Stress effects on astrocyte communication and regulation of adult hippocampal neural stem cells

By

David Covarrubias

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Molecular and Cell Biology

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Daniela Kaufer, Co-Chair
Professor Marla Feller, Co-Chair
Professor John Ngai
Professor David Schaffer

Spring 2015

Abstract

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The generation of new neurons in the hippocampus in adult mammals, or adult hippocampal neurogenesis, is a potent source of plasticity in the brain. Adult neurogenesis is highly regulated by the organism's environment, and stress is a powerful regulator of neurogenesis. Understanding the molecular basis of this stress-induced regulation helps us better appreciate the mechanisms and functional relevance of this fascinating process. Neurogenesis depends on the development of neural stem cells, which are tightly controlled by their surrounding cellular microenvironment, especially by a cell type of the brain that has shown to be highly important in regulating brain function, the astrocyte. The following studies describe how astrocyte-based signaling imparts key molecular regulatory mechanisms that are modulated by stress in the adult rat. Chapter 2 describes how an acute stressor enhances neural stem cell proliferation via astrocytic FGF2 signaling. Chapter 3 describes the suppression of stem cell proliferation by chronic stress via a late-onset astrocytic increase of BMP signaling. Taken together, these studies further advance our understanding of stress modulation of brain plasticity, and take into consideration an understudied cell type in stress biology, the astrocyte.

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Acknowledgements

I would like to begin by thanking my thesis committee. Throughout my time in Berkeley, they have always offered fantastic guidance, support and constructive criticism. With their input, I have become a better scientist, presenter and critical reviewer of scientific literature. In particular, I cannot speak highly enough of my advisor, Daniela Kaufer. She has been a wonderful and supportive mentor; she has supported both my scientific and personal endeavors with the highest of optimism and the most valuable of advice. I hold all of our conversations in the highest regard, and in particular I thank her for bringing me back from disappointment and disillusionment of the elusive and difficult endeavor that was the producing and understanding of the body of scientific data that I hereby present.

I would also like to thank the support of the whole MCB community, from Graduate Affairs Office and everyone else in the university administration I nagged with questions while trying to navigate the byzantine world of rules and obstacles that is obtaining a PhD. But in particular, I must thank my class of MCB 2009, where I found some of the best friendships who were a source of vital support. This voyage would not have been possible without some of the most intelligent, fun and strange people I have ever met. So many of us did so much to keep each other sane and able to power through it all, and I thank you all for being there through thick and thin. To Adam, Pengcheng and Mark, you guys kept me sane and at least marginally fit with our racquetball and board game times. To Anna, Adam, Livy, Nicole, Benjamin, Scott, Molly, Jenny and Dave, you fools were always around, be it as partners in cooking, drinking, sports watching and play, for games, movies or as outdoors enthusiasts; there are innumerable ways I want to thank you all. Also, to my old roommates Angela, Emily, Maddie, Matt and the little-sister-I-never-had Charlene, our first few years living together were some of my most fun, thank you for all our adventures and gratuitous food inventing and indulging. I wish you all the best always.

The entire Kaufer lab has been an incredibly fun and supportive group to work with. Our conversations have always been wonderfully silly and fun, and even every once in a while some of the most intellectually stimulating. I especially thank Anna, Liz, Jeremy, Kereshmeh, Soo, Sam, Sandra and Aaron. I've learned so much from all of you, and in particular I want to single out Liz for her mentorship, collaboration and guidance. I also need to thank the people I have mentored over the years, David, Jennifer, Annie, Shawn, Vivian, and everyone else whom I have taught; from you I've also learned so much from, you all have made me a better person. I hold a special place in my heart for Jennifer, whom I witnessed grow as a scientist and friend for three amazing years working together. This work would not have been the same without her.

My family has been the most amazing support for my whole life; needless to say I would have never reached this point without their love and help. You've taught and led me through example, I try to be the best person I can be because you all have made me such. Mom, Dad, Vicky, Guillermo and Hilda, this is for all of you. In here I also thank my brother by heart, if not by blood, David Ruiz. It's difficult to have a friendship as long lasting as ours, and I hold his with the utmost love, here's to many more years!

Finally, some say you have to save the best for last, and I definitely will hold this adage true in here. In grad school you find many things, you learn a lot about your science, life and yourself.

But for me, my greatest finding of them all was my love, my partner in everything, Anna Geraghty. She has been a part of so many facets of my life (as shown by her name being in many parts of my acknowledgements) and I really would not have it any other way. Anna, my love, you have made these the best years of my life, and I cannot wait to share the rest of my life with you. Thank you for everything, and I truly mean everything. You have been a lab-mate, a best friend, a partner, a roommate and soon you will be my lovely wife. You've helped me in my darkest of times, you've shared with me the brightest of moments, and my truly greatest, fondest and most lovely memories are with you. When I say you made this whole crazy thing possible, I mean it with all my heart.

So to you all, and to the beautiful city and campus of Berkeley, thank you. Now, with also endless appreciation and acknowledgement to the wonderful rats, astrocytes and stem cells that participated in these studies, I present and dedicate to you all, my dissertation.

Chapter 1

Introduction

A. Adult hippocampal neurogenesis

On adult neurogenesis

The generation of new neurons continues to occur in the adult mammalian brain in two regions: the dentate gyrus subgranular zone (SGZ) of the hippocampus[1,2] and the subventricular zone (SVZ) of the lateral ventricles[3]. These new cells originate from a pool of neural stem cells (NSCs) and in the dentate gyrus of the hippocampus, they are capable of self renewing and differentiating into new granule neurons, astrocytes, and oligodendrocytes[4,5].
These adult hippocampal stem cells were first observed *in vivo* by Joseph Altman in the 60s[6,7], but the field did not gain traction until the 90s, when neural stem cells were further characterized and eventually isolated and cultured *in vitro*[8,9]. Defined by their capacity to self-renew, proliferate and differentiate into neurons and glia, these stem cells can undergo self-renewal and differentiation into neurons and glia *in vitro*[2,10].

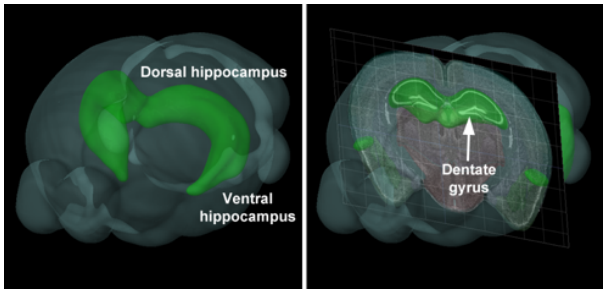
The life and times of neural stem cells

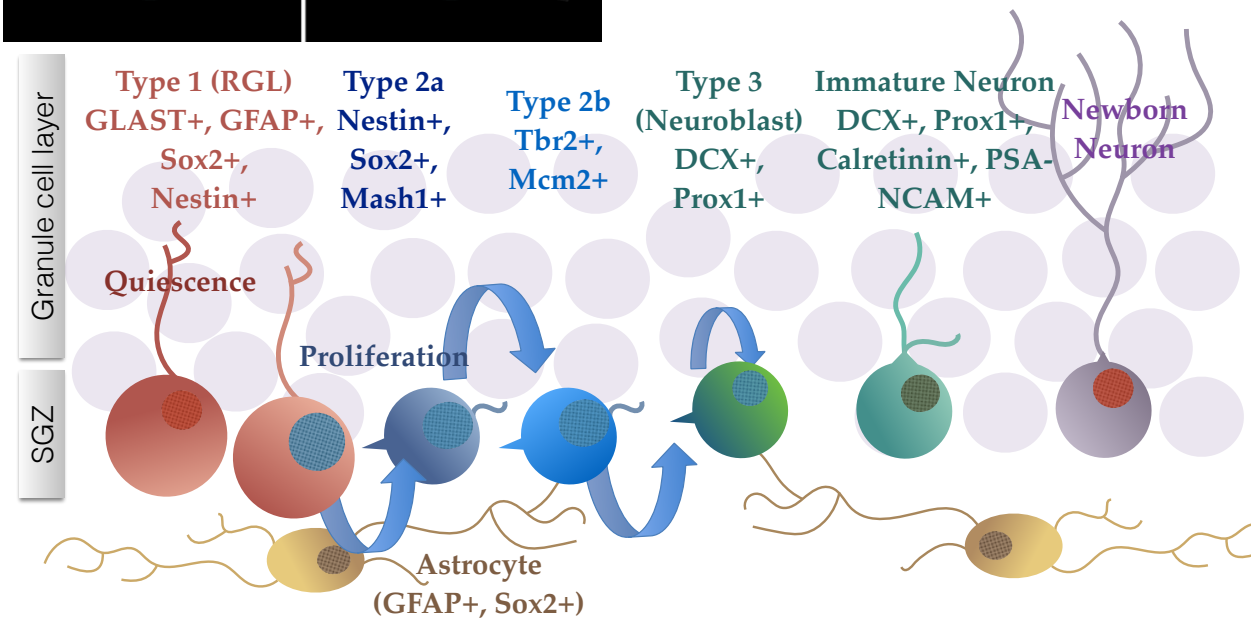
In the stem cell niche adult hippocampus, the SGZ of the dentate gyrus, NSCs go through a characteristic development composed of several stages, typically defined by their expression of certain immunohistochemical markers[11,12]. In the dentate gyrus, the putative resident stem cell is thought to be the Type-1 cell[13], also known as radial-glia like (RGL) cell. Type-1 cells are a mostly quiescent population that is slow to divide. They possess astrocyte-like properties[14,15] such as expression of glial fibrillary acidic protein (GFAP), glutamate aspartate transporter (GLAST) as well as NSC-specific markers Sox-2 and nestin. Next, Type-1 stem cells give rise to an intermediate transiently amplifying population of neural progenitor cells (NPCs) that are the main proliferative population; these include the type-2a cell, characterized by its expression of Nestin, Sox2 and Mash1, followed by the Tbr2- and MCM2-expressing type-2b cells. Type2 progenitors then give rise to doublecortin (DCX) and Prox1 expressing Type3 cells, or neuroblasts[13], which are slowly dividing. From the neuroblast stage, these cells become terminally differentiated and exit the cell cycle, becoming immature neurons that continue to express DCX, Prox1 and Calretinin, as well as PSA-NCAM[12,16–18] (Figure 1.1).
As in neurogenesis during early development, adult neurogenesis produces a surplus of transient amplifying progenitors and young neurons, more cells than what ultimately is integrated into the existing circuitry[19]. This means that cell survival is a key point in regulation for the final population of surviving newborn neurons. Cells that do not integrate with the functional hippocampal system undergo apoptosis[20,21], although the exact system for regulation is not clear. It is possible that the population of newly post-mitotic neurons undergo a competitive survival stage, where they compete for input and functional integration via synaptic input through the glutamate N-methyl-D-aspartate (NMDA) receptor[22]. Interestingly, the rates of cell death are significantly variable depending on the animal's genetic background[23], suggesting a that although cell survival and maturation can be affected by network activity[24], there is a different baseline of survival that is genotype-dependent rather than experience-dependent.
Young post-mitotic neurons expressing DCX and calretinin finalize migration into the granule cell layer (GCL), usually migrating the equivalent of 2-3 cell body diameter into the bottom third of the GCL. At this point, between 1-2 weeks post division, they begin neurite extension, and

while they are synaptically inactive and present only immature spines, they receive stimulation from ambient GABA and fire spontaneous action potentials[25]. Due to expression of the chloride importer NKCCl, GABA is depolarizing in the young neurons[26] and drives functional maturation of these cells[27,28]. The transition to glutamate becoming the main excitatory neurotransmitter occurs approximately 4 weeks after cell division due to the change in chloride transporters[25]. At this point, the immature neurons' dendrites begin to form spines and functional synaptogenesis around existing synapses[29].

Finally, the newborn neurons have distinct properties from mature neurons. They possess a lower threshold for synaptic plasticity in the form of long-term-potentialtion (LTP)[28], additionally, they receive almost no GABAergic inhibition, which is powerful in other mature granule neurons, meaning they are more constantly active than their mature counterparts[25]. Fine tuning of synaptic connectivity and dendritic morphology continues well into the second month of the new neuron's life[29], but by week 8-10 the cells become indistinguishable from their mature counterparts[30].

Figure 1.1. Adult neurogenesis in the dentate gyrus. Top: (Left) Image of the hippocampal formation within the rodent brain. (Right) Location of the dentate gyrus, the site of adult hippocampal neurogenesis. Images generated using Brain Explorer software from the Allen Institute for Brain Science. Bottom: Immunohistochemical markers distinguish development and subpopulations of neural stem cells in the subgranular zone (SGZ) of the granule cell layer of the adult dentate gyrus.





B. Function and Regulation of Adult Hippocampal Neurogenesis

Neurogenic niche and NSC regulation

Neurotransmitters. As mentioned before, the neurotransmitter gamma-aminobutyric acid (GABA) is excitatory in the early progenitor and young neuron stage. Activation of GABA-A receptors has been recorded from the early intermediate progenitor stage of NSCs [25,28] but it is unclear if the source of GABA comes from direct innervation from local interneurons or if it originates from ambient GABA spillover from nearby synapses. Also unclear is whether GABA promotes or represses proliferation due to the complexity of the inhibitory network in the dentate gyrus. For instance, forcing activation of GABA-A receptors to be inhibitory by early targeted overexpression of the chloride exporter KCC2 leads to premature and erroneous integration of the new neurons[26]. It is likely that GABA has several nuanced forms of control, since it is possible that it acts as a proxy for surrounding network activity, and that small changes in overall GABAeric and glutamatergic balance have specific effects in maintaining the orchestration of NSC development.

Like GABA, data on the effects of glutamate on neurogenesis are mixed. Extreme activation of glutamate receptors, as in a seizure event, leads to increased neurogenesis[31]. In contrast, lesioning excitatory input from the entorhinal cortex in one study also up-regulated cell proliferation[32], whereas a latter study failed to replicate this effect[33]. Furthermore, manipulation of different glutamatergic receptor systems has different effects on neurogenesis. NMDA receptor antagonism induces increased neurogenesis via an increased, or accelerated, differentiation program[32]. Kainate receptor activation leads to cell proliferation increases[34], and chronic administration of an AMPA receptor potentiatior also led to increases in neurogenesis, but these effects were likely via other mechanisms, such as possibly induction of BDNF expression in the hippocampus[35]. Further study with finer tools will be necessary to fully understand this complex system of NSC control by the main excitatory and inhibitory neurotransmitters.

The hippocampus receives serotonergic innervation from the dorsal raphe nucleus, and depletion of serotonin via lesion of serotonergic neurons leads to a decrease in adult hippocampal neurogenesis[36]. This work was followed up by a finding whereby re-engrafting fetal serotonergic neurons rescued the neurogenesis deficit[37]. A closer look at specific serotonin receptor influences seem to implicate serotonin receptors 1A and 2A (5HT-1A and 5HT-2A) on NSCs for these effects[38]. Furthermore, anti-depressant drugs that increase serotonergic activity also leads to increases in adult hippocampal neurogenesis[39,40], an effect that puts into question the role of neurogenesis in depression, stress and anxiety related disorders, a subject that will be further discussed below.

Growth factors and Morphogens. There are an astonishing variety of signals that tightly regulate the development of neural stem cells. If the wide array of neurotransmitters is complex, the milieu of cytokines, growth factors, morphogens, cell-adhesion signals and neurotrophic signals that control neurogenesis is vast. Fantastic reviews of the influence of these factors to neurogenesis are given by[12,41,42]. Here, I will describe a few factors that will appear within the scope of my studies. Growth factors of the receptor tyrosine kinase family such as fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) are all neurogenic to some extent, and can activate pro-proliferation programs in stem cells[43–47]. In fact, FGF2 or FGF-basic is a necessary component for cell proliferation in most

NSC cultures[2,8]. Brain derived neurotrophic factor (BDNF) is also pro-neurogenic[48,49]. Morphogens such as Wnt-3, secreted by astrocytes, is also neurogenic[50]. Finally, another morphogen, bone morphogenetic protein (BMP), a member of the transforming growth factor (TGF) signaling family, has been shown to be an important regulator of maintenance of "stemness" by inducing stem cell quiescence via BMP receptor type 1a (BMPR1a)[51,52]. In other words, this allows for these cells to maintain their stem-cell state without progressing through cell division and terminal differentiation. In the SGZ, proliferation and progression of NSC development is permitted to occur by suppression of BMP signaling via secretion of noggin[53] and neurogenesin-1 (aka chordin-like-1 or Chdl-1)[54].

The stem cell niche. The "niche" is the surrounding microenvironment where stem cells reside. It has been shown that cells in the stem cell niche control stem cell maintenance and development [42,55,56]. For instance, it has been shown that microglia have a role in clearing out, or phagocytosing, apoptotic cells during the phase of pruning of un-integrated newborn neurons[57]. Additionally, it has been well characterized that the stem cell niche is closely associated with surrounding vasculature[58], which is understood to have an important role not only in supplying the stem cell niche with trophic factors, but also serving as a window for connection with the organism's physiological state.

Astrocytes. In particular interest to the stem cell niche, astrocytes are a cell type that is physically closely associated to NSCs in the microenvironment[14,59]. Astrocytes actively control stem cell proliferation and fate specification through direct cell-cell contact through notch[60] or Eph-ephrin signaling[61], or a variety of secreted trophic factors[62]. In previous studies, it has been shown *in vitro* that secreted factors from astrocyte conditioned media (ACM) alone can significantly enhance neurogenesis by both increasing rates of NSC proliferation and instructing NSCs to adopt a neuronal fate[59,62,63]. These findings suggest that astrocytes have an array of signaling molecules that can actively regulate stem cell development and can modify the rate of neurogenesis in a variety of ways, suggesting that changes in astrocytic signaling can have a strong impact on regulation of neurogenesis.

Physiological/environmental regulation. As we've reviewed, hippocampal NSCs are highly controlled by their cellular environment. In addition to tight control of their development by their niche, NSC regulation is highly responsive to the organism's environmental and physiological conditions. It has been observed that several environmental challenges can be powerful modulators of neurogenesis, and can serve to enhance or suppress the formation of new hippocampal cells by affecting the progression of NSC development. Certain challenges to the organism, often related to positive outcomes, can lead to enhance neurogenic potential of the hippocampus. Exploring and living in an enriched environment[49,64,65], learning[66], voluntary exercise[65,67,68] and even sexual experience[69] are all known to increase neurogenesis. Interestingly, some of these interventions also enhance cognitive abilities related to hippocampal function, such as memory-associated tasks. On the other hand, challenges often viewed as having negative behavioral and cognitive consequences, such as several types of stress[70], social isolation or a deprived environment[71,72], sleep deprivation[73–75], jet lag[76] and aging[45,77,78] also lead to sharply decreased neurogenic potential of the hippocampus by affecting all points of NSC development, from proliferation, fate choice to cell survival. Whereas there are certain nuances and seemingly conflicting data regarding whether different stress protocols are indeed able to induce decreases of neurogenesis[79], most severe unpredictable and uncontrollable stresses are powerful inhibitors of neurogenesis. Protocols such as chronic restraint stress[80,81], social

defeat[82,83], early life stress[84–86], and mixed stressors such as cold swim, foot shock, etc[87,88] are able to effectively suppress neurogenesis.

Functional role of neurogenesis

The function of adult hippocampal neurogenesis is an active field of study, but studies have shed some light to several roles that newborn neurons could be playing. First, in order to understand what the newborn neurons are doing, we must understand the function of their resident structure: the dentate gyrus of the hippocampal formation.

The hippocampal formation. The hippocampus is thought to function in the encoding of spatial,[89,90] contextual or episodic memories,[90,91] and the ability of an animal to discern closely related events, termed pattern separation[92,93]. The hippocampus is structured as a trisynaptic circuit. First, layer 2 entorhinal cortex neurons send projections via the perforant pathway that synapse with dentate gyrus granule cells[94]. These dentate gyrus granule cells then integrate entorhinal input and are involved in a computation referred to as sparse coding, which involves coding similar inputs into outputs that are more distinct from one another[93]. This processing is key in what is thought to be an important role of the dentate gyrus: pattern separation. The second synaptic connection in the circuit is the output from the dentate gyrus via mossy fibers to the CA3 region that continues to concomitantly receive input from the perforant pathway, thus integrating information from both entorhinal and dentate inputs. Finally, the third synaptic connection is the CA3 projections to the CA1 via Schaffer collaterals, which in turn close the circuit by sending projections back to layer IV and V of the entorhinal cortex. The CA1 region has been well studied and is of interest for its role in spatial memory encoding via neurons that are termed "place cells"[89,90]. Additionally, a novel emerging functional difference between the dorsal and ventral sub-structures of the hippocampus has emerged, where the ventral hippocampus seems to be more functionally related to anxiety and emotional memory[95].

