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Stress effects on adult hippocampal neurogenesis and sexual reproductive function

By

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A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

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# Abstract

Stress can dramatically alter the brain. Comprised of both neural and hormonal components, response to a perceived stressor can reorganize neural circuits, disrupt cell cycle and shift central control of a variety of systemic regulatory systems. While highly adaptive in many acute scenarios, if too prolonged or too extreme, the brain and bodily response to stress can be detrimental, such as in post-traumatic stress disorder, anxiety disorders and depression. Understanding how the basic environmental and psychological challenge of stress affects the brain is therefore important not only as a window into the basic neurobiology of adaptation to a changing environment, but also as an exploration into neuropathology. The following studies all address the issue of how the stress response alters the brain and thereby the body. Chapter 2 addresses the promotion of adult hippocampal neurogenesis by the basolateral amygdala, a brain region key to fear processing. Chapter 3 addresses how stress can interact with amygdala control of adult neurogenesis, depending on amygdala input for acute stress effects on neurogenesis but not for chronic stress effects. Chapter 4 focuses on how stress regulates sexual reproduction, exploring the role of a novel neuropeptide, gonadotropin-inhibitory hormone, in stress-induces suppression of sexual reproduction. All of the studies confirm the powerful effect of stress on the brain, and furthermore emphasize the complex nature of the relationship.

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# Chapter 1: Introduction

## A. Stress and adult neurogenesis in the mammalian central nervous system.

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### Introduction

While the developing brain often receives the most credit for ability to change in response to the environment, the adult CNS is also characterized by a great deal of plasticity. The ability to create new neurons— known as neurogenesis— is one of the most intriguing aspects of adult CNS plasticity both because it reveals a unique cell population that continues to proliferate beyond development and because it is highly sensitive to environmental input. One of the most powerful and ecologically relevant factors known to affect adult neurogenesis is the experience of a stressor [1, 2]. Indeed, stress and stress hormones were the first exogenous factors shown to influence the production of new neurons in the adult mammalian brain [1, 3, 4]. This chapter will review the nature of the stress effect on adult mammalian neurogenesis: the when, where, how and why of stress-related alteration in new neuron birth, survival and differentiation in the adult CNS. In brief, it appears that several aspects of adult mammalian neurogenesis are sensitive to environmental stress, primarily via exposure to the stress hormones, glucocorticoids. However, this effect depends on the neurogenic region, the timing and duration of the stressor, and the phase of neurogenesis being examined.

### Adult neurogenesis: a brief primer

First demonstrated by Altman and colleagues [5, 6], adult mammalian neurogenesis occurs in the dentate gyrus subgranular zone (SGZ) and the subventricular zone (SVZ) of every mammalian species investigated to date, including rats [5-7], mice [8, 9], tree shrews [4], monkeys [10] and humans [11]. The process of neurogenesis consists of at least three overlapping phases: proliferation, survival and differentiation/migration. In the SGZ, cells are born (proliferate) at the rate of approximately 9000 per day [12] and around half of these newly generated cells survive beyond one month [13], migrating a short distance out of the SGZ into the granule cell layer of the dentate gyrus. Of the surviving cells, typically between 80 and 90% become neurons under standard conditions [12]. The remainder of the cells differentiate into glial cells, oligodendrocytes or epithelial cells. In the SVZ, by contrast, about 30,000 cells are born per day [14]. These new cells migrate along the rostral migratory stream to the olfactory bulb. Of the approximately 50% of these cells that survive [14, 15], 75-99% differentiate into olfactory neurons [14, 15]. Importantly, new neurons in both the dentate gyrus and the olfactory bulb seem to integrate functionally into existing networks [16, 17].

### Measuring neurogenesis: how to find new neurons

There are three broad categories of method that have been used to identify new cells in the adult CNS: exogenous DNA replication markers, endogenous cell cycle-associated proteins and retroviruses.

Using DNA replication to detect new cells

The most common method of identifying new cells relies on the incorporation of labeled nucleotides during DNA replication. In early studies of neurogenesis, tritiated thymidine ([$^3$H]dT) was injected systemically and autoradiography was used to identify cells that had incorporated the tagged nucleotide into their DNA, presumably during S phase of cell division. More recently, [$^3$H]dT has been replaced by 5'-bromodeoxyuridine (BrdU), a thymidine analog which can be detected using immunohistochemistry. BrdU is typically preferred because it requires less tissue processing time than [$^3$H]dT labeling and because it can be more easily combined with antibody labeling of other proteins. However, the underlying principle of marking new cells by detecting DNA division remains the same.

The timing of BrdU (or [$^3$H]dT) injection and the chase period before sacrifice both must be optimized to assess changes in proliferation and survival/differentiation. For effects on proliferation, BrdU is injected immediately after the manipulation, allowing for an assessment of the pool of currently dividing cells. The animals are then sacrificed sometime between two hours and a few days later [1, 18]. To investigate effects on survival and differentiation, BrdU is typically administered before beginning an experimental manipulation and the animals are then sacrificed after the manipulation is complete [19, 20].

