaling.

Alterations of glucocorticoid signaling have been associated with many disease conditions. Of particular interest to our lab, HPA axis alterations are commonly reported in depression and stressful events often precipitate depressive episodes. This clinical inspiration for my thesis work may draw specific benefit from our findings. For instance, we found that chronic corticosterone exposure downregulated the mRNAs for the astrocyte gap junctions Cnx43; this finding is parallel to reports in postmortem studies of depression, identifying a potential mechanism responsible for the observed alterations. Extrapolating from these results and moving beyond correlations, I would be interested to see if glucocorticoid regulation of astrocyte-enriched mRNAs can cause alterations in mood. One way to approach this question would be to utilize transgenic mice lacking GR in astrocytes and testing whether these animals still show depressive-like behavioral responses to chronic stress or glucocorticoid stimulation. Given that glucocorticoids play a role in development and other cellular processes, we could use a conditional knockout knockout mouse design (e.g. tamoxifen-inducible CreER-Aldh1l1) to enable a temporally selective deletion of GR from astrocytes in adult mice. Further functional hypothesis testing using astrocyte cultures may be desired before pursuing these clinical connections.

Our findings also contain a cautionary note for studies of depression and astrocytes. The astrocyte marker used to measure astrocyte changes associated with depression (Gfap) is clearly regulated by glucocorticoids; since both depression and the

utilized animal models are associated with alterations in glucocorticoid signaling, these reported results may be confounded by glucocorticoid regulation that may not actually be related to astrocyte cell number. We find that the more recently identified astrocyte marker Aldh1l1 was not downregulated by glucocorticoids under any conditions in our study; indeed, Aldh1l1 was not regulated or was even upregulated in response to chronic corticosterone exposure. Assessing Aldh1l1-positive cells in depressed brains compared to control brains as well as Gfap-positive cells may address this potential confound. Studies of astrocytes in depression would likely benefit from using a cell marker not regulated by any associated physiology, and the previous studies would also profit by reexamining the experiments with a combination of multiple astrocyte markers (e.g. Aldh1l1, S100β, GLT-1).

If specific mRNAs regulated by glucocorticoids in astrocytes are altered in depression as well, our findings may implicate stress signaling as mechanism and inform treatment development and therapeutics. Long-term, astrocytes may be a future target of depression treatment. Recent studies are already speculating that specific drugs may be altering functions commonly associated with astrocytes. For example, increasing glutamate uptake via treatment with riluzole was recently found to decrease depressive-like behaviors in animal models (Banasr et al., 2010). Also, ketamine was recently found to act as a rapid antidepressant, and recent hypotheses have been made that astrocytes may be a target mediating that response (Mitterauer, 2012). In addition, very recent data has implicated adenosine signaling in astrocytes in mediating the antidepressant-like actions of sleep deprivation (Hines et al., 2013). Our work identifies

a series of mRNAs whose expression is enriched in astrocytes and regulated by glucocorticoids, a key stress hormone known to be elevated chronically in the majority of patients with depression. Taken together, these reports implicate astrocytes as potential therapeutic targets in the treatment of depression.

While the clinical connections to depression are speculative at this point, findings in this thesis indicate that astrocytes actively respond to glucocorticoids and are likely players in the classic stress response. Our data indicate that a variety of brain processes involving astrocytes may be altered by stress levels of glucocorticoids, including glutamatergic neurotransmission, energy metabolism, and gap junction connectivity. Astrocytes represent an additional component to understanding glucocorticoid signaling in the brain. Future basic science research and clinical investigations of glucocorticoid action in the brain and HPA axis signaling will likely be enhanced by considering the factor of cell type in data interpretation.

Overall Synopsis of Research Findings

*Glucocorticoids dynamically regulate hundreds of mRNAs in astrocytes *in vitro*

*Identified 36 novel mouse astrocyte-enriched GR-regulated mRNAs

*Glucocorticoids via yet unknown mechanisms selectively regulate astrocyte-enriched mRNAs in astrocytes

*Glucocorticoid regulation in astrocytes regulate astrocyte-enriched processes in a time-dependent fashion biased towards early events while less cell type specific responses are delayed.

*Acute corticosterone exposure in mice regulates many mRNAs regulated by glucocorticoids in astrocytes *in vitro*, including select astrocyte-enriched mRNAs

*Chronic corticosterone exposure in mice regulates mRNAs regulated both by glucocorticoids in astrocytes *in vitro* and acute corticosterone exposure *in vivo*, including select astrocyte-enriched mRNAs

*Chronic corticosterone exposure regulates additional mRNAs compared to acute exposure *in vitro* and *in vivo*, including astrocyte-enriched mRNAs

*Glucocorticoids regulate astrocyte-enriched mRNAs *in vitro* and *in vivo* associated with known astrocyte function, such as glutamate reuptake and metabolism and gap junction connectivity

*Glucocorticoids regulate astrocyte-enriched mRNAs that are also altered in human depression

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