Neural stem cells and pattern separation. The role of the dentate gyrus as a pattern separator can give us an inkling of what role the newborn neurons are playing. Computationally, the ability to discern similar information, or pattern separation, can be encoded via distinct populations responding to similar inputs (*spatial encoding)*, or by distinct firing outputs from similar inputs (*rate encoding).* The dentate gyrus is well suited to fulfill either strategy, as it contains a far greater number of neurons than its input, the entorhinal cortex[94], perfect for spatial encoding, as well as the fact that granule cells have many lateral inputs, mainly inhibitory from hilar interneurons or excitatory from hilar mossy cells, allowing for distinct firing patterns to emerge (rate encoding). This, at least theoretically, would allow sparse coding to occur both by distinct cell populations firing at inputs that are similar to each other as well as distinct firing patterns to emerge from distinct contextual inputs[94,96]. It can then be hypothesized that new born neurons, possessing a lower threshold for synaptic plasticity compared to their mature neuron counterparts, are then more reliably activated during a novel experience. This can then help encode new information more easily without interfering with old memory encoding, and by creating new functional units to be involved in sparse coding of information lead to improved pattern separation. Indeed, several studies have found that altering neurogenesis can result in interesting changes in a wide array of behaviors and functions. Some of the first inklings of the relationship between neurogenesis and hippocampal memory function came from the observation that pro-neurogenic regiments, like exercise[65,73], come with added memory benefits[97,98] with the

added note that abolishing the neurogenic potential also abolished the positive outcomes[97]. Additionally, other studies indicate that enhancing neurogenesis can improve hippocampal memory function[44,99,100]. Conversely, others have found that ablating neurogenesis leads to detrimental effects on performance of several hippocampal memory tests[101–104].

Stress, depression and neural stem cells. Aside from effects on memory, the hippocampus is also involved in the regulation of the stress axis. The hippocampus functions as a negative feedback controller, a responsibility that endows it with a high amount of receptors for the stress hormones, glucocorticoids (the relationship between stress and neurogenesis is discussed in detail in the following section). Thus, the hippocampus is particularly sensitive to stress, and as we've stated, adult hippocampal neurogenesis is particularly sensitive to the stress response. But some recent findings indicate that the stress response itself is receptive to regulation by neurogenesis. Ablating neurogenesis led to suppression of the feedback system that shuts the stress response down[105,106]. There's also an interesting link to the etiology of anxiety and depression as neurogenesis is augmented by anti-depressants[107–110], and conversely neurogenesis is also required for the beneficial effects of anti-depressants[101,106,107,109]. This means that a big function of the neurogenic dentate gyrus is to integrate the affective and homeostatic balance of the organism's mental state and environment while modulating its own function to fine-tune memory encoding.

C. Stress and neurogenesis

Now we turn to briefly review the biology of the stress response system and the effect of stress on brain plasticity and hippocampal function to further understand the impact of stress on neurogenesis and the current understanding of the interplay between stress and neurogenesis.

Stress and the glucocorticoid system. Stress can be defined as a negative experience disruptive to the homeostatic balance of an organism, leading to a physiological adaptive response to cope with the challenge[111,112]. The organism responds to stress partly by the activation of the hypothalamic-pituitary-adrenal (HPA) axis, which leads to the secretion of glucocorticoids (GCs) from the adrenal cortex[111]. First, a stressful event leads to a hormone cascade that begins with the activation of hypothalamic neurons that secrete releasing hormones corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP). This leads to activation of pituitary neurons that in turn release adrenocorticotropic hormone (ACTH) that finalizes the cascade by activating the adrenal cortex to release high levels of glucocorticoids into circulation[113].

At the cellular level, GCs bind primarily to two receptor types, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). The MR has a 10-fold higher affinity to GCs than the GR, and is thus bound to baseline levels of circulating GCs, which follow a daily circadian pattern of release under normal conditions. The MR functions to affect baseline long-latency broad changes in gene transcription and is bound to CORT even in the nadir of circadian release of GCs[113]. The GR, on the other hand, only becomes occupied by increased levels of GC release occurring in response to peaks of circadian GC release or at highest concentrations during the response to a stressor[113,114]. The receptors themselves are intracellular receptors that are sequestered by a complex composed of heat shock proteins Hsp90 and Hsp70. Upon GC binding, GRs dimerize, a nuclear localization signal is exposed, and the dimer form of the functional GRs migrate into the cell nucleus where they act as in different ways to alter gene expression[115]. For example, they can activate or inhibit transcription by direct binding DNA sequences known as

GRE (glucocorticoid response element), or alter transcription by binding to other transcription factors such as NF-kB and CREB and inhibiting or enhancing their activity[115,116].

Physiologically, GCs have a broad spectrum of activities that function to support and stimulate a stress response, to serve as a shut-off for stress-induced changes, or even to prepare for a future stressor[116,117]. However, if this response becomes dysregulated or overused, as in a severe or chronic stress, this adaptive response can have severe aversive effects. This duality of stress, has been well described as an inverted U-curve[118–120], where chronic stress can lead to adverse health conditions, including cardiovascular disease, obesity, and severe psychiatric disorders such as depression and anxiety[121]. Furthermore, GCs are powerful modulators of mammalian brain physiology. It is has been observed that in the hippocampus, a structure that contains a high degree of expression of glucocorticoid receptors[122], chronic GCs exposure can lead to behavioral changes, such as anxiety and depressive disorders[123] and changes in memory and cognitive performance[119,124]. Chronic GCs can also lead to structural and cellular changes, such as atrophy of dendritic spines[125,126], reduced synaptic long term potentiation (LTP)[112,127], enhanced synaptic long term depression (LTD)[128], and decreased adult hippocampal neurogenesis[70,129].

Stress and neurogenesis. Chronic stress or chronic glucocorticoid receptor activation has powerful effects at several points in the process of neurogenesis[129,130], as it as been shown to suppress NSC proliferation[131,132], decrease cell survival[133], and inhibit neuronal differentiation[134] in the adult dentate gyrus. These stress-induced negative consequences on neurogenesis are consistent with behavioral studies indicating that reduced neurogenesis, like extended exposure to chronic stress, leads to impairment of hippocampal dependent memory[135] and anxiety and depressive-like disorders[108]. This suggests that there is a very close relationship between stress-related disorders and neurogenesis. Interestingly, recent reports indicate that newborn neurons have an impactful role in controlling the negative feedback loop that shuts-off HPA axis activation[105], suggesting that proper regulation of hippocampal neurogenesis is as key in maintaining a healthy stress response as the stress environment is key in regulating neurogenesis[136].

Several molecular mechanisms for the way GCs regulate neurogenesis have been proposed. First, direct action of GCs on stem cells, as shown by suppression of cell cycle progression by high concentrations of glucocorticoids or glucocorticoid receptor agonists *in vitro*[137]. Second, mechanisms affecting the surrounding neural circuitry. For example, stress alters the excitation-inhibition balance of the neural network[138], which can have an effect on NSC development. Moreover, it has been reported that NMDA receptor activity is necessary in GC-mediated decrease of stem cell proliferation[139]. However, a well-dissected role of the stem cell niche, in particular, the astrocytic involvement in the stress effects on neurogenesis has not been elucidated. In the studies presented here, I have begun to answer this question by studying the roles of astrocyte signaling in the effects of acute and chronic stress paradigms on neurogenesis in a rat model.

Chapter 2

Acute stress enhances adult hippocampal neurogenesis and memory via secreted astrocytic FGF2 in rats.

From: Elizabeth D. Kirby, Sandra E. Muroy, Wayne G. Sun, David Covarrubias, Megan J. Leong, Laurel A. Barchas and Daniela Kaufer. eLife, 2013

A. Introduction

Stress is a powerful and essential mediator of mammalian behavior. Proper response to a perceived stressor facilitates survival at the individual level and species propagation at the population level. Despite this necessity, stress responses can become maladaptive. Chronic stress, for example, leads to a host of adverse health consequences, including cardiovascular disease, obesity, depression, and exacerbation of neurodegeneration[121]. Acute stress, defined as a single exposure on the scale of minutes to hours without cycles of recovery and re-exposure, has proven more enigmatic.

One model of stress effects on the brain explains the variable consequences of acute stress for brain health as following an inverted U function[119]. In this model, while severe or prolonged stressors are detrimental, brief or moderate stressors actually enhance neural function. Behavioral studies focusing on the memory functions of the hippocampus have demonstrated such a relationship in rodents, where moderate stress enhances memory performance yet more severe stress impairs it[140].

The hippocampus is exquisitely sensitive to stress and the primary stress hormone class, glucocorticoids (GCs). Within the dentate gyrus (DG) sub-region, in particular, there exists a high density of GC receptors that respond to elevated circulating GCs[122]. In addition, the DG is strongly connected via the entorhinal cortex and medial septum to the basolateral amygdala (BLA), a brain region involved in emotional processing and an important mediator of many stress effects on the hippocampus[141]. Both of these mediators of stress (GCs and BLA input) have been shown to regulate the unique population of neural progenitor cells (NPCs) that reside in the adult DG[130,142].

Dentate NPCs proliferate and give rise to new neurons throughout the lifespan in several mammalian species, including rats, mice and primates[12,130]. They become electrophysiologically active, integrate into local circuitry, play important modulatory roles in hippocampal memory function[12] and can respond to stress and stress hormones at multiple phases of development[130]. Moreover, recent work indicates that newborn, immature neurons integrate multiple signals more readily than mature neurons and that they have enhanced excitability, possibly contributing to a disproportionately large role in new memory formation[143].

Numerous studies show that chronic stress, in addition to impairing memory function, suppresses proliferation, survival and differentiation of new neurons in the adult DG[129,130,133]. The effect of acute stress on neurogenesis, however, is unclear. While early work indicated suppression of proliferation following acute stress or GC injection[144,145], subsequent studies have yielded mixed results, often reporting no change in proliferation following a variety of acute stressors[79,146–148]. In contrast, investigations of hippocampal growth factor secretion have shown that acute stress enhances expression of mitogenic growth factors such as basic fibroblast growth factor (FGF2) and nerve growth factor (NGF)[149,150], implying a potential for increased neurogenesis following acute stress. Indeed, a number of interventions that stimulate GC release such as acute exercise[68] and acute sexual experience[69] actually increase neurogenesis in the adult hippocampus. Combined, these studies suggest that adult hippocampal neurogenesis may follow an inverted U function similar to hippocampal memory, decreasing following chronic stress yet increasing in response to acute stressors.

We examined the effect of several forms of acute stress on adult hippocampal neurogenesis, seeking to resolve the contradictory evidence for enhanced versus impaired hippocampal plasticity. We found that acute stress or corticosterone (CORT, the primary rat GC)

administration increased dorsal but not ventral hippocampus cell proliferation in adult rats. This increase was not dependent on input from the BLA and was accompanied by an increase in FGF2 expression in dorsal hippocampal astrocytes. Furthermore, we show that astrocyte-secreted FGF2 is necessary for CORT-induced enhancement in NPC proliferation *in vitro*. Two weeks after acute stress, when newborn neurons are first becoming functional, we also find enhancement in hippocampus-dependent memory accompanied by enhanced activation of newborn neurons. These findings have important implications for understanding regulation of hippocampal plasticity in the face of environmental challenge and in distinguishing adaptive versus pathological stress responses. Moreover, they suggest that stress effects on adult neurogenesis may follow an inverted U function similar to that already demonstrated for hippocampal memory function[119].

B. Results

Acute stress increases dorsal hippocampus NPC proliferation. To investigate the effect of acute stress on adult neurogenesis in the DG (Fig. 2.1), we chose three common models of acute stress in rodents: 30 min novel environment, 30 min footshock or 3 hours immobilization. Rats were handled for 5 days prior to stress exposure then perfused 3 hours after the beginning of the stressor (Fig. 2.2a). Immobilization stress significantly increased the number of cells immunopositive for the proliferation marker Ki67 in the dorsal DG 3.23 fold above control (27.51± 2.74 control versus 88.99 ±12.49 immob) while novel environment or footshock did not significantly alter Ki67+ count compared to control (Fig. 2.2b,c). Plasma CORT was significantly elevated above control levels both 30 min and 3h after stressor initiation in immobilized rats but not in novel environment or footshock-exposed rats (Fig. 2.2d). This finding suggested that an increase in CORT might underlie the immobilization-induced increase in DG proliferation. To test that hypothesis, we next habituated handled rats to daily oil injections for three days and then injected them with exogenous CORT (0, 5 or 40 mg/kg body weight) and assessed cell proliferation 3 hours later (Fig. 2.2a). 40 mg/kg CORT significantly increased the number of Ki67+ proliferative cells in the dorsal DG 1.92 fold above oil-injected controls (26.19 ± 2.83 0 mg/kg CORT versus 50.29 ± 7.74 40 mg/kg CORT) (Fig. 2.2c,e). Plasma CORT levels were also consistently elevated 30 min and 3 hours after injection (Fig. 2.2f), similar to the levels seen in immobilized rats. Notably, injection of 5 mg/kg CORT yielded similar plasma CORT levels to footshock (approximately 119 ng/ml and 121 ng/ml, respectively) and also did not produce an increase in the numbers of Ki67 positive cells. If rats were not habituated to injection, no difference in cell proliferation was found (Fig. 2.2j,k). We next assessed short-term survival of newly born cells following acute stress. Three hours after the start of immobilization or CORT injection, rats were injected with the proliferative marker 5-bromodeoxyuridine (BrdU) then perfused 24 hours later. Both immobilization (Fig. 2.2g,i) and 40 mg/kg CORT injection (Fig. 2.2h,i) significantly increased the number of cells immunopositive for BrdU surviving 24 hours after termination of the stressor in the dorsal DG by 1.92 and 1.48 fold, respectively (34.88 ± 8.70 control versus 68.96 ± 10.23 immob; 39.80 ± 6.89 0 mg/kg CORT versus 58.95 mg/kg CORT).

Acute stress does not increase ventral hippocampus NPC proliferation. Recent work suggests different functional roles for the dorsal and ventral hippocampus in mediating spatial memory versus emotion regulation, respectively[151]. Adult neurogenesis may also be differentially

regulated in dorsal versus ventral hippocampus; while environmental enrichment enhances neurogenesis in both dorsal and ventral DG, chronic mild stress preferentially suppresses proliferation in the ventral subregions[152]. In our acute stress models, we found no effect of novel environment, footshock, immobilization or CORT injection (Fig. 2.3a-c) on proliferative Ki67+ cell number in the ventral hippocampus (Fig. 2.1b).

BLA input does not modulate acute stress-induced proliferation. Work by McGaugh and colleagues shows that acute stress-induced alterations in hippocampal memory and long-term potentiation depend on input from the BLA[153–155], a fear and emotion processing center. In addition, we have previously shown that BLA input regulates adult DG neurogenesis under basal conditions and is required for the activation of newborn neurons in a fear conditioning paradigm[141]. To test whether BLA input is necessary for the acute stress-induced increase in proliferation, we performed unilateral excitotoxic lesion of BLA in adult rats (as described in[142,156]). Animals were then exposed to immobilization stress or no stress control (Fig. 2.4a). At the end of the stressor, each rat received a BrdU injection and was perfused 2 hours later. Plasma CORT response to immobilization was similar in lesioned and sham-operated rats (Fig. 2.4b), suggesting intact hormonal stress response in BLA lesioned, immobilized rats. Consistent with our findings in intact rats, sham-operated immobilized rats had a significant 2.6-fold increase in BrdU+ proliferative cells over no stress controls (6.37 ± 1.14 control versus 16.69 ± 2.11 immob) (Fig. 2.4c). BLA lesion suppressed proliferation ipsilateral to the lesion by approximately 1.5 fold (1.66 fold control, 1.44 fold immob), as we have previously reported, but did not block the stress-induced increase in proliferation (Fig. 2.4d). These findings suggest that input from the BLA is not necessary for acute stress effects on adult hippocampal cell proliferation.

CORT-induced increase in isolated NPC proliferation is dependent on astrocyte signaling. Given that the stress-induced increase in proliferation was not dependent on BLA input, we next tested whether stress-induced proliferation is a cell autonomous phenomenon. This was accomplished by quantifying the response of isolated adult rat hippocampal NPCs grown *in vitro* (as described in[10]) to acute CORT exposure. NPCs were FGF2 deprived for 24 hours then treated with 1 μM CORT (equivalent to approximately 350 ng/ml, the level measured in plasma of immobilized rats) or EtOH vehicle in either high (20 ng/ml) or low (0 ng/ml) FGF2. After 3 hours, BrdU was added to the cells and they were fixed 2 hours later (Fig. 2.5a). Cultured rat hippocampal NPCs depend on FGF2 signaling for proliferation[157]. We found that while high FGF2 increased the percentage of proliferating BrdU+ NPCs over low FGF2 (1.21 fold in EtOH, 1.48 fold in 1 μM CORT), exposure to CORT did not affect the percent of proliferative cells in either high or low FGF2 media as compared to vehicle (Fig. 2.5b). These data indicate that NPCs do not respond to acute CORT in isolation, but likely rely on input from another cell type in the neurogenic niche. A growing body of work indicates that astrocytes can strongly regulate NPC dynamics through secreted factors[59], so we next tested whether astrocytes might participate in regulation of NPCs in response to CORT. We treated primary hippocampal astrocytes cultured as described in[158] with 1 μM CORT or EtOH vehicle for 3 hours then extracted astrocyte conditioned media (ACM). When administered to isolated NPCs, CORT-treated ACM increased the percent of BrdU+ NPCs significantly over EtOH-treated coculture media (CoC) control by 1.52 fold (29.56 ± 2.81 CoC-EtOH versus 44.93 ± 2.82 ACM-1 μM CORT) (Fig. 2.5c,d). These

findings suggest that astrocytes could mediate the *in vivo* increase in NPC proliferation following acute stress through secreted factors.

Acute stress increases dorsal hippocampus FGF2 expression. Given that astrocytes secrete a variety of growth factors that support cell proliferation, we next investigated growth factor expression in the hippocampus of stressed rats. We quantified levels of the following growth factors previously reported to be mitogenic and/or elevated by acute stress[149,150,159] in the dorsal hippocampus of stressed and control rats: fgf2, brain derived neurotrophic factor (BDNF), nerve growth factor (NGF), fgf receptor 1 (FGFR1), FGFR2, FGFR3, FGFR4, vascular endothelial growth factor (VEGF), growth arrest and DNA damage–inducible 45β (GADD45β) as well as the early immediate gene, CFOS. Acute stress caused a significant increase in FGF2 mRNA and protein levels in the dorsal hippocampus (Fig. 2.6a-d,i). Immobilization increased fgf2 mRNA 1.77 (± 0.20) fold over control while 40 mg/kg CORT increased fgf2 mRNA by 1.70 (± 0.23) fold over 0 mg/kg CORT (Fig. 2.6a,b). Immobilization and 40 mg/kg CORT also significantly increased FGF2 protein levels in the dorsal hippocampus by 1.59 (± 0.24) fold and 2.54 (± 0.34) fold, respectively (Fig. 2.6c,d,i). In contrast, bdnf exon IV mRNA levels were significantly decreased by immobilization and CORT (Fig. 2.6e,f). BDNF protein level, however, was unchanged by either manipulation (Fig. 2.6g-i). Acute stress did not consistently alter mRNA levels of ngf, fgfr1, fgfr2, fgfr3, fgfr4, vegf, bdnf exon IX, or gadd45β (Fig. 2.7a-p). mRNA of the immediate early gene cfos was generally increased at 30 minutes after any manipulation, including oil injection (Fig. 2.7q,r).

Acute stress does not increase FGF2 expression in the ventral hippocampus. We next quantified FGF2 expression in ventral DG where we previously saw no effect of stress on cell proliferation. Immobilization and 40 mg/kg CORT injections significantly increased fgf2 mRNA levels in the ventral hippocampus, similar to the dorsal hippocampus (Fig. 2.6j,k). However, protein levels of FGF2 were not increased by any of the acute stressors in the ventral portion of the hippocampus (Fig. 2.6l-n). These results provide intriguing correlative evidence that increased FGF2 protein levels following acute immobilization or CORT exposure could underlie the observed increase in dorsal hippocampal proliferation.

Acute stress increases FGF2 expression in hilar astrocytes of the dorsal hippocampus. To determine whether astrocytes were the source of increased dorsal hippocampal FGF2 *in vivo*, we used confocal microscopy to quantify double immunohistochemical labeling for FGF2 and the astrocyte marker, glial fibrillary acidic protein (GFAP) in adult male rats treated as in Fig 2a. Consistent with previous reports[160], FGF2-immunoreactive cells were found throughout the DG and the hilus, with staining primarily in the cell body and nucleus. The DG is primarily composed of granule neurons and while almost every cell expressed FGF2, we found very few GFAP+ cells within the DG as expected. Mean optical density (with background correction) throughout the Z-stack of FGF2 expression in the DG revealed no effect of stress or CORT injection on DG FGF2 expression (Fig. 2.8a,d). In the hilus, a mixture of GFAP+ and GFAP- cells was found, with almost all GFAP+ cells being FGF2+. By quantifying integrated optical density of individual FGF2+ cells that were either GFAP+ or GFAP-, we found that both immobilization and 40 mg/kg CORT injection increased FGF2 signal in GFAP+ cells significantly over their respective control groups (Fig. 2.8b,d). No effect of immobilization or CORT was observed in GFAP- cells in the hilus (Fig 2.8c,d). These findings suggest that the

stress-induced increase in FGF2 levels in the DG most likely comes from neighboring astrocytes in the hilus.