There are several potential pitfalls of using BrdU to label new cells. First, neurotoxicity of even relatively low doses of BrdU has been reported [21], suggesting that BrdU incorporation might alter cellular function. However, many other studies have used high, repeated doses of BrdU without any reported ill-effect. Second, it is possible that BrdU might be incorporated into cells during DNA repair, but the size of this repair-related contribution to the population of labeled cells is likely very small [22]. Third, the bioavailability of BrdU could change in response to an environmental manipulation through changes in blood flow or blood-brain-barrier perrmeability. Such a change in bioavailability could either create false or mask real group differences in proliferative cell labeling. One way to control for this limitation of BrdU is through the use of endogenous markers of cell division, as described below.

Endogenous markers of cell cycle

A number of proteins are expressed exclusively during the cell division cycle and can be used as markers to quantify the number of dividing cells. The most commonly used marker is Ki-67, a nuclear protein expressed throughout the cell cycle, which has been successfully combined with BrdU to assess proliferation levels in the adult rodent hippocampus [13, 23]. Proliferating cell nuclear antigen (PCNA), a DNA polymerase-associated protein, has also been used to evaluate proliferating cell number [24]. While these markers and others like them have the advantage of being endogenous, they only allow for the assessment of proliferation and, within that, only the subset of cells that are within S phase at the very moment of sacrifice are marked.

Retroviral labeling of new cells

Newly born cells can also be detected through retroviral infection. Stereotaxically injected retrovirus preferentially infects cells undergoing division and if the virus carries a reporter gene such as GFP, it can be used to visualize mitotically active cells at the time of infection. This approach has been most powerful when used to investigate the electrophysiological and morphological properties of newly born cells [16]. While staining for BrdU or other cell cycle markers requires processing that destroys neuronal function, retroviral labeling with direct visualization of a fluorescent reporter protein allows for the targeting of newly born cells in tissue slice preparations for electrophysiology. Unfortunately, retroviral infection is somewhat sporadic and unpredictable, making this method inappropriate for quantification of neurogenesis.

Determining cell fate

The most common method of demonstrating the fate of new cells is by combining staining for BrdU with staining for lineage-specific proteins. Markers such as doublecortin (DCX), neuron-specific class III β-tubulin (TUJ1), polysialylated-neuronal cell adhesion molecule (PSA-NCAM) and turned on after division (TOAD-64) are commonly used as markers of immature neurons [18, 25-27]. To mark mature neurons, the most common proteins used are neuronal specific nuclear protein (NeuN), neuron specific enolase (NSE) and microtubule-associated protein (MAP-2) [18, 28, 29].

**Stress-induced alteration in cell proliferation**

The effects of stress on cell proliferation can be divided into those due to acute stress and those due to repeated, chronic stress. This division is based on experimental evidence that stress has different effects on cell division depending on whether exposure is short-term or long-term (see Table 5.1).

Acute stress

Acute stress has been shown to inhibit cell proliferation in the adult SGZ in a number of species, including rat [30-32], mouse [32], tree shrew [4] and marmoset monkeys [33] and using a number of different stressors, including brief restraint [32], exposure to fox odor [30] and subordination stress [4, 33]. This acute stress-induced suppression of proliferation appears to be driven by exposure to high levels of glucocorticoids (GCs)—while acute stress effects on SGZ proliferation are blocked by removing GCs through adrenalectomy [30], acute corticosterone injections decrease cell proliferation in the SGZ much as acute stress does [1, 33]. Moreover, we have shown that treating neural precursor cells with GCs in vitro leads to a dose-dependent reduction in proliferation [34]. By contrast, cell proliferation in the SVZ does not appear to be sensitive to stress [35, 36], suggesting that the differences in BrdU labeling found in the SGZ following acute stress/GC exposure reflect true proliferation differences, not just changes in systemic label bioavailability.

The acute stress-induced suppression of adult hippocampal proliferation rate is relatively short lived, dissipating within 24 hours in rodents [31, 37]. The reduction in new cell number due to this brief suppression of proliferation rate remains detectable for up to one week [30, 35]. By three weeks, however, the number of surviving BrdU positive cells is similar in stressed animals and controls [30], implying either a floor effect in number of surviving cells over the long-term, or an enhanced resistance of cells born during acute stress exposure to later cell death.

It should be noted that a few studies have not found an acute stress effect on adult SGZ proliferation [38-41]. Some of these studies can be explained by the timing of the BrdU injection—by administering BrdU before the stressor, labeled cells were those dividing immediately before stress began. This timing makes the BrdU+ population a more appropriate measure of short-term survival than proliferation [39, 40]. It is also possible that control animals experienced some environmental stressor that the investigators were unaware of or that the stressor used was not severe enough (i.e. did not lead to a great enough rise in corticosterone) to affect proliferating cells.