FGF2 neutralization in CORT-ACM blocks increased NPC proliferation. To determine whether FGF2 was the astrocyte-derived factor driving NPC proliferation, we next examined the role of FGF2 in the effect of CORT-treated ACM on isolated NPCs. Consistent with previous work[161], media from untreated astrocytes showed no detectable FGF2 (0.12 ± 0.46 pg/ml). In contrast, astrocytes treated with 1 μM CORT for 3 hours secreted 3.5 (± 0.68) pg/ml FGF2 protein. We next tested whether levels of FGF2 as low as 4 pg/ml were sufficient to stimulate NPC proliferation. Treating with 4 pg/ml rat recombinant FGF2 caused a significant 1.39 fold increase in BrdU labeling compared to vehicle (0 pg/ml FGF2), suggesting that the levels of FGF2 present in CORT-ACM were sufficient to stimulate NPC proliferation (11.95 ± 1.22% BrdU+, 0 pg/ml; 16.65 ± 1.72% BrdU+, 4 pg/ml, $p = 0.04$). We next investigated whether blocking FGF2 function could prevent acute stress-induced enhancement of NPC proliferation using a neutralizing antibody against FGF2 (nAb). An ELISA for rat FGF2 revealed that the nAb decreased available FGF2 over a wide range of concentrations (Fig. 2.9b). The nAb did not, however, affect the availability of FGF1 (Fig. 2.9c), a closely related member of the FGF family. When tested *in vitro*, we found that FGF2 neutralization blocked FGF2-induced stimulation of NPC proliferation without affecting proliferation in FGF2-free conditions (Fig. 2.9d). These findings suggest that the nAb blocks FGF2 signaling specifically and does not have nonspecific toxic effects on NPC growth. We then pretreated ACM from EtOH- or CORT-treated astrocytes with FGF2 nAb before exposing NPCs to the treated media. Pretreatment with the FGF2 nAb blocked the CORT-ACM induced increase in NPC proliferation (30.67 ± 3.44% BrdU positive in EtOH, no nAb versus 30.33 ± 2.59% BrdU positive in 1 μM CORT with nAb) (Fig. 2.9e), suggesting that increased astrocytic FGF2 is the driving signal for CORT-ACM-induced proliferation of adult hippocampal NPCs.

Acute stress leads to a delayed enhancement of fear extinction retention. Several studies suggest that newly born neurons play an important functional role in hippocampal memory during an immature, highly plastic phase of their development[100,162–164]. However, newborn neurons in the adult rat require 2 or more weeks to mature and become physiologically active[165], implying that the enhancement in neurogenesis we observed following acute stress should require several weeks to influence behavior. We therefore assessed hippocampal memory using a fear conditioning task either 2 days or 2 weeks after acute stress. Immobilized and control rats were given 10 unsignaled, 1s, 1 mA shocks in a fear conditioning chamber. The next day, they received five 10 min extinction trials where they were exposed to the fear conditioning chamber without shock. On the third day of testing, they received a single 10 min extinction probe trial without shock. When rats were tested 2 days after immobilization, control and immobilized rats showed similar freezing behavior during training, extinction and the 24 hour extinction probe (Fig. 2.10a-c). When rats were tested 2 weeks after immobilization, immobilized rats did not differ from controls in percent time freezing during training or during the 5 extinction trials (Fig. 2.10d,e). During the extinction probe, however, immobilized rats froze significantly less than control rats, indicating better retention of the fear extinction (Fig. 2.10f). These results suggest that an incubation time is necessary for acute immobilization to improve fear memory and demonstrate enhanced hippocampal memory at a time when newborn neurons are highly plastic and sensitive to environmental input.

Acute stress leads to enhanced activation of newly born neurons. To determine if the neurons born 2 weeks before fear conditioning might play a role in the enhancement of fear memory retention, we quantified cell fate and activation in a subset of rats perfused 1 hour after the fear extinction probe. The number of surviving BrdU+ cells (Fig. 10g) and the percent of BrdU+ cells expressing the immature neuronal marker doublecortin (DCX+) or the astrocytic marker GFAP+ (Fig. 2.10h) were similar in immobilized and control rats. However, immobilized rats had significantly more immature (DCX+) cells expressing the immediate early gene cfos than control rats (1.57 ± 0.52% cfos+ versus 5.39 ± 1.40% cfos+) (Fig. 2.10i,j), suggesting greater activation of immature neurons in immobilized rats. These results indicate enhanced utilization of the highly plastic pool of new neurons born around the time of an acute stressor and suggest that stress-stimulated proliferation may support later memory benefits.

C. Discussion

The present study demonstrates that acute stress or exposure to the stress hormone CORT induces an increase in proliferation of hippocampal NPCs via increased secretion of astrocytic FGF2. This increase in proliferation is correlated with selective activation of the hyper-plastic newborn neurons and enhanced retention of fear extinction 2 weeks after the stressor. Taken together, these findings suggest a beneficial role for acute stress on hippocampal plasticity. Consistent with our findings, previous studies using similar stress paradigms show stress-induced enhancements in memory consolidation and growth factor expression[149,153,154]. Acute stress may also support hippocampal LTP, another factor implicated in stimulating adult hippocampal neurogenesis[166]. Notably, other manipulations that increase stress hormone secretion, such as exercise, sexual experience and mild immune challenge, similarly stimulate adult neurogenesis[68,69,167,168]. Recent work in adult squirrel monkeys also shows that coping with intermittent social stress through multiple pair separations and new pair formations both stimulates adult hippocampal neurogenesis and improves hippocampal memory[169]. These studies, along with our findings, evoke the interesting possibility that acute stress may be beneficial for brain health in general and hippocampal plasticity in particular.

Previous studies of adult NPC response to acute stress have yielded mixed results. While some initial work suggested that acute stress causes a decrease in NPC proliferation[133,144,145], more recent publications have not replicated these findings[79,146–148]. Notably, while we found that acute CORT enhanced proliferation in well-handled, habituated rats, we did not find any effect of acute CORT on neurogenesis in rats that were not habituated to injection. Our findings therefore demonstrate that factors present before the stressor begins may alter the effect of acute stress, perhaps by changing the susceptibility of controls or stressed animals to manipulation. Not surprisingly, a rich literature exists regarding the role of handling on altering the rodent stress response[84]. Such experimental differences could explain discrepancies in the literature.

Previous research concerning the long-term effects of acute stress in rodents focuses primarily on models of traumatic stress and the resultant PTSD-like symptoms. Most prominently, the single prolonged stress model of PTSD uses three acute stressors in series (restraint, forced swim and ether) and results in delayed deficits in fear extinction, as well as enhanced anxiety[170,171]. However, if any one of the three components of this stressor protocol are eliminated, deficits are no longer evident[170], suggesting that stressor effects depend on the severity and length of the stressor, perhaps following the inverted U function previously described[119].

Previous work in primates has provided additional evidence for an inverted U relationship between stress and neural function. In multiple non-human primate species, for example, mild early life stress can lead to resilience against stress later in life, a phenomenon referred to as stress inoculation[172,173]. In contrast, more severe early life stress can have the opposite outcome, increasing stress vulnerability later in adulthood. When combined with our current findings, these studies fit well with the proposed inverted U function for stress effects on brain health[174,175]. At high, traumatic levels, acute stress may result in maladaptive pathology (e.g. PTSD-like symptoms in the single prolonged stress model). At more moderate levels, though, such as immobilization in well-handled rats, acute stress may actually enhance function. Future research will be needed to more precisely define the limits of stimulating versus detrimental acute stress.

Many acute stress-induced changes in hippocampal plasticity rely on functional input from the BLA. For example, while stress enhances hippocampal memory consolidation and LTP, lesion of the BLA blocks these enhancements[154,155,176]. We have recently reported that hippocampal cell proliferation and the activation of the newborn neurons in a fear-condoning paradigm both depend on BLA neural input[156]. In the current study, we found that while BLA lesion suppressed hippocampal cell proliferation as we have shown before[142], it did not affect the acute stress-induced increase in neurogenesis. These findings imply that acute stress regulation of neurogenesis may not rely on the same systems-level *in vivo* circuitry that mediates acute stress regulation of memory consolidation and LTP. In support of this hypothesis, we were able to model CORT-induced NPC proliferation in isolated NPCs. We found that while NPCs did not respond to acute CORT treatment independently *in vitro,* they did proliferate more in response to conditioned media from CORT-treated primary astrocytes, suggesting a role for secreted factors from local astrocytes in mediating stress effects on NPCs.

The dynamic role of astrocytes in facilitating neuronal function through secreted factors has gained much recognition[177]. In addition to aiding in synaptic glutamate recycling, astrocytes secrete several factors such as thrombospondins[177,178], Hevin, SPARC[179] and glypicans [180] that regulate synaptic formation and function in mature neurons[177]. Astrocytes express the glucocorticoid receptor, GR, and when they are exposed to high levels of GCs, GC-bound GR translocates to the nucleus and enhances FGF2 gene transcription[149,181,182]. FGF2 is a potent and necessary proliferative factor in adult NPCs[157]. We found that acute stress stimulated FGF2 expression in the dorsal hippocampus and in primary hippocampal astrocytes. We further showed that in the DG and hilus, the enhancement in FGF2 levels following stress is largely restricted to GFAP+ astrocytes, Notably, changes in FGF2 protein levels following acute stress closely paralleled the neurogenic response; that is, FGF2 levels were increased only in stress conditions that also stimulated enhanced neurogenesis. Neutralizing the astrocyte-secreted FGF2 prevented enhanced proliferation in cultured NPCs. These findings suggest a novel role for astrocytes in supporting hippocampal plasticity in response to an environmental stressor through secreted FGF2. Further research will be required to fully dissect the molecular mechanisms by which stress induces FGF2 secretion from astrocytes.

Newly-born neurons are implicated in numerous hippocampal memory functions[96], particularly contextual fear conditioning. Contextual fear conditioning activates immature neurons and when new neurons are selectively knocked down, fear extinction is impaired[142,163,164]. Sahay et al. have further shown that prolonging survival of newly-born neurons through targeted knockdown of apoptotic pathways enhances discrimination between similar contexts in a fear conditioning task [99]. We found that 2 weeks, but not 2 days, after acute stress, immobilized rats showed enhanced

contextual fear extinction retention compared to controls. We also found that immobilized rats had greater activation of newly born neurons in response to fear extinction recall. This time window coincides with the period in which the newborn cells are hyper-plastic and more likely to be recruited to active circuitry[142,162,163]. Given that immobilized rats had a similar number of newborn neurons as controls, these data suggest that immobilized rats better utilize the pool of immature neurons, possibly contributing to their enhanced memory. However, this connection does not preclude the potential contribution of other aspects of hippocampal plasticity to the enhanced memory. Future research will be necessary to fully determine the role of immature neurons versus existing circuitry in enhancement of hippocampal memory.

A recent hypothesis posits that the dorsal and ventral hippocampus support different behavioral functions, with the dorsal region being important for spatial and declarative memory while the ventral region facilitates affective regulation[151]. This division may also apply to neural stem cell regulation within the DG of each region where chronic stress preferentially effects the ventral over the dorsal hippocampus, perhaps reflecting the negative affective consequences of chronic stress[152]. In the present study, we found that acute stress or stress hormone exposure increased neurogenesis and FGF2 expression in the dorsal, but not in the ventral, hippocampus. Moreover, the memory benefits observed 2 weeks after stress were in contextual fear extinction, a task that relies on the kind of spatial memory hypothesized to depend on the dorsal DG. This selective involvement of the dorsal hippocampus implies that our acute stressor model functions as a cognitive stimulant for the declarative domains of hippocampal function rather than as a modulator of emotional responsivity.

The brain's response to acute stress can define the line between life-saving adaptation and long-term pathology. The current study suggests that moderate, acute stress may stimulate heightened brain plasticity via increased neurogenesis. These findings have important implications for understanding adaptive versus pathological responses to stress.

D. Materials and Methods

Animals: Adult male Sprague-Dawley rats (Charles River) were pair-housed on a 12h light dark cycle with lights on at 700h. Rats were allowed to acclimate to the animal facility for one week before handling began. All animal procedures were approved by the UC Berkeley Animal Care and Use Committees.

Stressors: Novel environment, footshock, immobilization and control rats were all handled for 5 days. On the 6th day, they were exposed to a stressor or left undisturbed in the case of controls. The novel environment and footshock exposure both lasted 30 min and occurred in fear conditioning chambers described in the fear conditioning section. For footshock, rats were exposed to 1 mA, 1 s duration unsignaled shock 30 times. Rats were returned to their home cage after the 30 min exposure until the time of sacrifice. Immobilized rats were confined for 3 hours in decapicone bags (Braintree Scientific).

Corticosterone injection: For most experiments, rats were handled for two days, given a subcutaneous (SC) needle stick on day 3 and then injected with sesame oil (SC) for two days (days 4 and 5). On the 6th day, rats received 0, 5 or 40 mg/kg corticosterone (SC, Sigma) suspended in sesame oil. CORT was either at 5 mg/ml or 40 mg/ml such that rats receiving 5 or 40 mg/kg corticosterone received equal volumes of oil relative to body weight. In the case of the rats not pre-injected, rats were handled for three days and then injected with 0, 5 or 40 mg/kg corticosterone as above on the 4th day.

BrdU injection: 5-Bromo-2'-deoxyuridine (BrdU, Sigma) was dissolved in physiological saline. Rats were injected with BrdU (intraperitoneally, 200 mg/kg) 3 hours after the beginning of the stressor or injection unless otherwise noted.

BLA lesion: Excitotoxic lesions of the BLA were performed using unilateral stereotaxic infusion of N-methyl-d-aspartate (Sigma) as per[142,156]. Coordinates for BLA infusion were: -2.8 mm anterior/posterior (A/P), +/-5.1 mm medial/lateral (M/L) relative to bregma; -6.8 mm (2 min) and -6.5 mm (1 min) relative to dura. Following three weeks of recovery, during which rats were handled regularly, rats were immobilized for 3 hours (n = 7 each, sham and unilateral lesion) or left undisturbed in their home cage (n = 7 each, sham and unilateral lesion, respectively). Tail vein blood samples were taken at the beginning and end of immobilization for plasma corticosterone quantification. At the end of immobilization, all rats received one injection of 100 mg/kg BrdU and were perfused two hours later.

Immunohistochemical staining. Rats were anesthetized with Euthasol euthanasia solution and transcardially perfused with ice cold 0.1M phosphate buffered saline (PBS) followed by 4% paraformaldehyde in 0.1M PBS. Brains were post-fixed for 24 h at 4ºC, equilibrated in 30% sucrose in 0.1M PBS and then stored at -20ºC. Immunostaining was performed on a 1 in 6 series of free-floating 30 µm cryostat sections.

Ki67 staining was done for sections from control (n = 10), novel environment (n = 6), footshock (n = 4), immobilized (n = 6), 0 mg/kg (n = 6), 5 mg/kg (n = 6) and 40 mg/kg (n = 6) CORT-injected rats as per[7] with a few additions. Sections were antigen-retrieved using 10mM citrate buffer, pH 8.0 at 95ºC for 20 min prior to peroxidase blocking and the primary antibody used was rabbit anti-Ki67 (1:500, Novus).

BrdU staining was done as per[7] for sections from control (n = 6), immobilized (n = 5), 0 mg/kg (n = 6), and 40 mg/kg (n= 5) rats who were perfused 24 hours after the BrdU injection, which occurred 3 hours after the start of the stressor. Sections from control (n = 10) and immobilized (n = 8) rats who were perfused two weeks after immobilization/BrdU injection, 1 hour after the final fear extinction probe (see fear conditioning section), were also stained for BrdU using the same procedure. All stained BrdU and Ki67 sections were mounted on gelatin-coated slides, dehydrated in alcohols and coversliped with permount.

Double immunohistochemical labeling for FGF2 quantification in GFAP+ cells was done on sections from control (n = 2), immobilized (n = 6), 0 mg/kg CORT injected (n = 6) and 40 mg/kg CORT injected (n = 6) rats as per[142] with the following deviations. Primary antibodies were rabbit anti-FGF2 1(:100, Abcam), and mouse anti-GFAP (1:1000, Cell Signalling). Secondary antibodies were AlexaFluor 488 donkey anti-rabbit (1:200, Invitrogen) and Cy3 donkey anti-mouse (1:200, Jackson ImmunoResearch). Following secondary incubation and rinsing, sections were mounted on gelatin-coated slides and coverslipped with Vectashield mounting medium with DAPI (Vector).

Triple immunohistochemical labeling for cell fate analysis was done on sections from control (n = 6) and immobilized (n = 6) rats two weeks after immobilization/BrdU injection, after the final fear extinction probe (see fear conditioning section) as per[142] with a few exceptions. Primary antibodies were goat anti-DCX (1:200, Santa Cruz Biotechnology), mouse anti-GFAP (1:100, Cell Signalling) and rat anti-BrdU (1:500, Abcam). Secondary antibodies were AlexaFluor 594 anti-goat, AlexaFluor 647 anti-mouse and biotin anti-rat (1:500, Jackson ImmunoResearch). Tertiary antibody was Streptavidin Alexa Fluor 488 (1:1000, Jackson ImmunoResearch). Double immunohistochemical staining for DCX and cfos was performed similarly, (n = 5 control, n = 7 immobilized) with goat anti-DCX as above and mouse anti-cfos (1:50, Santa Cruz

Biotechnology). Secondary antibodies were as above for anti-goat and AlexaFluor 647 anti-mouse (1:500, Jackson ImmunoResearch). Sections were mounted on gelatin-coated slides and coversliped with DABCO antifading medium.

Quantification of Ki67+ and BrdU+ cells. Ki67- and BrdU-positive cells were counted in the dorsal and ventral dentate gyrus and subgranular zone using a 40x air objective (Zeiss). The area sampled was calculated using StereoInvestigator software (MicroBrightfield) and used to calculate the number of positive cells per μm^2.

Confocal analysis. For quantification of FGF2 immunoreactivity, 18 μm Z-stacks of 1 μm slices in the dorsal DG and hilus were acquired using a 20x air objective. Mean DG optical density was measured in ImageJ software using the summed Z-stack of FGF2 immunoreactivity. Mean optical density of FGF2- areas of tissue were subtracted from the DG intensity value to correct for background. Integrated optical density of GFAP+ and GFAP- negative cells in the hilus were determined by confirming GFAP expression in the Z-stack and then acquiring integrated optical density from the summed Z-stack of each individual cell. Fifty one to 97 GFAP+ cells and 16 to 57 GFAP- cells were sampled per rat. To quantify cFos expression in new neurons, BrdU positive cells were located in the dorsal dentate gyrus for each animal using a 40x oil objective and assessed in z-series of <1.0 μm slices to determine if other markers (DCX, GFAP) were co-expressed. Confocal images were captured on a Zeiss 510 META/NLO confocal microscope with a 40x oil objective.

Real time quantitative PCR. Rats were lightly anesthetized with isoflurane and rapidly decapitated 30min or 3h after the beginning of their respective stressors (n = 6/grp). Bilateral hippocampi were dissected and flash frozen in liquid nitrogen. Trunk blood was collected for plasma corticosterone quantification. One hippocampus per rat was used for mRNA expression quantification. The other was used for western blot analysis, right and left side being counterbalanced among groups. Real-time reverse transcriptase PCR was run on Trizol-extracted RNA with primers detailed in the table below.

gene	direction	sequence
bdnfexonIV	+	GGAGTGGAAAGGGTGAAACA
	-	GGATTCAGTGGGACTCCAGA
bdnfexonIX	+	GAGAAGAGTGATGACCATCCT
	-	TCACGTGCTCAAAAGTGTCAG
cfos	+	GGCAAAGTAGAGCAGCTATCTCCT
	-	TCAGCTCCCTCCTCCGATTC
fgf2	+	CGGTACCTGGCTATGAAGGA
	-	CTCCAGGCGTTCAAAGAAGA
fgfr1	+	ACCTGAGGCATTGTTTGACC
	-	GTGAGCCACCCAGAGTGAAT
fgfr2	+	GGCCTCTCTGAATGCTAACG
	-	ACGAGACAATCCTCCTGTGG
fgfr3	+	TCTGGTCCTTTGGTGTCCTC
	-	TGAGGATGCGGTCTAAATCC

fgfr4	+	GTGGCTGTGAAGATGCTGAA
	-	GAGGAATTCCCGAAGGTTTC
gadd45β	+	GTCACCTCCGTCTTCTTGGA
	-	GAGGCGGTGGGACTTACTTT
ngf	+	GGACGCAGCTTTCTATCCTGG
	-	CCCTCTGGGACATTGCTATCTG
rplp	+	CCAAAGGTTTGGGAGAACAA
	-	GGGTCATGGCATAGAGCAAT
vegf	+	GAGGAAAGGGAAAGGGTCAAA
	-	CACAGTGAACGCTCCAGGATT

Primer sequences were designed using Primer1 software and checked for specificity using BLAST. Extracted RNA was treated with DNase (DNA-free, Ambion), and two-step PCR was used, following manufacturer instructions for iScript cDNA synthesis kit (BioRad) and then iQ SYBR Green Supermix. Samples were run in a BioRad IQ5 real-time PCR machine. After the PCR was complete, specificity of each primer pair was confirmed using melt curve analysis. Ct values were determined using PCR miner[183] and normalized to the reference ribosomal RNA, RPLP. Fold change in mRNA expression is relative to no stress control rats.

Western blot. Protein was extracted by homogenizing in RIPA buffer with protease inhibitor (Calbiochem, 1:100) and phosphatase inhibitor (Roche, 1:10). Following 30 min incubation on ice, samples were centrifuged at 12,000g for 30 min at 4ºC. The extracted protein was stored at -80 ºC. Total protein content was assessed using a BCA kit (Pierce). Samples were diluted 1:1 in laemmli buffer (Biorad) + 5% β-mercaptoethanol (Fisher) and run on 4-20% Mini-PROTEAN TGX gels (Biorad) at 100V for 1.5 hours in 1x Tris-glycine-SDS buffer. They were then transferred to nitrocellulose membrane (Biorad) at 100V for 1 hour in 1x Tris-glycine-SDS buffer with 20% methanol. Membranes were blocked for 1 hour with 5% milk in 0.1M Tris buffered saline with 1% Tween-20 (Fisher)(TBS-t). Membranes were incubated in primary (rabbit anti-FGF2, 1:100, abcam; mouse anti-actin, 1:10,000, Roche, rabbit anti-bdnf, 1:500, abcam) in blocking solution overnight at 4ºC. The next day, membranes were rinsed 3 times with TBS-t then incubated in secondary (LiCor IRDye 680LT Donkey anti-mouse or LiCor IRDye 800CW Donkey anti-rabbit, 1:20,000) for 1 hour. After three final rinses, membranes were visualized using a LiCor Odyssey scanner. The correct band size was found relative to a LiCor IRDye (680/800) protein marker ladder. All bands were quantified using LiCor Odyssey software, corrected for background and expressed relative to their corresponding actin band. Fold change in protein expression was then calculated relative to no stress control.

Plasma corticosterone sampling. All blood samples were centrifuged at 2000g for 15 minutes and plasma was extracted and stored at -20°C until assayed. Corticosterone was measured using a Corticosterone EIA kit (Cayman Chemical).

Culturing of hippocampal NPCs. Isolation of neural stem/progenitor cells from adult rodents are described in detail in[28]. Progenitors used in these experiments were purchased from Millipore (SCR022). Cells were cultured under standard conditions (37°C, 5% CO2) on poly-ornithine (Sigma) and laminin (Invitrogen) coated plates in N2-supplemented (Invitrogen) Dulbecco's modified Eagle medium (DMEM)/F-12 (1:1) (Invitrogen) with 20 ng/mL recombinant human FGF-2 (PeproTech).

Culturing of primary hippocampal astrocytes. Primary astrocyte cultures were prepared from P1-2 day old Sprague Dawley rat pup hippocampi using the method described by McCarthy and Vellis[158]. Briefly, hippocampi were dissected in ice-cold media, chopped and digested using papain from papaya latex extract (Sigma) in HBSS (Invitrogen) for 20 minutes at 37°C. Papain was inactivated using 10% horse serum, cells were centrifuged for 1 min at 1000 rpm and resuspended in HBSS and triturated by passing through serological and flame-polished pipettes of progressively smaller bores. Cells were then plated in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Axenia BioLogix) and 1% Penicillin/Streptomycin (Invitrogen) at a density of $3x10^6$ in T75 flasks. After reaching confluency, flasks were shaken on an orbital shaker at 225 rpm for 2 hours at 37°C. Cells were then washed 5x with warm PBS to remove suspended microglia. Astrocytes were then trypsinized and re-plated in 100mm dishes. 24 hours after plating, astrocytes were treated with 1 μM CORT or equivalent volume of EtOH vehicle for three hours. ACM was then collected, filtered with a 0.2 μm sterile filter and stored at -20°C.
Cell treatment for BrdU-labeling. In all studies, NPCs were FGF2 deprived for 24 hours then treated for 3 hours with the appropriate media. They were then pulsed with 30 μM BrdU and fixed 2 hours later with 4% paraformaldehyde for 10 min. Cell treatments were: 0 ng/ml FGF2 (+EtOH, n = 6; +CORT, n = 5), 20 ng/ml FGF2 (+EtOH, n = 5; +CORT, n = 5), CoC media (+EtOH, n = 6; +CORT, n = 6), ACM (+EtOH, n = 6; +CORT, n = 6). Treatments for the rat recombinant FGF2 experiment were 0 pg/ml FGF2 (n = 8) and 4 pg/ml FGF2 (n = 7).
Immunocytochemical BrdU staining and quantification. Fixed cells were rinsed with 0.1 M PBS, denatured in 1 N HCl at 37°C, rinsed and blocked in 5% normal donkey serum, 0.3% triton-100 in PBS. Cells were then incubated overnight at 4°C in mouse anti-brdu (1:500, BD Biosciences) in 2% normal donkey serum in PBS. Cells were then rinsed and incubated in Cy3 anti-mouse (1:500, Jackson Immunoresearch) in 2% normal donkey serum in PBS, rinsed, counterstained with DAPI (1:20,000 in PBS) and coverslipped with DABCO anti-fading medium. BrdU+ and DAPI+ cells were counted in randomly sampled sites within each well using StereoInvestigator software (Microbrightfield) and a 20x air objective (Zeiss).
FGF2 neutralization. CoC media or ACM from primary astrocytes treated with EtOH or CORT was incubated with neutralizing FGF2 antibody (Millipore) for 1 hour at 37°C prior to use on NPCs. For nAb dose testing, NPCs were treated with 0 ng/ml FGF2 (0 μg/ml nAb, n = 8; 10 0 μg/ml nAb, n = 5, 20 μg/ml nAb, n = 3) or 20 ng/ml FGF2 (0 μg/ml nAb, n = 8; 10 0 μg/ml nAb, n = 7, 20 μg/ml nAb, n = 8). For ACM treatment with the FGF2 nAb, all n = 6.
FGF2 ELISA for ACM. For nAb specificity tests, the recommended standard curves of FGF1 and FGF2 from their respective rat-specific ELISAs (Antibodies Online) were preincubated in 0, 10 or 20 μg/ml nAb at 37°C for 1 hour before being quantified in the ELISA according to manufacturer's instructions. ACM from CORT (n = 3) and EtOH (n = 3) treated astrocytes was analyzed using the same rat FGF2 ELISA (Antibodies Online).
Fear conditioning. Rats were immobilized for 3 hours or left undisturbed, then injected with 200 mg/kg BrdU. Either 2 days or 2 weeks after immobilization, rats were trained in contextual fear conditioning as per[7] (2 days: n = 10 con, n = 10 immob; 2 weeks: n = 20 con, n = 18 immob). The next day, rats were exposed to the fear conditioning context 5 times for 10 min each time with no shock (extinction). 24 hours later, they were re-exposed to the conditioning chamber for 10 min with no shock (extinction probe). A subset of rats (2 days: n = 10 con, n = 10 immob; 2 weeks: n = 10 con, n = 8 immob) was tested on an elevated plus maze 1 day prior to contextual fear conditioning (data not shown).

Statistics. In most studies, data were analyzed using a one-way ANOVA followed by Dunnett's posthoc tests with the appropriate control group as reference. For *in vivo* CORT injection studies with 30 min and 3 h time points, data were analyzed using two-way ANOVA followed by Dunnett's posthoc test (0 mg/kg-30min used as reference). For cell culture studies, data were analyzed using two-way ANOVA followed by Dunnett's posthoc tests. For BLA lesion studies, a repeated measures two-way ANOVA was used with hemisphere being the paired variable. When only two groups were being compared, unpaired t-tests were used in all cases. $P \leq 0.05$ was considered significant.

E. Figures

Figure 1: Dorsal versus ventral DG.

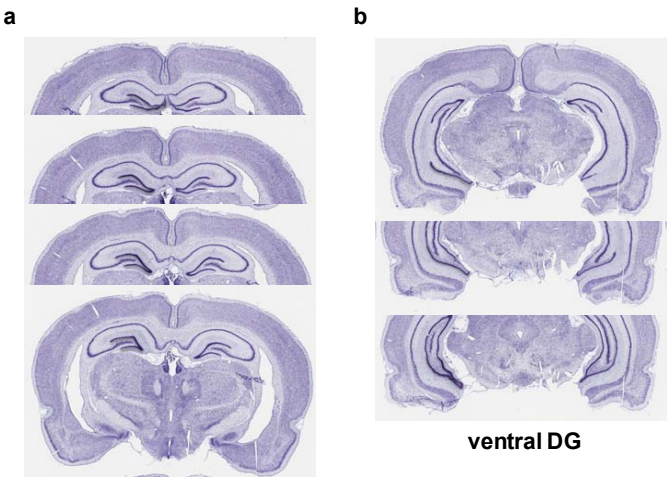


Figure 2.1 (above): Dorsal versus ventral DG. Areas of dorsal (a) and ventral (b) dentate gyrus used for cell proliferation quantification are highlighted in red. Images are adapted from brainatlas.org.

Figure 2.2 (below): Acute stress increases adult cell proliferation in dorsal hippocampus. a) Experimental timeline. b) Acute immobilization increased Ki67+ cell count in the adult dorsal DG while exposure to novel environment or footshock did not significantly change Ki67+ cell count. One-way ANOVA, $p < 0.0001$; ***$q = 5.975$, $p < 0.0001$. c) Representative images of Ki67+ cells (black arrows) in the dorsal DG (dashed outline) of control, immobilized, 0 mg/kg and 40 mg/kg CORT-injected rats. d) Acute immobilization increased plasma CORT levels 30 min and 3 h after the stressor began. CORT elevations caused by novel environment and footshock were not significant. One-way ANOVA, $p < 0.0001$; ***$q = 5.56$, $p < 0.0001$; **$q = 4.02$, $p < 0.001$. e) Acute injection of 40 mg/kg CORT increased Ki67+ cell count in the adult dorsal DG compared to 0 mg/kg oil control while 5 mg/kg CORT did not significantly alter Ki67+ cell count. One-way ANOVA, $p = 0.007$. *$q = 3.15$, $p < 0.05$. f) 40 mg/kg CORT injection led to a sustained increase in plasma CORT 30 min and 3 h after injection. The change in plasma CORT following 5 mg/kg CORT injection was not significantly different from oil injection. Two-way ANOVA, effect of CORT dose $p < 0.0001$. **$q = 3.62$ and 3.61, $p < 0.001$, 40 mg/kg 30 min and 3 h, respectively. g) The number of BrdU-labeled newborn cells surviving 24 hours after the end of immobilization was greater in immobilized rats than controls. *$p = 0.03$ h) The number of BrdU-labeled newborn cells surviving 24 hours after CORT/oil injection was

greater in rats given 40 mg/kg CORT compared to 0 mg/kg CORT. *p = 0.04. i) Representative images of BrdU+ cells (black arrows) in the dorsal DG (dashed outline) of control, immobilized, 0 mg/kg and 40 mg/kg CORT-injected rats. j) Experimental timeline. Rats were handled for three days, injected with CORT or oil vehicle then perfused 3h later. k) No difference in Ki67+ cell number was found in the adult dorsal DG with increasing CORT dose. All values are average ± SEM.All values are average ± SEM. Scale bar is 100 μm.

Figure 2: Acute stress increases adult cell proliferation in dorsal hippocampus.

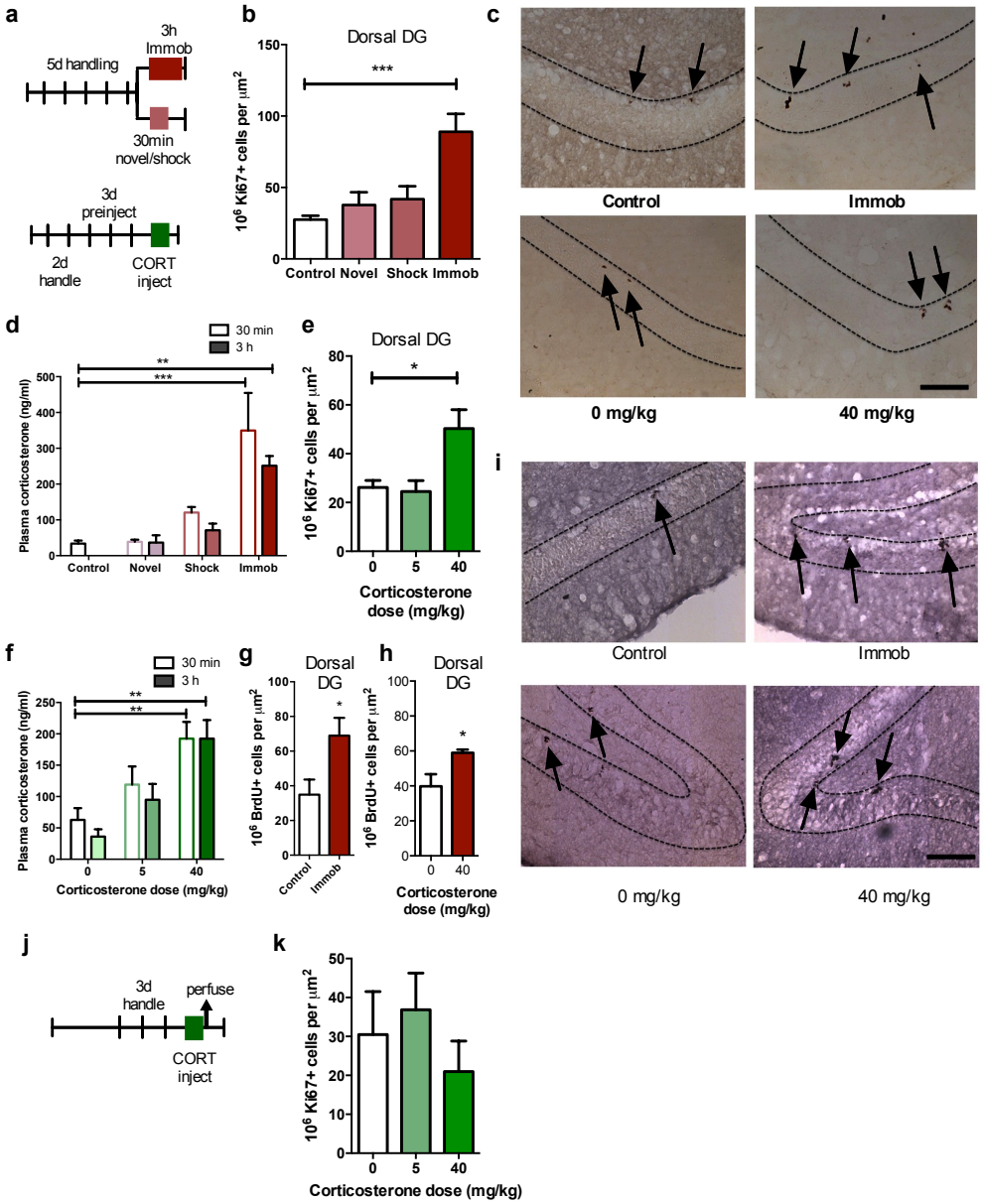


Figure 3: Acute stress does not increase adult cell proliferation in ventral hippocampus.

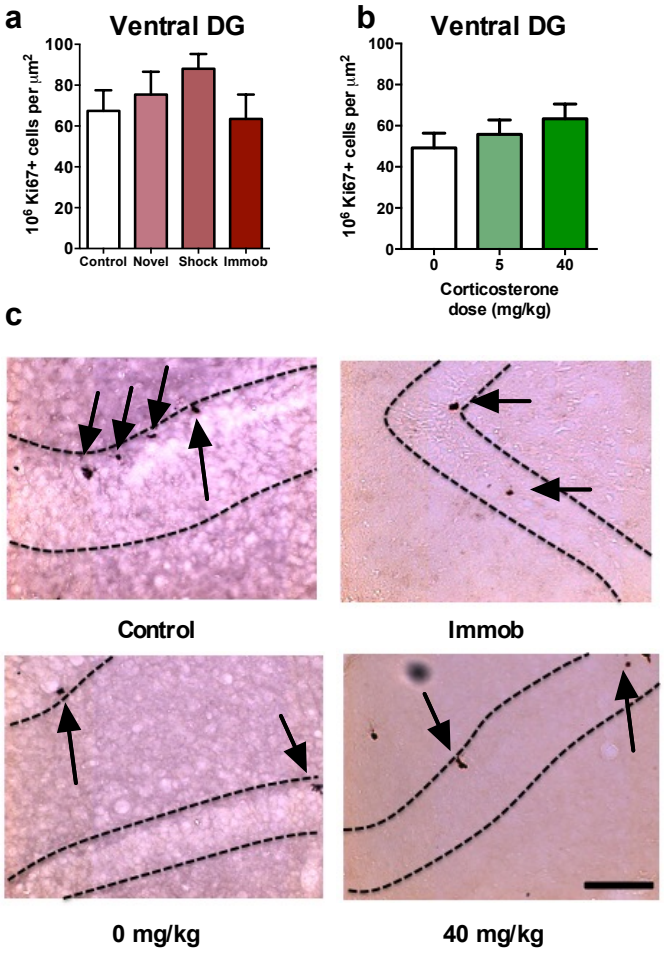


Figure 2.3: Acute stress does not increase adult cell proliferation in ventral hippocampus. a) None of the stressors affected Ki67+ cell count in the ventral DG. b) CORT did not affect Ki67+ cell count in the ventral DG. c) Representative images of Ki67+ cells (black arrows) in the ventral DG (dashed outline) of control, immobilized, 0 mg/kg and 40 mg/KG CORT-injected rats. All values are average ± SEM. Scale bar is 100 μm.

Figure 4: Acute stress increases cell proliferation independent of BLA input.

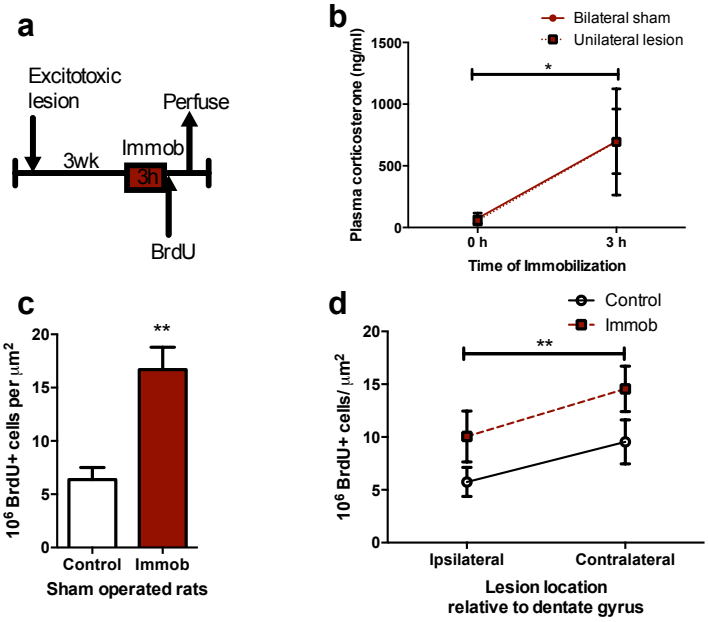


Figure 2.4: Acute stress increases cell proliferation independent of BLA input. a) Experimental timeline. b) Plasma CORT elevation after immobilization was similar between sham-operated and unilaterally BLA-lesioned rats. Two-way ANOVA effect of time, *p = 0.04. c) In sham-operated rats, acute immobilization increased the number of BrdU+ cells in the adult DG. **p = 0.001. d) Unilateral excitotoxic lesion of the BLA decreased the number of BrdU+ cells in the ipsilateral DG, but did not interact with stress. Two-way ANOVA, effect of lesion, **p = 0.002. All values are average ± SEM.

Figure 5: ACM from CORT-treated astrocytes increases NPC proliferation.

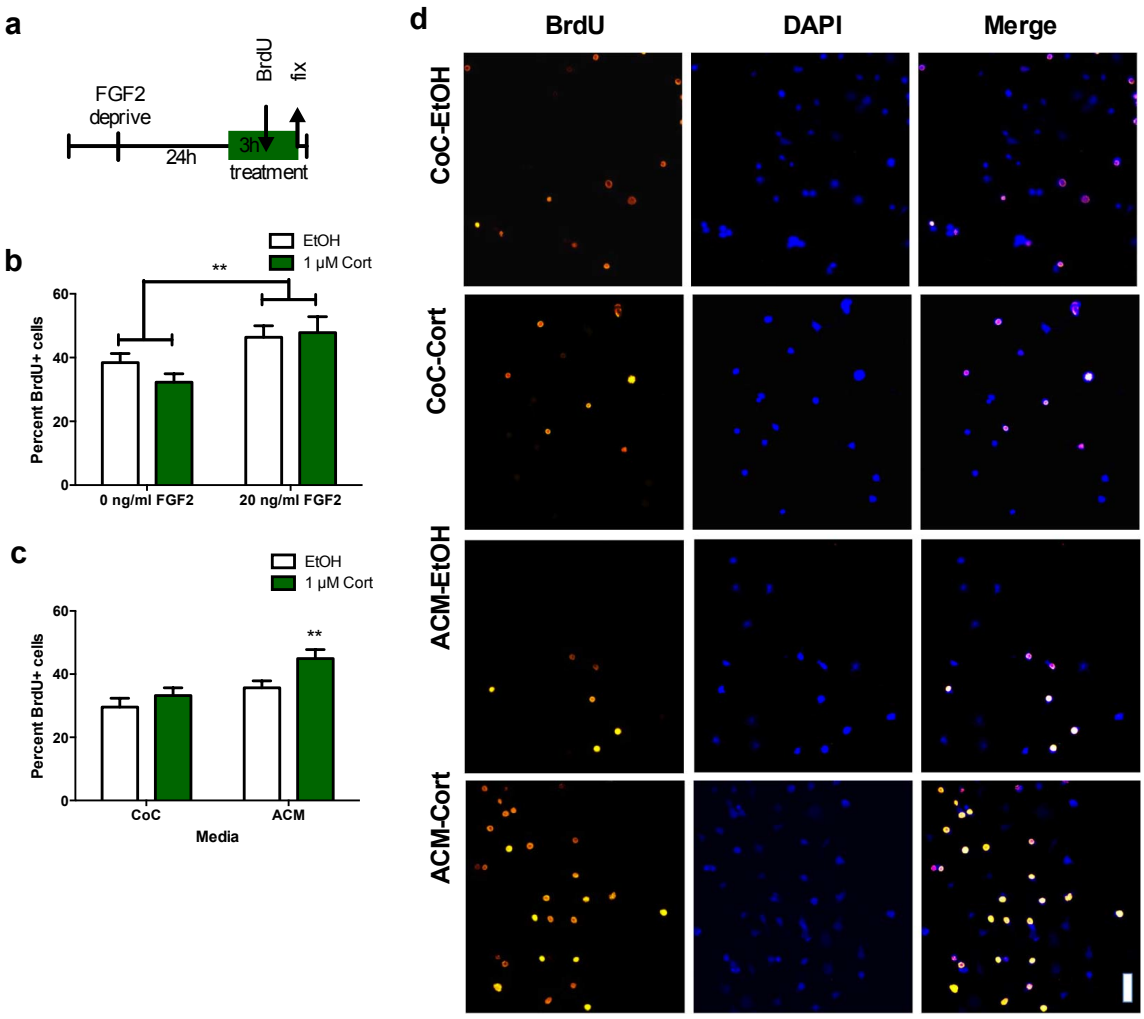


Figure 2.5: ACM from CORT-treated astrocytes increases NPC proliferation. a) Experimental timeline. b) Treatment of isolated hippocampal NPCs with 1 μM CORT for 3 hours did not alter the percent of proliferating BrdU+ cells compared to EtOH vehicle. Three hours of treatment with 20 ng/ml human recombinant FGF2 increased the percent of proliferative BrdU+ cells. Two-way ANOVA, effect of FGF2, **p = 0.005. c) ACM was extracted from astrocytes treated with 1 μM CORT or EtOH vehicle. Treatment of NPCs with ACM from CORT-treated astrocytes increased the percent of proliferative BrdU+ cells compared to EtOH, CoC-treated control NPCs. Two-way ANOVA, effect of CORT, p = 0.02; effect of media, p = 0.0024. **q = 4.23, p <0.001 vs. EtOH-CoC. d) Representative images of NPCs treated with CoC or ACM, EtOH or CORT. BrdU+ cells are orange and DAPI is blue. All values are average ± SEM. Scale bar is 10 μm.

Figure 6: Acute stress increases FGF2 expression in dorsal hippocampus.

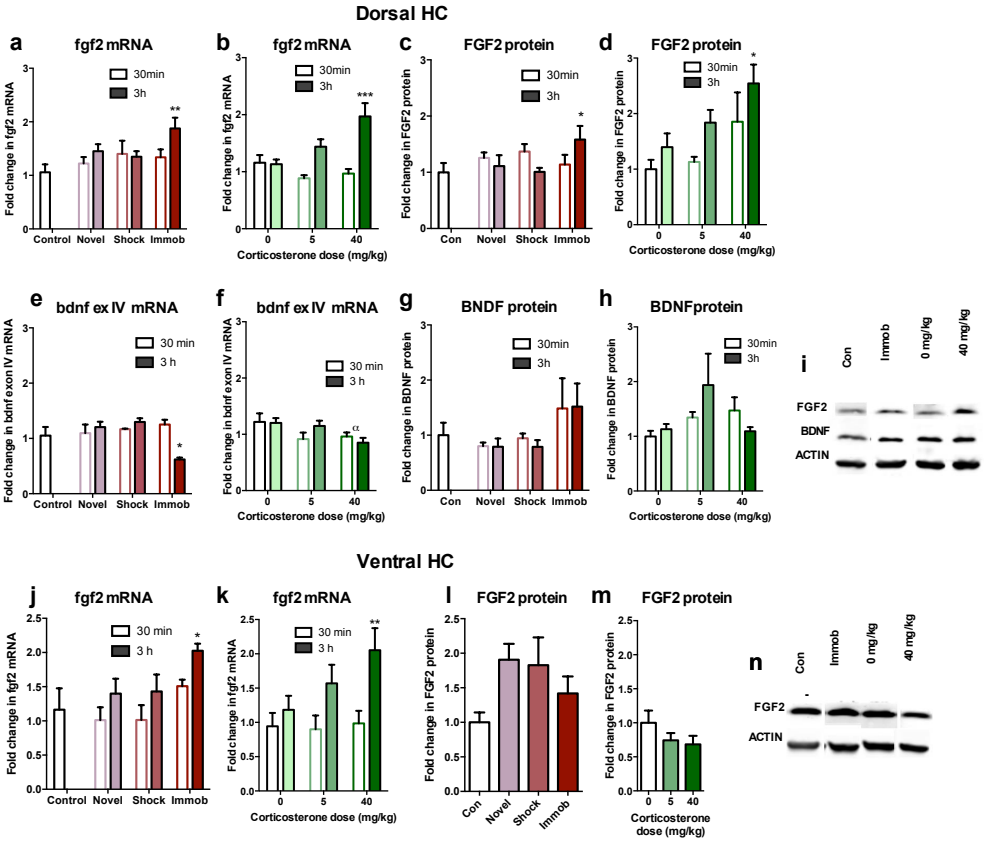


Figure 2.6: Acute stress increases FGF2 expression in dorsal hippocampus. a) Three hours of immobilization increased fgf2 mRNA expression over control in dorsal hippocampus. Other groups did not significantly differ from control. One-way ANOVA, $p = 0.05$. $^{**}q = 3.54$, $p < 0.01$. b) 40 mg/kg CORT increased fgf2 mRNA expression in the dorsal hippocampus 3 hours after CORT injection compared to 30 min after 0 mg/kg CORT injection. Other groups did not significantly differ from oil-injected controls. Two-way ANOVA, effect of CORT, $p = 0.03$; effect of time, $p < 0.0001$; interaction, $p = 0.0021$. $^{***}q = 4.34$, $p < 0.001$. c) FGF2 protein levels in dorsal hippocampus increased with 3 hours of immobilization over control. Other groups did not significantly differ from control. One-way ANOVA, $p > 0.05$. $^{*}q = 2.79$, $p < 0.05$. d) FGF2 protein levels in dorsal hippocampus increased 3 hours after 40 mg/kg CORT injection compared to 30 min after 0 mg/kg vehicle injection. Two-way ANOVA, effect of CORT, $p = 0.01$; effect of time, $p = 0.03$. $^{*}q = 3.18$, $p < 0.05$. e) Three hours of immobilization decreased bdnf exon IV expression over control in dorsal hippocampus. Other groups did not significantly differ from controls. One-way ANOVA, $p = 0.0007$. $^{*}q = 3.05$, $p < 0.05$. f) There was an overall significant decrease in bdnf exon IV mRNA expression with increasing CORT dose in dorsal hippocampus. Two-way ANOVA, effect of CORT, $^{*}p = 0.02$. g) BDNF protein levels in dorsal hippocampus did not change with immobilization, novel environment or shock compared to control. h) BDNF protein levels did not change compared to 0 mg/kg vehicle with increasing CORT dose. i) Representative western bands of FGF2, BDNF and ACTIN from the 3h time point in dorsal hippocampus. j) Three hours of immobilization increased fgf2 mRNA expression over control in ventral hippocampus. Other groups did not significantly differ from controls. One-way ANOVA,

p = 0.03. *q = 2.87, p < 0.05. k) 40 mg/kg CORT increased fgf2 mRNA expression in the ventral hippocampus 3 hours after CORT injection compared to 30 min after 0 mg/kg CORT injection. Other groups did not significantly differ from oil-injected controls. Two-way ANOVA, effect of time, p = 0.002. **q = 3.34, p < 0.01. l) Immobilization, novel environment and shock do not alter FGF2 protein levels in ventral hippocampus. m) CORT does not alter FGF2 protein levels in ventral hippocampus 3 hours after injection. n) Representative western bands of FGF2, BDNF and ACTIN from the 3 hour time point in ventral hippocampus. All values are average ± SEM.

Figure 7: mRNA expression levels in dorsal hippocampus following acute stressors.

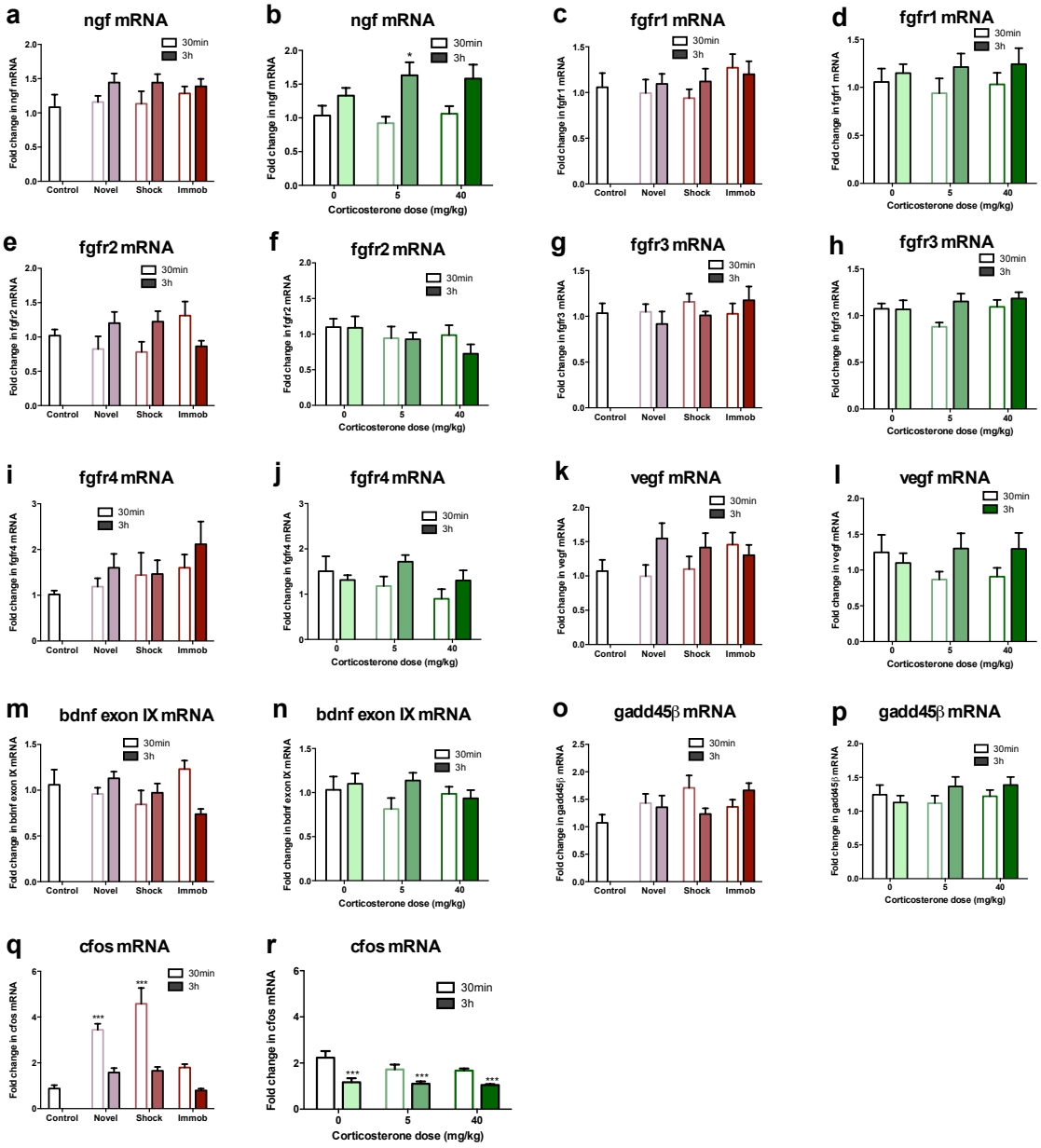


Figure 2.7. Acute stress increases FGF2 expression in GFAP+ cells in the dorsal hilus. a) There was no change in mean optical density of the DG following 3 hrs immobilization or CORT

injection. b) Both immobilization and 40 mg/kg CORT injection significantly increased integrated optical density of GFAP+ cells in the hilus. One-way ANOVA, p = 0.0024. *p = 0.04 and 0.05, con v immob and 0 v 40 mg/kg CORT, respectively. (c) Integrated optical density in GFAP- cells of the hilus did not change. d) Representative images of a FGF2+ cells (green) that are GFAP+ (red) or GFAP- in DG and hilus of a control and an immobilized rat. DAPI is blue. Scale bar = 10 μm.

Figure 8: Acute stress increases FGF2 expression in GFAP+ astrocytes

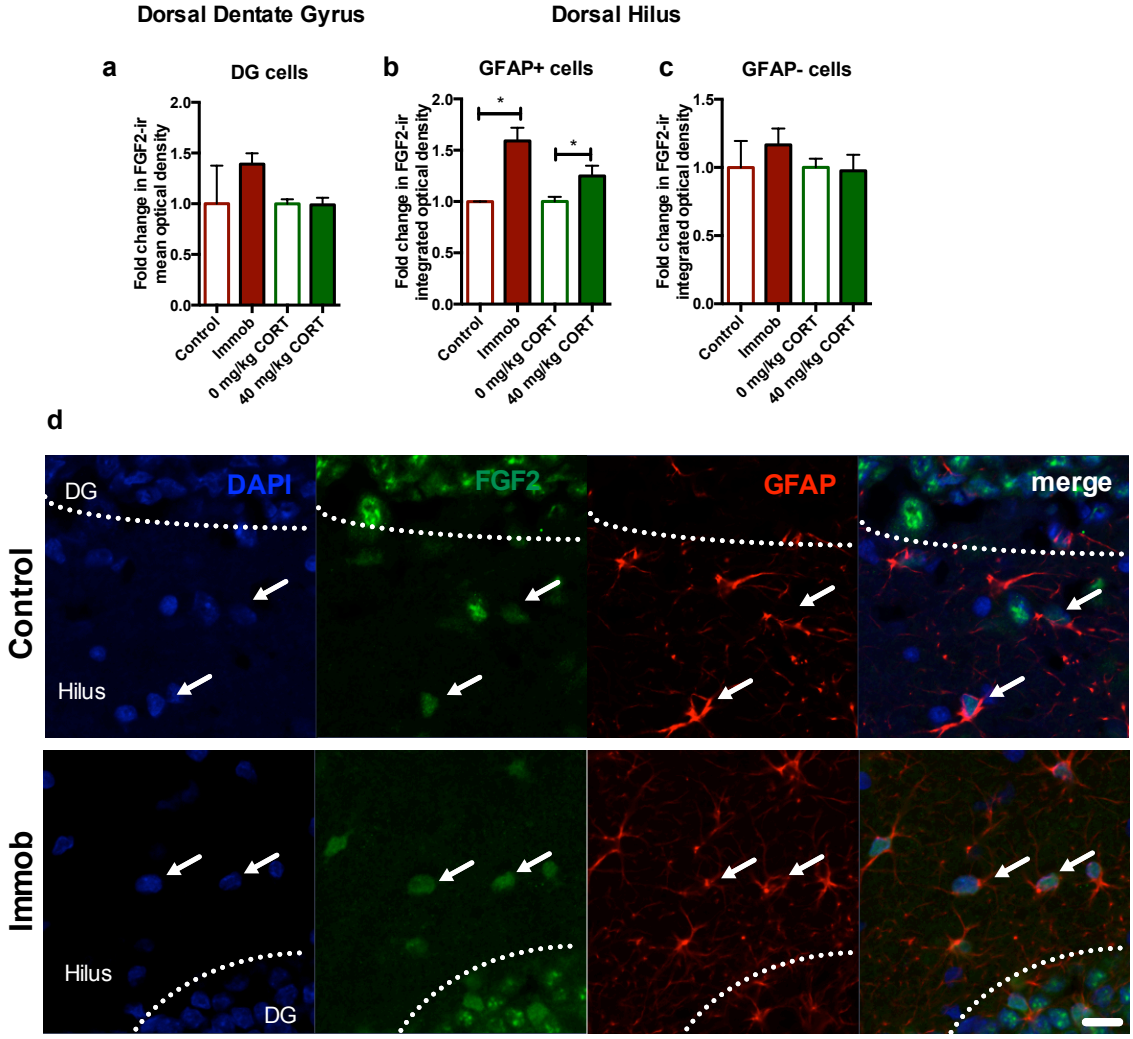


Figure 2.8. mRNA expression levels in dorsal hippocampus following acute stressors. a) There was no change in ngf mRNA with novel environment, shock or immobilization. b) 5 mg/kg CORT significantly increased ngf mRNA at 3 hours over 0 mg/kg CORT, 30min. *q = 2.79, p < 0.05. There was no change in fgfr1 (c,d), fgfr2 (e,f), fgfr3 (g,h), fgfr4 (i,j), vegf (k,l), bdnf exon IX (m,n) or gadd45β (o,p) mRNA expression in dorsal hippocampus. q) Exposure to novel

environment or footshock increased cfos expression in the dorsal hippocampus. One-way ANOVA, $p < 0.0001$. ***$q = 5.92$ and $q = 8.54$, novel and shock, respectively, $p < 0.001$. All values are average ± SEM. r) All injection conditions showed a decrease in cfos mRNA over time. Two-way ANOVA, effect of time, $p < 0.0001$. ***$q = 4.42, 4.68, 4.91$, 0 mg/kg 3 h, 5 mg/kg 3 h, 40 mg/kg 3 h, respectively, $p < 0.001$. All values are average ± SEM.

Figure 9: Blocking FGF2 prevents CORT-ACM induced increase in NPC proliferation.

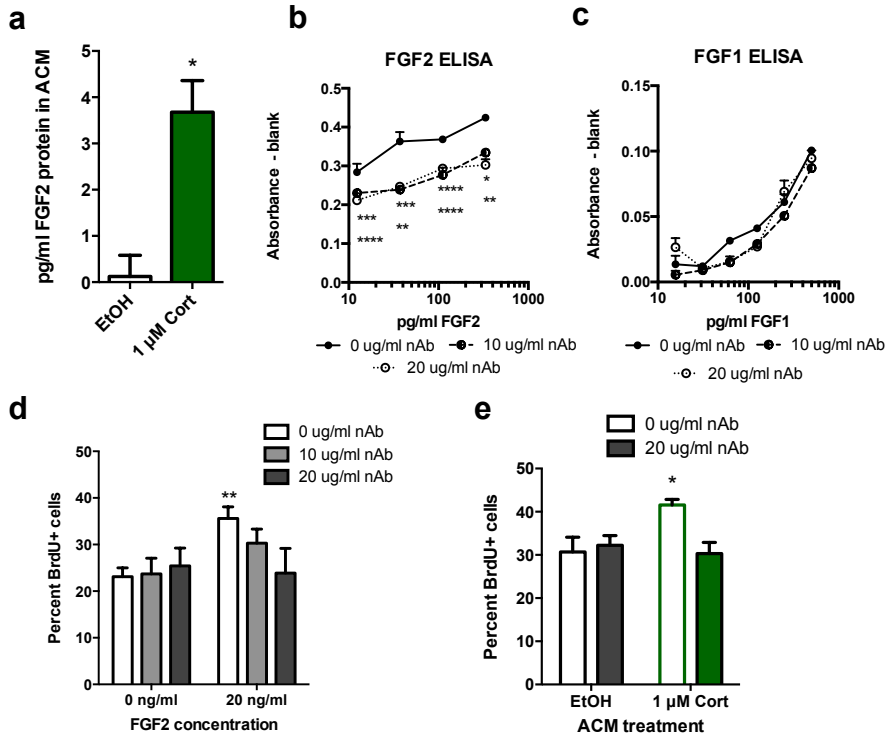


Figure 2.9: Blocking FGF2 prevents CORT-ACM induced increase in NPC proliferation. a) ACM from EtOH-treated primary astrocytes had no FGF2 protein (relative to blank) while CORT-treated ACM had 3.5 pg/ml FGF2. *$p = 0.01$. b) Availability of rat FGF2 was dramatically reduced by pretreating FGF2 protein with an FGF2 neutralizing antibody. Two-way ANOVA, $p < 0.0001$ main effects of nAb and FGF2 concentration. Post-hoc Dunnett's multiple comparison tests with 0 μg/ml as control shown for 10 μg/ml (upper row *s) and 20 μg/ml (lower row *s): *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. c) Availability of FGF2 was not affected by pretreating FGF1 protein with the same FGF2 neutralizing antibody. d) Isolated NPCs were treated with the FGF2 nAb in either high (20 ng/ml) or low (0 ng/ml) FGF2 conditions. 20 μg/ml nAb effectively blocked the FGF2-induced increased in percent BrdU+ proliferating cells. Two-way ANOVA, effect of FGF2, $p = 0.04$. **$q = 3.37$, $p < 0.01$. The nAb did not affect proliferation in low FGF2 conditions. c) Pretreatment with FGF2 nAb prevented the increase in percent BrdU+ proliferating NPCs caused by ACM from CORT-treated astrocytes. Two-way ANOVA, interaction, $p = 0.02$. *$q = 3.06$, $p < 0.05$. All values are average ± SEM.

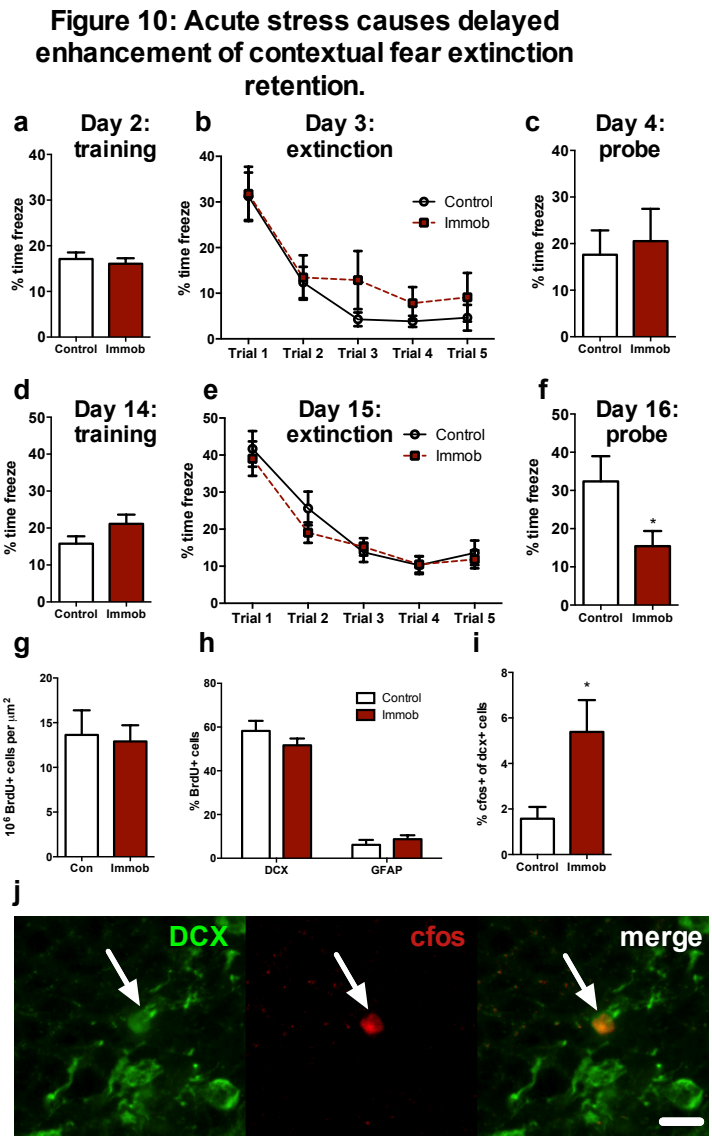


Figure 2.10: Acute stress causes delayed enhancement of contextual fear extinction retention. Acute immobilization 2 days prior to contextual fear conditioning did not change percent time freezing during training (a), extinction (b) or 24-hour extinction probe (c) compared to control. Two-way ANOVA for extinction, effect of trial, $p < 0.0001$. Acute immobilization 2 weeks prior to contextual fear conditioning did not change percent time freezing during training (d) or extinction (e) compared to control. Two-way ANOVA for extinction, effect of trial, $p < 0.0001$. f) Immobilized rats froze significantly less than controls in the 24-hour extinction probe. *$p = 0.04$. g) The number of surviving BrdU+ cells in the dorsal DG of immobilized rats did not differ from controls. h) Immobilization does not alter the percent of BrdU+ cells co-expressing doublecortin (DCX) or glial fibrillary acidic protein (GFAP). i) The percent of DCX+ immature neurons expressing cfos was greater in immobilized rats compared to controls. *$p = 0.02$. All values are average ± SEM.

Chapter 3

Chronic stress suppresses adult hippocampal neurogenesis via astrocytic BMP signaling

A. Introduction

Bone morphogenetic protein (BMP) signaling, a member of the transforming growth factor (TGF) signaling family, has been shown to be an important regulator of NSC maintenance by inducing stem cell quiescence via BMP receptor type 1a (BMPR1a)[51,52]. Both *in vitro* and *in vivo,* NSCs receiving BMP stimulation become quiescent via activation of the canonical BMP pathway, where binding of BMP to BMPR1 leads to the formation of an active heterodimer with BMP receptor type II that phosphorylates SMAD1/5/8 protein, which in turn forms a dimer with the co-SMAD SMAD4 that is able to migrate into the nucleus to serve as a transcription factor and modulator[184]. In the SGZ of the dentate gyrus this program leads to stem cell quiescence[51]. However, in order for stem cells to proliferate suppression of BMP signaling is necessary, and it is thus inhibited via secretion of BMP antagonists noggin[53] and neurogenesin-1 (aka chordin-like1, or Chrdl1)[54]. This system allows for points of regulation of stem cell activity. One instance that this has been observed is during voluntary exercise[97], a paradigm that is known to enhance NSC proliferation and overall neurogenesis[98]. During exercise, Gobeske and colleagues observed a decrease in expression of BMP4 concomitant with an increase in expression of noggin and a decrease in SMAD1/5/8 phosphorylation[97]. Moreover, when direct transgenic manipulation of BMP4 or noggin expression was done, they observed an either abolishment or a recapitulation of the pro-neurogenic program, respectively. Interestingly, BMP regulation of neurogenesis is not only able to induce quiescence of early NSCs, but by inducing quiescence in the early stem cell, the Type-1 radial glia-like cells, and the highly proliferative intermediate progenitors, Type2a and Type2b cells, BMP signaling actually modulates the tempo of adult neurogenesis, slowing down the progression of NSC development and neurogenesis when active, or speeding up the tempo of neurogenesis when inhibited[185]. This finding sheds light to one of the conundrums of neurogenesis, namely that the cognitive benefits often attributed to interventions that increase neurogenesis follow a time line that is a bit too fast for the baseline timeline of NSC development. By being able to regulate the tempo of this development, BMP signaling is a potentially powerful candidate for environmental regulation of neurogenesis, and may play a part on the repression of neurogenesis induced by stress and glucocorticoids.

Chronic stress or chronic glucocorticoid receptor activation is shown to suppress NSC proliferation[131,132], decrease cell survival[133], and inhibit neuronal differentiation[134] in the adult hippocampus. These stress-induced negative consequences on neurogenesis are consistent with behavioral studies indicating that reduced neurogenesis, like during extended exposure to chronic stress, leads to impairment of hippocampal dependent memory[135], anxiety and depressive-like disorders[108]. This suggests that there is a very close relationship between stress-related disorders and neurogenesis.

Several molecular mechanisms have been proposed to explain how GCs regulate neurogenesis, from the direct action of GCs on stem cells, as shown by suppression of cell cycle progression by high concentrations of glucocorticoids or glucocorticoid receptor agonists *in vitro*[137], to mechanisms impacting the excitation-inhibition balance of the neural network[138], as well as the importance of NMDA receptor activity in GC-mediated decrease of stem cell proliferation[139]. However, the role of astrocyte regulation of the chronic stress effect on neurogenesis has not been elucidated. It has been shown that cells in the stem cell niche regulate stem cell maintenance and development[42,55,56]. In particular, astrocytes are closely associated to stem cells in the microenvironment[14,59] and they interact with NSCs to actively regulate stem cell

proliferation and fate specification through direct cell-cell contact[60,61] or secreted trophic factors[62]. In previous studies, it has been shown *in vitro* that secreted factors from astrocyte conditioned media alone can significantly enhance neurogenesis by both increasing rates of NSC proliferation and instructing NSCs to adopt a neuronal fate[59,62,63]. These findings suggest that astrocyte signaling actively regulates stem cell activity and modifies the rate of neurogenesis, suggesting that changes in astrocytic signaling can have a strong impact on regulation of neurogenesis. We have previously reported that acute stress, delivered as a single stressful experience lasting three hours, increases hippocampal NSC proliferation and may have a beneficial role in CNS plasticity and adaptation[44]. This effect was found to be due to GC-induced secretion of astrocytic FGF2, opening the possibility that GC effects on astrocyte regulation of NSCs is also involved in the negative neurogenic effect of chronic stress.
In this study, we provide evidence that astrocyte signaling is necessary for GC-induced reduction of NSC proliferation, and that this effect is mediated through increased signal-to-inhibition ratio of BMP signaling due to a decrease in astrocytic noggin expression and secretion, leading to increased phosphorylated SMAD1 signaling and reduced proliferation of NSCs. These findings have important implications for distinguishing a major mechanism by which stress acts on the mammalian brain. Moreover, they reinforce the active role of astrocytes as a crucial mediator between environmental stress and regulation of adult hippocampal neurogenesis.

B. Results

Astrocyte secreted factors mediate NSC proliferation decline in response to glucocorticoids. In order to investigate whether physiological concentrations of CORT lead to a decrease in NSC proliferation *in vitro*, we treated cultured adult rat NSCs in co-culture media with 1 μM CORT[44,186], (equivalent to approximately 350 ng/ml, the level measured in plasma of immobilized rats). No difference in stem cell proliferation as measured by incorporation of a 24 hour BrdU pulse (Fig 3.1A) was detected between control NSCs and CORT treated NSCs (27.01±1.239% vehicle vs 30.55±2.044% CORT, Figure 3.1B). This suggests that NSC proliferation is not affected by glucocorticoid exposure in these conditions in a cell-autonomous manner. However, when NSCs were co-cultured with astrocytes in a no-contact Banker culture system[63,187], astrocytic 24 hr CORT treatment (Fig 3.1C) led to a significant 40.6% decrease in proliferation in response to CORT treatment (32.28 ± 2.554% vehicle vs 13.11 ± 1.146% CORT, Figure 3.1D), indicating that astrocytes are necessary for regulating the CORT-induced NSC proliferation deficit.
In order to assess whether the anti-proliferative effect of astrocytes is due to a secreted factor that can be found in the media, we treated NSCs with conditioned media from astrocyte cultures treated with CORT (ACM-CORT) or vehicle (ACM), in which GC treatment was washed out so that NSCs aren't exposed to CORT, but only secreted factors from astrocytes (Fig 3.1E). Interestingly, the significant drop in proliferation was also observed when incubating NSCs with ACM-CORT, with a 51.96 % decrease in proportion of $BrdU^+$ cells (29.84 ± 2.225% vehicle ACM vs 15.51 ±1.254% CORT ACM, Figure 3.1F). Taken together, results suggest that, at physiological levels of glucocorticoids, cultured NSCs require astrocytic secreted factors in order for the negative proliferation effect of long-term glucocorticoid exposure to occur.

Downregulation of astrocytic noggin in response to glucocorticoids leads to increased BMP signaling in neural stem cells. Because BMP signaling has been found to be involved in inducing

quiescence of NSCs, and changes to BMPs and BMP inhibitor noggin have been implicated during conditions that regulate neurogenesis, such as exercise[97,185], we wanted to measure what BMP signaling molecules were released from astrocytes, and whether they are affected by stress. Using quantitative PCR, we found changes in BMP-signaling related genes from astrocyte cultures treated with CORT for 24 hours. Astrocyte cultures treated with CORT show downregulation of BMP2 and BMP4 (0.58 ± 0.07 and 0.50 ± 0.03 fold change, respectively), and no change in levels of BMP5, BMP6 and BMP7 (Figure 3.2A). Notably, treatment of astrocytes with CORT led to a 0.51 ± 0.06 fold change in mRNA expression of BMP-inhibitor noggin while mRNA for chordin-like 1 (aka neurogenesin-1[54]) was not changed (Figure 3.2D). Finally, in order to assess whether the observed downregulation of noggin gene expression led to observable decreases in noggin protein secretion, we measured the concentration of noggin in ACM from control astrocytes vs. astrocytes treated with CORT. We found a decrease in noggin protein concentration from 85.58 ± 11.97 pg/mL in control ACM to 50.19 ± 5.824 pg/mL in ACM-CORT (Figure 3.2E).

Since BMP signaling is shown to lead to NSC quiescence, we hypothesized that the decrease in NSC proliferation in response to ACM-CORT could be due to a decrease in total BMP inhibition compared to BMP signal. Because there are multiple BMPs that are secreted by astrocytes, we wanted to understand which BMP is the most highly expressed by these cells, and hence could be the highest contributor to the BMP signal to NSCs. We found that BMP6 is the most highly expressed BMP in cultured astrocytes (Figure 3.2B) and in adult rat hippocampi (Figure 3.2C). We calculated the level of expression using the DeltaCT value of real time PCR quantification, where an estimation of the levels of starting template were calculated using single-curve analysis via the PCR-Miner method[183]. We found that under baseline conditions, BMP6 is the most highly expressed sub-type of BMP by astrocyte cultures, being expressed about 30 times more highly than BMPs 2 and 4 and accounting for 96% of the measured mRNA transcripts, and with mRNA expression of BMPs 5 and 7 only slightly above detection (Figure 3.2B). Interestingly, relative BMP mRNA expression in the hippocampus shows 51% of the BMP expression being contributed by BMP6, and 41% of expression is accounted by BMP4 (Figure 3.2C). This suggests that the main source of BMP6 in the hippocampus is astrocytic, whereas a different cell type secretes BMP4. Furthermore, changes in noggin expression in response to CORT might have a stronger impact on BMP signaling than Chrdl1, as it has been reported that noggin possesses a 10-fold higher affinity than chordin[188], which is highly homologous to Chrdl1. Noggin is also the most powerful inhibitor of BMP signaling in digit morphogenesis, being effective at inhibiting BMP-inducible markers at concentrations 10-fold lower than Chrdl1[189]. Taken together, these CORT-induced changes in astrocyte BMP and noggin transcripts would predict increased BMP signaling in the NSCs in response to CORT.

To test this prediction, we measured BMP signaling activation in NSCs in response to ACM-CORT. We treated NSCs with ACM from astrocytes treated with CORT or vehicle (Figure 3.3A) to measure changes in phosphorylation of the BMP signal transducer SMAD1/5/8 (pSMAD1/5/8) via western blot. Supporting our hypothesis of ACM-CORT dependent increased activity of BMP receptor, we found a 2.25 fold increase of pSMAD1/5/8 optical intensity in western blots from NSCs treated with ACM-CORT compared to NSCs treated with control ACM (Figure 3.3B,C). Finally, we saw that re-introduction of noggin to ACM-CORT prior to NSC treatment led to the level of optical intensity of pSMAD1/5/8 signal to return to 1.115 fold relative to control, suggesting that addition of noggin was sufficient to induce BMP signaling of NSCs back to, and not below, control levels.

Glucocorticoid-induced astrocytic anti-proliferative effect can be blocked by re-introduction of BMP inhibitor noggin. We next assessed whether the decrease in BMP signaling via re-introduction of noggin could be sufficient to rescue the ACM-CORT induced decrease in stem cell proliferation, which would indicate that increased BMP signaling is necessary for the astrocyte mediated decrease in NSCs proliferation in response to CORT. We tested NSC cultures treated with non-conditioned co-culture media (CoCM) plus or minus CORT or ACM from astrocytes treated with or without CORT, and supplemented with noggin (or vehicle) for a 24-hour period (Figure 3.4a). At the time of NSC treatment, all groups were pulsed with 30 μM BrdU to determine proliferation rates. Quantification of BrdU+ cell counts normalized to total DAPI cell counts showed once again that ACM-CORT reduced NSC proliferation by 33.24% compared to control (50.33 ± 1.956% BrdU+ cells ACM-control vs 33.60 ± 1.480 %BrdU+ cells ACM-CORT, $P < 0.001$, Figure 3.4C). Remarkably, NSCs treated with the noggin-supplemented ACM-CORT restored proliferation to levels similar to control, whereas noggin-supplemented ACM-control had no effect on increasing proliferation compared to ACM-control (50.33 ± 1.956% BrdU+ cells ACM-control vs 43.51 ± 1.434% BrdU+ cells ACM-CORT+nog vs 46.81 ± 1.523% BrdU+ cells ACM-control+nog, Figure 3.4C). Furthermore, in NSC cultures treated with non-conditioned CoCM, neither CORT treatment nor noggin supplementation altered levels of NSC proliferation (49.12 ± 1.763% BrdU+ control vs 44.98 ± 1.9% BrdU+ control-nog and 44.11 ± 1.626% BrdU+ CORT vs 46.12 ± 1.576% BrdU+ CORT-nog, Figure 3.4B), strongly suggesting that noggin specifically blocks the increase in overall astrocytic BMP signaling in response to glucocorticoids, and that this blockade is sufficient to rescue NSCs from astrocyte-mediated, glucocorticoid-induced suppression of proliferation.

Chronic, but not acute stress, changes gene expression of hippocampal noggin and serotonin receptors 1A, 2A and monoamine oxidase genes. We recently reported that acute stress is able to enhance adult hippocampal neurogenesis in rats, and that this effect is mediated by astrocytic secretion of FGF2[44]. Thus, we asked what are the changes in BMP signal molecules in the hippocampi of rats exposed to an acute (Figure 3.6A) vs. a chronic stress paradigm (Figure 3.6B). We found that BMP 2 (Figure 3.5 C,D) , BMP4 (Figure 3.5 E,F), and BMP6 (Figure 3.5 G,H) do not significantly change in response to either chronic or acute stress. However, noggin expression was reduced by almost half of the level of expression in the chronically stressed animals compared to control (0.56 ± 0.28 fold change, $p = 0.0087$, Figure 3.5J), but not in the acute stress rats (Figure 3.5I). This level of downregulation was similar to the one found in cultured astrocytes (Figure 3.2D). In studying the diverging proliferation effects of acute and chronic stress conditions on neurogenesis, we decided to look at changes in serotonergic genes, since serotonin has also been implicated in the neurogenic effects from exercise[190], environmental enrichment[191], and perhaps even the anti-neurogenic effect of stress[192]. So we measured changes in gene expression in acute vs. chronic stress paradigms for the primary serotonin receptors in NSCs, receptors 5HT-1A and 5HT-2A[193], and monoamine oxidases A and B. We found that all mRNA from all these genes in the hippocampus decreased in response to chronic, but not acute, stress (Figure 3.6), highlighting the contrasting different brain-states under acute and chronic stress. Further investigation will be necessary to find a causal relationship between the serotonergic and astrocytic-signaling systems, and whether changes in serotonergic activity leads to a change in astrocytic regulation of neurogenesis.

C. Discussion

We previously reported that astrocytes mediate an increase in hippocampal NSC proliferation in response to an acute stressor via FGF2 signaling. In this study, we present evidence that a longer glucocorticoid exposure also affects astrocytic regulation of NSCs, this time through changes in BMP signaling. Furthermore, gene expression analysis of adult rat hippocampi seems to corroborate the changes that were observed *in vitro.* Astrocytes seem to be directing a switch in the transition from acute to chronic stress proliferation modulation by using two different signals. We propose that pro-proliferation FGF2 may be acting on the mid- to latter stage intermediate progenitor pool, increasing a cohort of possible new neurons immediately following the stressor. It is possible, however, that the pool of proliferative cells becomes exhausted as repeated stressors in the form of chronic stress lead to a later-onset reduction in secretion of noggin and followed by BMP-signaling mediated quiescence of Type1 and Type2a NSCs, leading in the long term to an overall slow-down of stem cell proliferation. In combination with lower survival and differentiation of NSCs under chronic stress[133,134], this leads to an overall decrease in neurogenesis. Further studies will be necessary to determine how these findings fit in with other evidence of factors affecting environmentally induced changes in neurogenesis. Stress and glucocorticoids are known to increase glutamatergic signaling in the hippocampus, and whether that affects astrocytic trophic signaling remains to be studied, as it is known that astrocytes express several glutamatergic receptors and are highly responsive to network activity[194–196]. Blockade of NMDARs blocks the negative effect of chronic stress on neurogenesis[139], but whether that effect is directly through stem cell NMDAR activity, or through signaling of the stem cell niche is not clear. Moreover, recent reports suggest that BMP signaling is able to modulate the tempo of neurogenesis[185], and that BMP signaling may be necessary for the enhanced neurogenesis occurring in response to other environmental factors such as voluntary exercise[97]. However, a paradoxical release of glucocorticoids accompanies exercise, despite the fact that glucocorticoids lead to decreased neurogenesis in a stressful environment. It may be that this apparent discrepancy could be explained by compounding other network activity that occurs only under stress. For instance, other studies have shown that serotonergic signaling is necessary for the enhancement of neurogenesis following voluntary exercise[190]; while others have shown that serotonin, via activity of 5-HT1A and 5-HT2A receptors is neurogenic[38], opening up the possibility that exercise, through increased serotonergic input to the hippocampus, alters the way astrocytes respond to glucocorticoids, changing their modulation of BMP signaling through inhibitors such as noggin. It has been previously reported that stress modulates serotonergic activity in the hippocampus[40,192] (Fig3.6), which could link these two mechanisms. Further studies will be necessary to elucidate how these systems interact.

D. Materials and Methods

Culturing of primary astrocyte cultures. Astrocytes were isolated from P1-P2 day old Sprague Dawley rat pup hippocampi using the method described by McCarthy and Vellis (McCarthy and de Vellis, 1980). Briefly, hippocampi were dissected in ice-cold dissection media (HBSS, 0.02M glucose, 10mM HEPES), minced and digested using 10 U/mL papain derived from papaya latex extract (Sigma P4762) in HBSS (Invitrogen) for 20 minutes at 37°C with frequent shaking. After papain-inactivation with 10% horse serum, cells were spun down at 1000 rpm for 1 min, resuspended and triturated in HBSS. Cells were then plated in Dulbecco's modified Eagle

medium (DMEM) (Invitrogen), supplemented with 10% fetal bovine serum (Axenia BioLogix) and 1% Penicillin/Streptomycin (Invitrogen) at a cell density of about $3x10^4$ in T75 flasks (Nunc). After growing to confluency, microglia were removed by shaking on an orbital shaker at 225 rpm for 2 hours at 37°C and washed 5x with warm PBS to remove suspended microglia. Astrocytes were then trypsinized, re-plated in 100mm dishes, and cultured in incubators under standard conditions of 37°C and 5% CO2. Cells are grown to confluency before treatment and conditioned media collection.

Culturing of hippocampal NSCs. Progenitor cells from adult rodents were purchased from Millipore (Billerica, MA; SCR022). NSC cells were cultured as previously described [2,44] in a 37°C, 5% CO2 incubator environment on 10 μg/mL poly-ornithine (Sigma P1399) and 5 μg/mL laminin (Invitrogen 23017) coated plates. Growth medium consisted of N2-supplemented (Invitrogen 17502-048) DMEM/F-12 (Invitrogen 11330) with 20ng/ml recombinant human FGF-2 (PeproTech 100-18). Cells were plated at $5x10^5$ cells per plate and grown to 70-80% confluency before treatment or co-culture media change.

Cell treatments. Twenty-four hours prior to experimental treatments, astrocytes and NSCs were allowed to stabilize in a NSC-astrocyte co-culture media (CoCM) consisting of MEM (Invitrogen 11090) supplemented with N2, 2mM glutamine, 0.2M glucose, 0.05% Ovalbumin (Sigma A2512), and 1mM Sodium Pyruvate (Invitrogen 11360-070). Cells were then treated with 1 μM CORT (Sigma C2505), dissolved in 100% ethanol or vehicle (an equivalent volume of 100% EtOH, usually about 1-10 microliters) for 24 hours.

BrdU-labeling. For the proliferation experiments, NSC proliferation was quantified by cell incorporation of the thymidine analog Bromodeoxyuridine (BrdU). At the time of NSC treatment, all groups were pulsed with 30 μM BrdU for 8 or 24 hours before fixation.

Immunocytochemical BrdU staining and quantification. Fixed cells were rinsed with 0.1 M PBS, denatured in 1 N HCl at 37°C, rinsed and blocked in 5% normal donkey serum, 0.3% triton-100 in PBS. Cells were then incubated overnight at 4°C in rat anti-brdu (1:500, Abcam ab6326) in 2% normal donkey serum in PBS. Cells were then rinsed and incubated for two hours at room temperature in secondary donkey biotinylated anti-rat antibody (1:500, Jackson Immunoresearch) in 2% normal donkey serum in PBS, rinsed and incubated 1hr at room temperature with terciary strep-alexa 594 or 488 (1:1000 Jackson Immunoresearch). Finally the cells were counterstained with DAPI (1:20,000 in PBS) and coverslipped with DABCO anti-fading medium. BrdU+ and DAPI+ cells were counted in randomly sampled sites within each well using Metamorph software and a 20x air objective (Zeiss).

Noggin ELISA for ACM. ELISA for rat noggin (Biomatik EKU06325) was done on undiluted ACM-CORT (n=4) and ACM-Veh (n=4) according to manufacturer's instructions.

Conditioned media experiments. ACM was collected and filtered with a 0.2 μm sterile filter, and 80% of NSC media was changed to ACM (or CoCM for no-astrocyte controls). For noggin supplementation, 200 ng/ml recombinant human noggin (ScienCell 108-09) was added immediately before treating NSCs for an incubation period of 24 hours.

Protein extraction. Protein was extracted from NSC cultures with radioimmunoprecipitation assay (RIPA) buffer. Treated NSCs were washed with ice cold 1x PBS, scraped and transferred to a 15 ml conical, and then centrifuged at 1000 rpm for 1 min. Protease inhibitor (1:100, CalBiochem/EMD Millipore) and phosphatase inhibitor (1:10, Roche) were added to RIPA buffer made with 50 mM Tris HCl (pH 8, Invitrogen), 150 mM NaCl (Fisher Scientific), 1% NP-40 (US Biological), 0.5% Sodium Deoxycholate (Fisher Scientific), and 0.1% SDS (Fisher Scientific). Cells were incubated in RIPA buffer with inhibitors for 30 min to lyse cells and

extract protein. Samples were centrifuged for 30 min at max speed, and then supernatant was collected.

BCA Protein Assay. Pierce BCA Protein Assay Kit was used to quantify protein concentration in each sample. Kit standards were made with stock albumin following the kit protocol. 25 µl of 1:10 diluted samples are required per well, and each sample was run in duplicates. Samples were diluted in RIPA buffer with protease and phosphatase inhibitors at concentrations stated before and incubated with assay reagents for 30 min at 37°C. Colorimetric detection was then used to determine protein concentration.

Western Blot Analysis. Protein (40 µg), normalized to equal volumes with RIPA buffer and protease and phosphatase inhibitors, was prepared in a 1:1 ratio with 5% β-mercaptoethanol in lamelli loading dye (BioRad). Samples were boiled at 95°C for 5 min, and 20 µg of protein was loaded and electrophoresed on SDS/4-20% PAGE precast gels (BioRad). Gels were then transferred onto nitrocellulose membranes and blocked with TBS/5% powdered milk (Apex) at room temperature for 1 hour. After, membranes were incubated overnight at 4°C in primary antibodies: rabbit polyclonal anti-phosphorylated-SMAD1/5/8 (Cell Signaling, 1:1000) and mouse monoclonal anti-actin (Abcam, 1:400). Membranes were washed three times for 10 min with TBS/0.1% Tween 20 and incubated at room temperature for 1 hour in secondary antibodies: 680RD donkey anti-mouse IgG (LiCor, 1:10,000) and 800RD donkey anti-rabbit IgG (LiCor, 1:10,000). Membranes were washed again three times for 10 mins before fluorescence was visualized with LiCor Odyssey CLx infrared imaging system. pSMAD signal intensity was analyzed using Image Studio software and normalized to actin loading control.

Animals: Adult male Sprague-Dawley rats (Charles River) were pair-housed on a 12h light dark cycle with lights on at 700h. Rats were allowed to acclimate to the animal facility for one week before handling began. All animal procedures were approved by the UC Berkeley Animal Care and Use Committees.

Immobilization Stress: All rats were all handled for 5 days. On the 6th day, they were exposed to a stressor (30 minutes or 3 hour immobilization stress) or left undisturbed in the case of controls. Rats' weights were monitored for the duration of the entire experiment. Immobilized rats were confined for 3 hours in decapicone bags (Braintree Scientific). For acute stress experiment, rats were sacrificed within 30 minutes of the stressor. For chronic stress experiment, stress was repeated at the same time at the beginning of the light cycle for 7 days, and sacrificed at day 7 within 30 minutes after the stressor.

Real Time Quantitative PCR. For animal experiments, rats were lightly anesthetized with isoflurane and rapidly decapitated 30min or 3h after the beginning of their respective stressors (n = 6/grp). Bilateral hippocampi were dissected and flash frozen in liquid nitrogen. One hippocampus per rat was used for mRNA expression quantification.

For cell culture experiments, cell media was aspirated and cells were scraped with 1mL Trizol (Invitrogen), and proceded to extract RNA according to manufacturer's instructions. Real-time reverse transcriptase PCR was run on Trizol-extracted RNA with primers detailed in the table below.

Gene	Direction	Sequence
BMP2	+	CCGTTTGCTGGCTGCTCTCACA
	-	GAGTGCCGGCGGTACAGGTC

BMP4	+	GACCCTGGTCAACTCCGTTA
	-	CTCCACCACCATCTCCTGAT
BMP5	+	GCAGCCGTTCATGGTGGCCT
	-	CCGTTTGCTGGCTGCTCTCACA
BMP6	+	TAGCAATCTGTGGGTGGTGA
	-	GAAGGGCTGCTTGTCGTAAG
BMP7	+	AGGGTGGGCGCTTACGTCTGT
	-	GAAGGGCCTCTCCATCCTCCACA
Noggin	+	CGGCCAGCACTATCTACACA
	-	GCGTCTCGTTCAGATCCTTC
Chrdl-1	+	TCTCGCCACAACCTGCGAGC
	-	CCACGCTTGCACACGCTCTG
5HT-2A	+	CTTCCAACGGTCCATCCACA
	-	CACGCCTTTTGCTCATTGCT
5HT-1A	+	TACTCCACTTTCGGCGCTTT
	-	ACCTTCCTGACAGTCTTGCG
MAO-A	+	ACTGCTCGGGAATTTGCGTA
	-	CAAATTTCCGTTCCTGGCCG
MAO-B	+	GGTCAAGTGAGTGAGCGGAT
	-	TGGGCAGAGGAGGACTATGG
RPLP	+	CCAAAGGTTTGGGAGAACAA
	-	GGGTCATGGCATAGAGCAAT

Primer sequences were designed using PrimerBlast software on the NCBI website[197]. Extracted RNA was treated with DNase (DNA-free, Ambion), and two-step PCR was used, following manufacturer instructions for iScript cDNA synthesis kit (BioRad) and then SsoAdvanced Universal SYBR Green Supermix (BioRad). Samples were run in a BioRad IQ5 real-time PCR machine. After the PCR was complete, specificity of each primer pair was confirmed using melt curve analysis. Ct values were determined using PCR miner[183] and normalized to the reference ribosomal RNA, RPLP. Fold change in mRNA expression is relative to no stress control rats. For relative expression of different BMPs, the relative nRNA transcript value was estimated using the DeltaCT method and normalized to RPLP.

Statistics

In most studies, when only two groups were being compared, data were analyzed using two-tailed unpaired T-tests, and when noted, with Welch's correction for experimental groups that showed unequal variances. When comparing more than one group, one-way ANOVA followed by Dunnett's posthoc tests with the appropriate control group as reference, or Tukey's multiple comparison test when comparing all columns (western blot and noggin rescue experiments). $P \leq 0.05$ was considered significant.

E. Figures

Figure 3.1. Astrocyte secreted factors mediate NSC proliferation decline in response to glucocorticoids. A) Experimental time-line. NSCs were FGF2 deprived for 24hours prior to 24hr

treatment with 1 μM CORT. B) No difference in BrdU incorporation was detected in NSCs treated with CORT compared to vehicle. C) Experimental time line. NSC and astrocyte co-culture were treated 24-hrs with 1 μM CORT. D) We found a significant decrease in proliferative BrdU+ NSCs in the CORT treatment (32.28 ± 2.55% vehicle vs 13.11 ± 1.15% CORT, $p < 0.001$). E) ACM from astrocytes treated with 1 μM CORT lead to F) a significant decrease in proliferative BrdU+ NSC (29.84 ± 2.225% vehicle ACM vs 15.51 ±1.25% CORT ACM, $p < 0.001$). All values are average ± SEM, compared with two-tailed unpaired T-tests with Welch's correction for unequal variances.

Figure 3.2 Glucocorticoid reduce astrocyte expression and secretion of the BMP-inhibitor noggin. A) mRNA expression levels of different BMPs in cultured astrocytes treated for 24hrs with 1 μM CORT (n = 6) relative to vehicle (n = 6). A CORT-induced reduction in mRNA expression of BMP2 (0.58 ± 0.07 fold change, *p = 0.0164) and BMP4 (0.50 ± 0.03 fold change, *p = 0.0179), but no difference in BMPs, 5, 6 and 7 was detected. B) Relative mRNA expression levels of all the different BMPs in cultured astrocytes and in C) adult rat hippocampi. BMP6 mRNA is expressed primarily by astrocytes and it is the most abundant BMP mRNA expressed in the hippocampus. D) mRNA expression levels of BMP inhibitors noggin and chrdl-1 in cultured astrocytes treated for 24hrs with 1 μM CORT relative to vehicle. Noggin mRNA expression significantly decreased in response to CORT (0.51 ± 0.06 fold change, ***p = 0.0005). E) Protein secretion of noggin in ACM is also reduced by CORT treatment (n = 4), as measured by rat noggin ELISA (85.58 ± 11.97 pg/mL in control ACM to 50.19 ± 5.82 pg/mL in ACM-CORT, *p = 0.0376). All values are average ± SEM, compared with two-tailed unpaired T-tests.

Figure 3.3 Conditioned media from astrocytes treated with CORT increases canonical BMP signaling via phosphorylation of SMAD1/5/8 in NSCs. A) Experimental time line. B) Western blot of NSCs treated with ACM, ACM-CORT and ACM-CORT+noggin. C) Fold difference in optical density of western blot signals normalized by actin. *p = 0.0205, one-way ANOVA with Tukey's multiple comparison test.

Figure 3.4 Noggin is sufficient to inhibit the ACM-CORT induced suppression of NSC proliferation. A) Experimental time line. We tested proliferation of NSC cultures treated for 24-hours with non-conditioned co-culture media (CoCM) ± CORT or ACM from ± CORT-treated astrocytes, then supplemented ± noggin. B) When treating NSCs alone, no changes in proliferative BrdU+ NSCs were detected with any treatment. C) When exposed to ACM factors from astrocytes treated with CORT, BrdU+ cell counts went from 50.33 ± 1.956% BrdU+ cells ACM-control down to 33.60 ± 1.480 %BrdU+ cells ACM-CORT, (***P < 0.001, one-way ANOVA with Tukey's multiple comparison test). However, addition of noggin rescued NSC proliferation (43.51 ± 1.43% BrdU+ cells ACM-CORT+nog). All values are average ± SEM.

Figure 3.5 Chronic, but not acute stress reduces noggin mRNA expression in the hippocampus of adult rats. A) Acute stress paradigm experimental time line. B) Chronic stress paradigm experimental time line. C/E/G/I) Hippocampal mRNA expression levels relative to control of 30min or 3hr immobilization stress rats for BMP2, BMP4, BMP6, noggin, respectively. D/F/H/J) mRNA expression levels relative to control of chronic stress rats for BMP2, BMP4, BMP6 and noggin, respectively. A significant decrease in noggin was found in chronically stressed rats

(0.56 ± 0.28 fold change, **p = 0.0087, two-tailed unpaired T-test) compared to control. All values are average ± SEM. Statistics for acute stress, one-way ANOVA with Dunnet's multiple comparison post-hoc test against controls. Statistics for chronic stress, two-tailed unpaired T-tests.

Figure 3.6 Chronic, but not acute stress, reduces mRNA expression of serotonin receptors 1A and 2A and monoamine oxidases A and B in the adult rat hippocampus. A) Acute stress paradigm experimental time line. B) Chronic stress paradigm experimental time line. C/G/E/I) Hippocampal mRNA expression levels of 30min or 3hr immobilization stress rats relative to control for 5HT-1A, 5HT-2A, MAO-A and MAO-B, respectively. D/F/H/J) mRNA expression levels relative to control of chronic stress rats for BMP2, 5HT-1A, 5HT-2A, MAO-A and MAO-B, respectively. All values are average ± SEM. Statistics for acute stress, one-way ANOVA with Dunnet's multiple comparison post-hoc test against controls. Statistics for chronic stress, two-tailed unpaired T-tests.

Figure 3.1 Astrocyte secreted factors mediate NSC proliferation decline in response to glucocorticoids

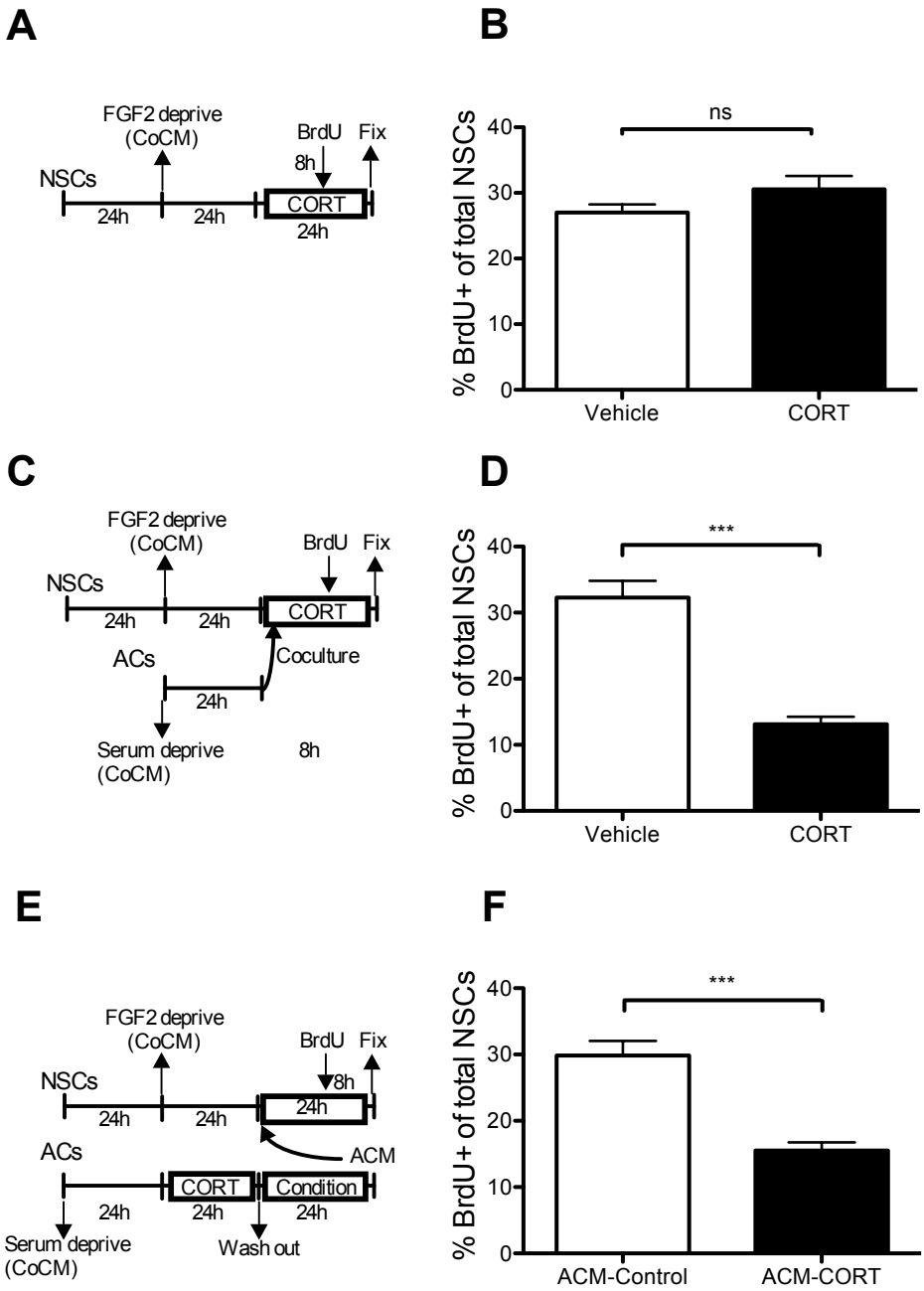


Figure 3.2 Glucocorticoids reduce astrocyte expression and secretion of the BMP inhibitor noggin

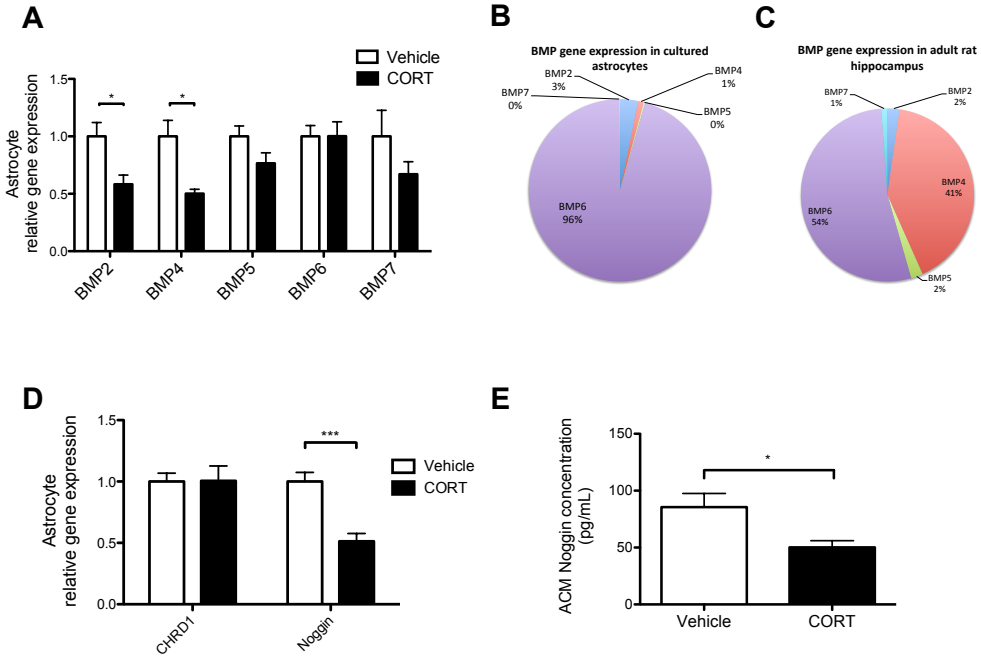


Figure 3.3 Conditioned media from astrocytes treated with CORT increases canonical BMP signaling via phosphorylation of SMAD1/5/8 in NSCs.

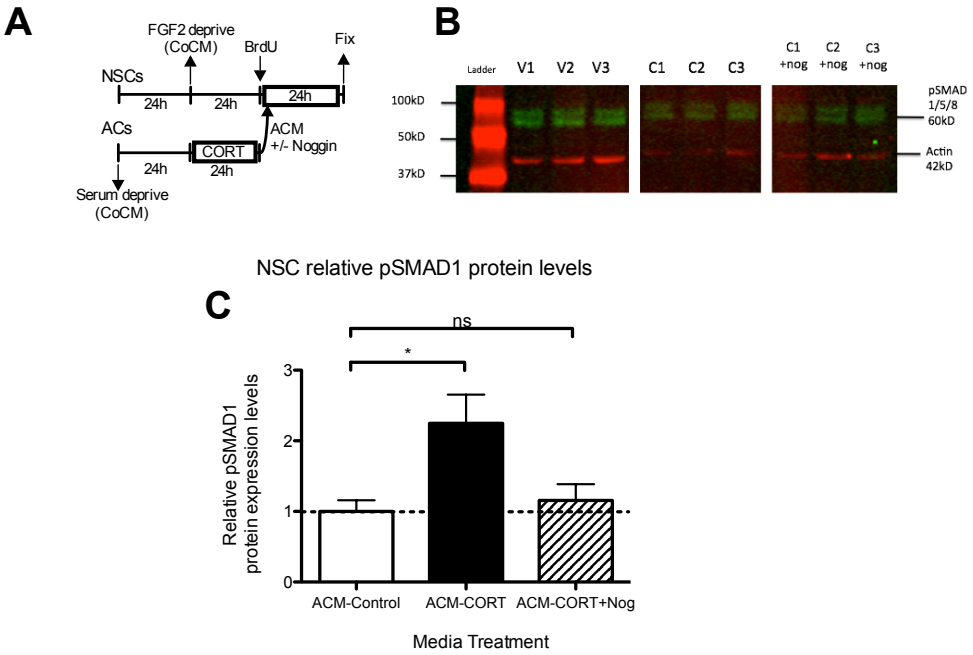


Figure 3.4 Noggin is sufficient to inhibit the ACM CORT-induced suppression of NSC proliferation

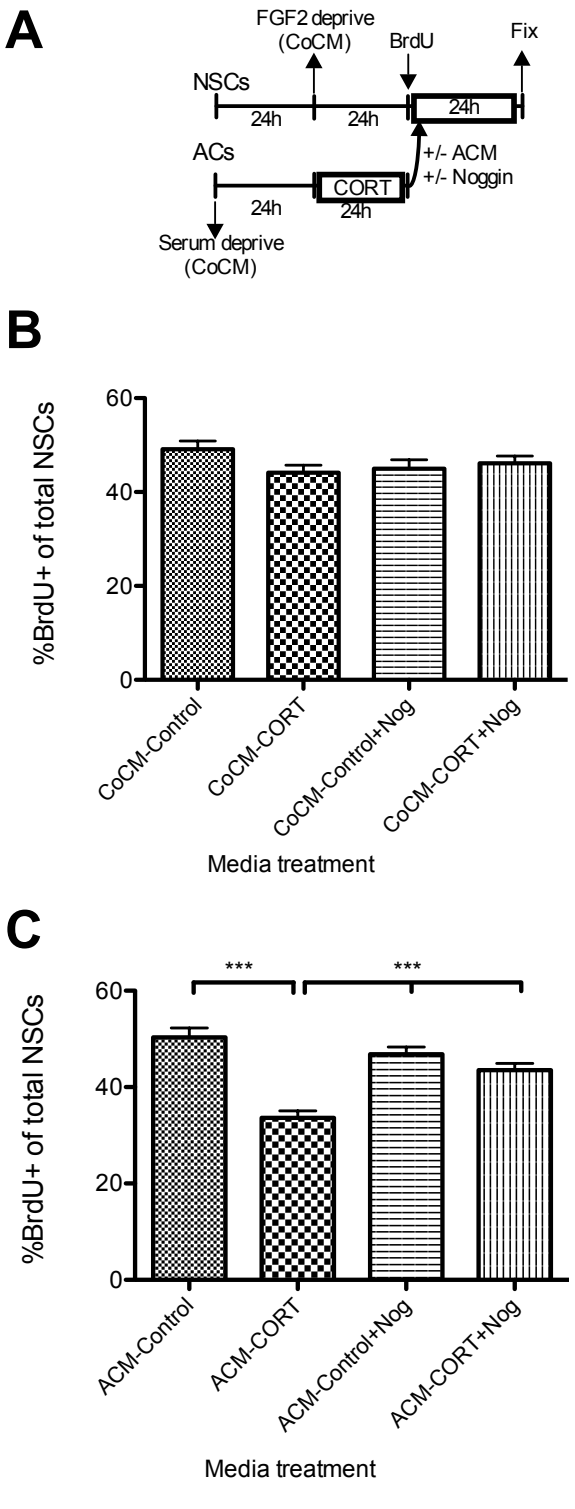


Figure 3.5 Chronic, but not acute stress reduces noggin mRNA expression in the hippocampus of adult rats

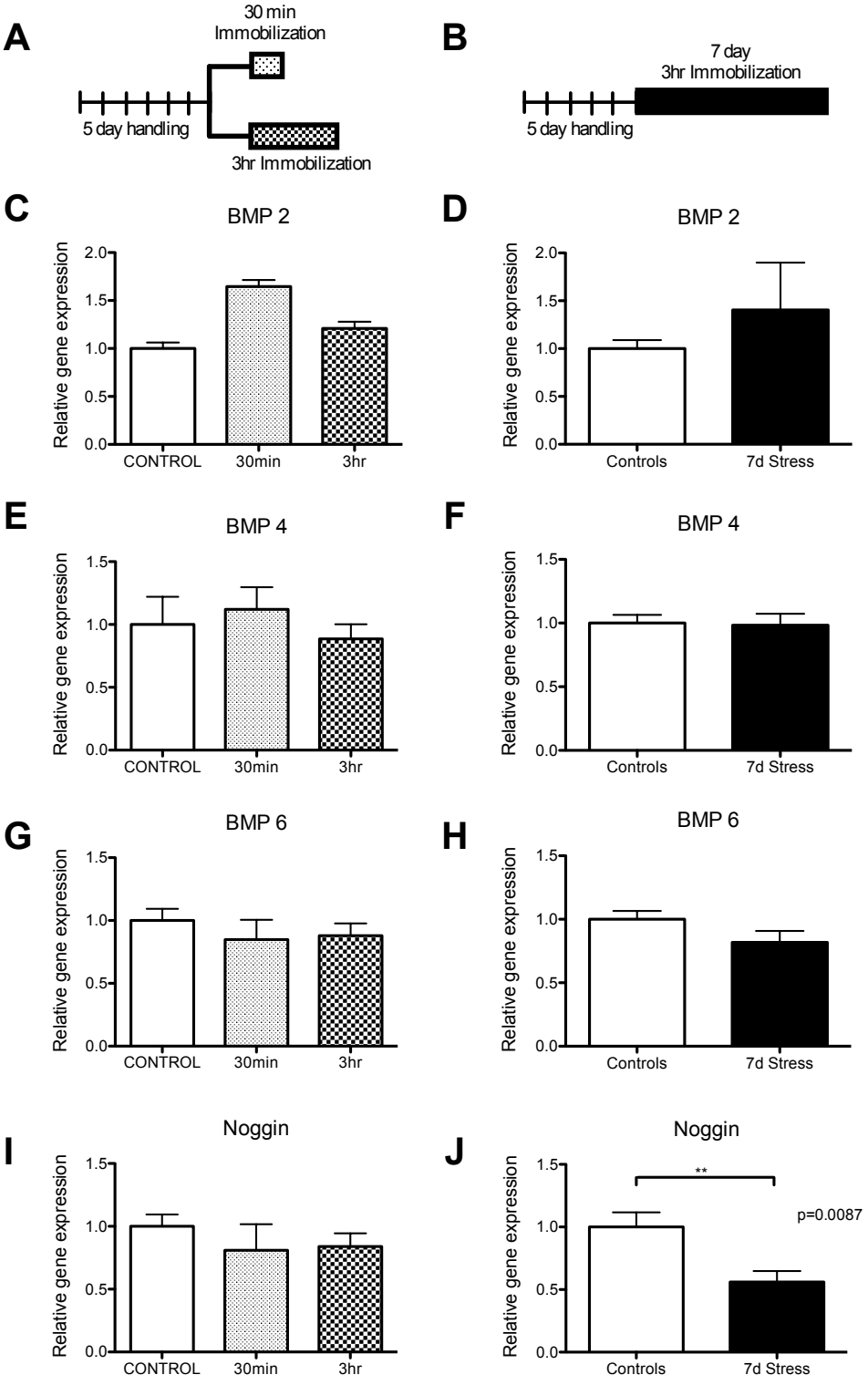
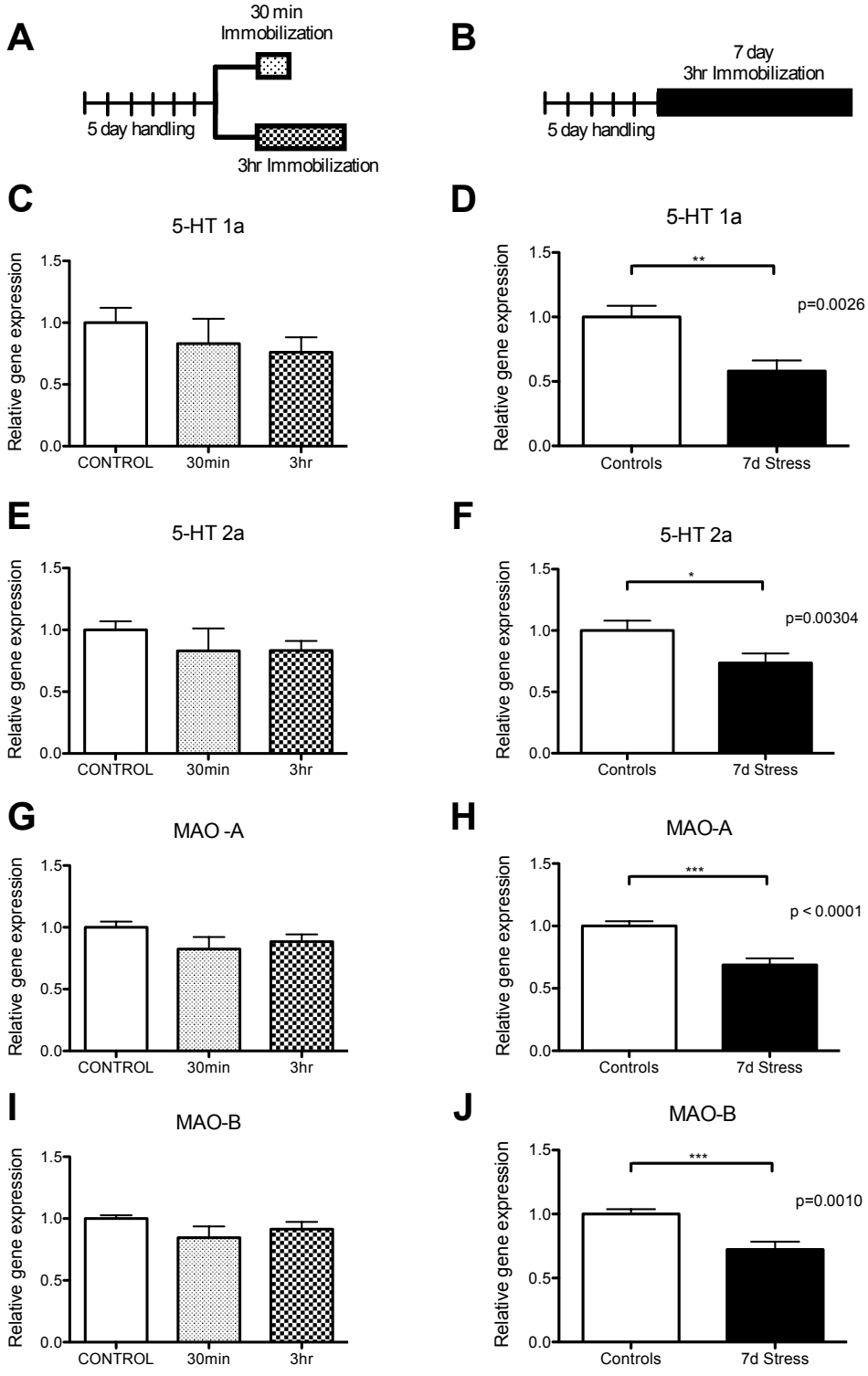


Figure 3.6 Chronic, but not acute stress reduce mRNA expression of serotonin receptors 1A and 2A and monoamine oxidases A and B in adult rat hippocampus



Chapter 4

Discussion

A. Acute vs. chronic stress and neurogenesis

There are many types of events that can affect neurogenesis, some increasing, some suppressing neurogenesis. As we have reviewed, chronic stressors can decrease neurogenesis[129,198], but other events that also induce the release of glucocorticoids, such as exercise[199], sexual experience[69] and even an acute stressor[44] have a positive neurogenic effect. An interesting developing view of these events affecting the organism is that even though they could seem to fit into "positive" or "negative" categories, they all in fact lead to activation of the stress response and lead to glucocorticoid release. This makes sense, since the stress response comes into play whenever there's an event that affects in one way or another the homeostasis of the organism[111,121]. However, their distinct effects in both neurogenesis as well as cognition, suggest that positive associations to certain challenges, such as perceived rewarding experiences, can lead to powerfully different outcomes.

In these studies presented here, my colleagues and I find evidence of astrocytes responding differentially to two types of stress, acute vs. chronic, by utilizing two different molecular signals: pro-proliferative FGF and anti-proliferative BMP, respectively (Figure 4.1). The question then, is what are the different upstream molecular mechanisms that separate these two opposing results. Two different factors might come into play. 1) That the timing of exposure to glucocorticoids brings about a gradual change in the output from astrocytes (i.e. changes in expression and secretion of different signaling molecules), and 2) that FGF and BMP signaling act differentially on distinct sub-populations of stem cells. Thus, at first, short bouts of glucocorticoid exposure push sub-populations of intermediate progenitors to proliferation via FGF2 secretion from astrocytes (Figures 2.6, 2.7, 2.9), but then prolonged glucocorticoid exposure brings about a late-onset reduction of astrocytic noggin secretion (Fig 3.5), which in turn increases BMP signaling, ultimately leading to a quiescent population of NSCs (Summarized on Figure 4.1). Furthermore, a compelling possibility would be that a neural circuit coordinates the mental state (reward or punishment) with the status of endocrine and local molecular signals. One potential neural signal is serotonin, which has been associated with affective disorders[40,200]. Serotonin input in the hippocampus is downregulated with stress[192,200] (Figure 3.6), and has recently been implicated with reward experience and behaviors, being able to respond to reward/punishment paradigms[201–203]. Interestingly, serotonin has also been implicated in exercise; a physiological state that brings upon enhanced neurogenesis yet induces a concomitant glucocorticoid release. Serotonin increase has been measured in the hippocampus after exercise[204], and it has been reported that serotonin is necessary for the pro-neurogenic exercise effect[190]. At the same time, the pro-neurogenic effect of exercise has also been tied with VEGF signaling[205] and, interestingly, a reduction BMP signaling[97], opposite to the effect we observe with stress. It is then theoretically possible that the positive, rewarding experience of exercise brings about a cognitive state, mediated in part by serotonergic activity, which coordinates other molecular signals to bring about this shift.

When considering the potential functional relevance or evolutionary reasons for this stress-neurogenesis interaction, one has to consider the function of neurogenesis. The first obvious consideration is its role on memory and improvement in pattern separation and spatial memory tasks[94]. But one also has to consider the link between neurogenesis and mood. There exists an ample literature linking depressive/anxiety related conditions with neurogenesis. Just as anti-depressants increase neurogenesis[107–110], neurogenesis is also required for the beneficial effects of anti-depressants[101,106,107,109]. Moreover, the link between an individual's affective state and

neurogenesis is also strongly tied together with the bi-directional regulation between stress and neurogenesis. In other words, while stress can affect neurogenesis in many ways[44,129,198], neurogenesis also seems to modulate the stress axis itself, as it helps to improve the negative feedback loop that shuts down the stress response[105,106].

All this evidence points towards a view that the neurogenic hippocampus serves as a grand integrator of the organism's mental state and its surrounding environment, all while being plastic and adaptable to fine-tune cognitive function. In terms of what the potential adaptive effects these phenomena have, we have to consider both the memory and the mood functions of neurogenesis together. An emerging view discussed here[136,206] suggests that neurogenesis serves as a substrate for functional changes in hippocampal function that fits with whatever environmental challenges are currently experienced by the organism. By modulating memory function as well as anxiety and stress, adult hippocampal neurogenesis is likely involved in impacting the behavior of an animal, such as improvements in mobilizing, locating resources, and exploring an environment. In a challenging but not prohibitive environment, fine-tuned neurogenesis could therefore be a powerful asset. However, during a time of severe and chronic stress, it could be maladaptive to be overtly bold at exploration and resource utilization. Thus, the short-term inhibition of neurogenesis can be beneficial for 1) diverting of metabolic resources to more essential functions. 2) Leading to a modification in the behavior of the animal to enter survival-mode. But as we have come to understand in the many examples of the stress response gone awry, overshooting the stress response in the realm of neurogenesis can also lead to maladaptive consequences, such as affective disorders like depression and anxiety. Thus, the studies discussed here bring about better understanding of the molecular mechanisms that bring about these subtle changes in hippocampal function that have a major impact in an organism's adaptability, and could be relevant when understanding the state of affairs during affective disorders.

Figure 4.1 Summary of astrocyte mediated effects of acute and chronic stress on adult hippocampal neurogenesis

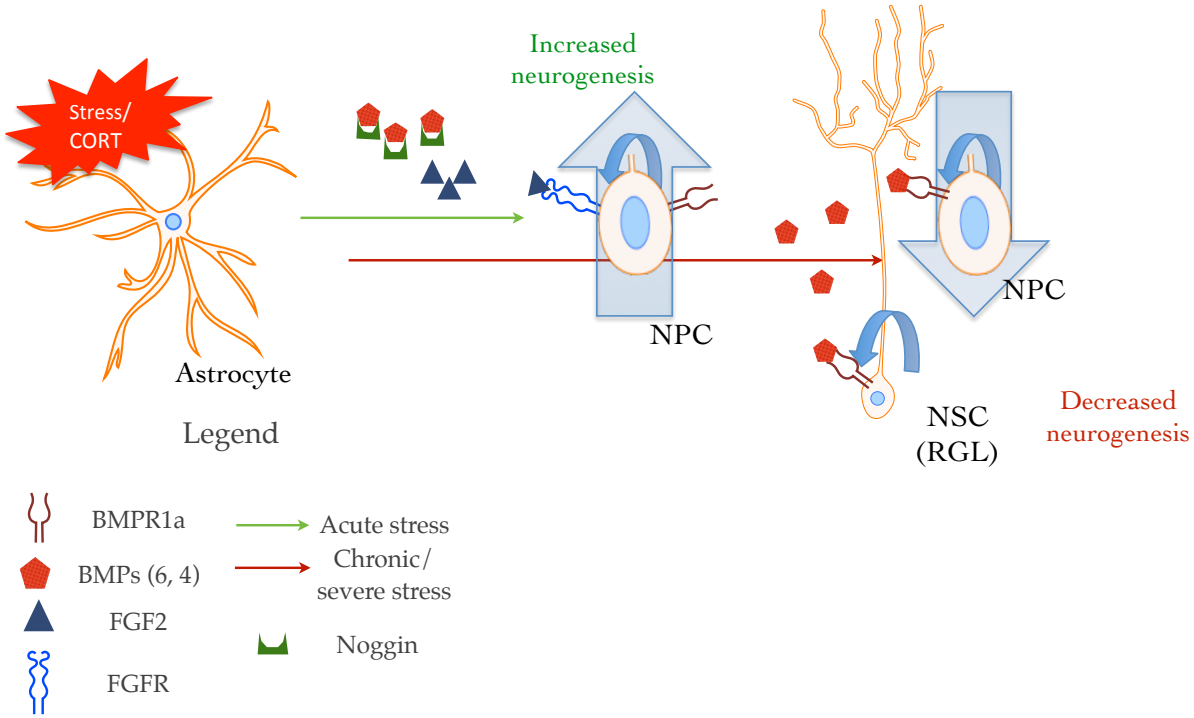


Figure 4.1 Summary of astrocyte mediated effects of acute and chronic stress on adult hippocampal neurogenesis. First, an acute stressor (green arrow) leads to a short-term release of glucocorticoids, which lead to astrocytic release of FGF2. FGF2 is pro-proliferative, and is likely acting on intermediate neural progenitor cells (NPCs) to boost their proliferation in an environment permissive for cell cycle progression, where noggin is able to inhibit BMP-induced stem cell quiescence. However, repeated chronic stressors (red arrow), lead to a downregulation of astrocytic noggin, leading to inhibition of NSC and NPC proliferation and hence stem ell quiescence and an overall decrease in neurogenesis.

B. Future directions on the biology of stress of glial cells

Similar to the field of adult neurogenesis, neuroscience's interest in astrocyte biology has increased substantially in the past couple of decades, to the degree that we've come to see astrocytes as much more than just "passive support cells." Astrocytes are involved in almost every process that is vital for the functioning of the mammalian CNS[207], from synaptic activity (the so-called tripartite synapse)[195], synaptogenesis[208], blood-brain barrier regulation[209], and metabolite clearing.[210] Furthermore, their heterogeneity in functionality as well as cell sub-types is at least as large as that of neurons. In terms of stress biology, it is possible that astrocytes play a large part in a lot of the processes that are affected during the stress response.

Stress responses throughout different anatomical regions in the brain are also heterogeneous. An excellent example of these differences is the opposing effects of stress on the amygdala and the hippocampus. The amygdala shows larger LTP and enhanced dendritic arborization upon stress, while the opposite is true of the hippocampus. One interesting question would be to better understand how the different subtypes of astrocytes in different brain regions respond to stress, and how their activity impact brain function.

An interesting direction for future study of the changes experienced by astrocytes in response to stress would be a finer tool of dissection for transcriptome analysis of astrocytes. *In vitro* cultures of astrocytes are significantly different in phenotype and gene expression than *in vivo* astrocytes[211]. Several techniques that use cell isolation based on antigen recognition for better culturing of mature adult brain astrocytes have been innovated[211,212], but the best way to study astrocytic responses to stress would be within the context of the intact tissue. An ideal way would be to specifically pull down tagged astrocytes after stress. Functionally, this could be done using FACS sorting of GFP-tagged astrocytes using the Aldh1L1[213] and other astrocyte specific promoters. Additional dissection of the role of molecular players, such as astrocytic GR, could be done using specific mouse genetic tools, or the use of virus-directed genetic therapy. With the advent of genetic manipulation tools such as the CRISPR/Cas9 system and our growing understanding of astrocyte markers and function, we can continue to gain deeper knowledge of the huge part that astrocytes play in regulating brain function and cognition, especially during stress, a condition that is highly relevant to human physiology and brings about large changes to the brain.

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