The majority of work on acute stress and adult SGZ cell proliferation has used male subjects. However, basal rates of adult neurogenesis differ in male and female rodents [42], suggesting that neurogenic stress response may also vary by sex. Indeed, it has been reported that while acute fox odor exposure suppresses cell proliferation in the SGZ of male rats, it does not do so in females [43]. Given that administration of estradiol to female rats increases SGZ cell proliferation [42], high circulating estrogen levels in female animals may be neuroprotective for

mitotic cells, preventing GC-induced suppression of cell division. More research is needed to probe this potential sex difference in proliferative response to stress and what mechanism(s) might underlie it.

Chronic stress

As with single, acute stress exposure, repeated exposure to a variety of stressors (social defeat [19, 44], 2-3 weeks of daily restraint [38, 41] or 3 weeks of daily exposure to rotating stressors such as cold swim, shock, etc [31, 37, 45]) has also been shown to suppress cell proliferation in the adult SGZ of many species, including rats [19, 41], mice [46] and tree shrews [44]. Like acute stress, the effect of chronic stress on dividing cells also appears to be driven by GC exposure [47]. Importantly, the effectiveness of chronic stress at reducing proliferation suggests that proliferating cells are not capable of a great degree of adaptation to high circulating glucocorticoids over time.

The effect of chronic stress is more enduring than that of acute stress. While the suppressive effects of acute stress and GC treatment seem to be alleviated within 24 hours, 24 hours following the end of chronic stress, the proliferative capacity of the SGZ is still diminished [37, 38, 41]. Indeed, chronic stress-induced suppression of SGZ proliferation may extend up to three weeks after the end of the stressor [31, 37]. Importantly, at these later time points following stress, circulating GCs have returned to normal levels [48], indicating that prolonged exposure to high levels of GCs leads to a change in the proliferating pool of cells that persists even when hormone levels have returned to baseline.

Research on sex differences in the effect of chronic stress on SGZ proliferation is more limited than with acute stress. One study has found that females are resistant to chronic stress effects of cell proliferation/survival [49]. However, this effect was dependent on the social environment and the BrdU injection regimen used in that study made disentanglement of proliferation and survival effects difficult. More research is needed to determine how chronic stress effects female SGZ cell proliferation.

Chronic stress may also suppress proliferation in the adult SVZ. Using BrdU labeling and in vitro colony forming assays, Hitoshi et al. [50] recently showed that the number of rapidly dividing progenitor cells in the adult mouse SVZ is decreased by three weeks, but not one week, of daily exposure to forced cold swim. Moreover, in vitro, they showed that corticosterone treatment can suppress proliferation of SVZ neural precursors, suggesting that the stress effect is driven by GCs. They also showed that the chronic-stress induced suppression of proliferation was still present after three weeks recovery, much as is the case with chronic stress effects in the SGZ. More research is needed to expand upon these initial findings, but it seems that the SVZ may be sensitive to GCs when exposure is prolonged.

Cell cycle arrest versus progenitor death

There are two main possibilities for how stress, acute or chronic, might impact the number of newly born cells in the adult dentate gyrus: GCs could cause a slowing of the cell cycle of progenitors/stem cells or they could eliminate progenitor/stem cells by causing them to exit the cell cycle permanently. The rapid time course of onset of stress effects on proliferation and the speed of recovery of proliferative ability following acute stress both suggest that stress acts through a slowing of the cell cycle rather than by causing a reduction in the progenitor pool. In support of this hypothesis, one study using adult hippocampal neural progenitors in vitro showed that 24 hours of treatment with a synthetic GC receptor (GR) agonist, dexamethasone (DEX), led not only to a decrease in new cell production, but also to an upregulation in p21, a cdk-inhibitor that is associated with cell cycle arrest in G1 [47]. Upregulation of p21 and

accumulation of cells in G1 was also found when HT-22 cells, a neuronal mouse cell line, were treated with DEX [51]. Moreover, in vivo, chronic stress has been shown to cause upregulation of p27Kip1, another cdk-inhibitor associated with cell cycle arrest at the G1/S checkpoint [52]. All together, these studies suggest that GCs somehow block the transition from G1 to S phase.

**Stress-induced alteration of new cell survival**

Approximately half of newly born cells in the adult dentate gyrus die within the first few weeks after birth [12, 13]. This pruning of new cells, while dramatic, is also highly sensitive to environmental input [26, 53]. Though less research has been done on the effects of stress and GCs on new cell survival than on proliferation, stress-related suppression of new cell survival has been well demonstrated (see Table 5.2).

Daily stress in the form of social defeat [19] or variable stressors such as cold exposure, shock, overcrowding, etc. [54, 55] have both been shown to decrease the number of surviving cells from those born prior to stress exposure in the adult dentate gyrus (though see [37, 41]). As with stress effects on proliferation, stress-induced suppression of cell survival appears to be mediated by GC secretion; while adrenalectomy leads to an increase in cell survival rates that is eliminated by low level corticosterone replacement, injections of high levels of corticosterone suppresses new cell survival [20]. However, the sensitivity of newly born dentate cells to GCs appears to be somewhat temporally limited; in rats, administration of corticosterone after cells are 18 days old does not lead to changes in number of surviving BrdU positive cells [20]. More work is needed to determine whether these findings extend to females and/or cell survival in the SVZ/olfactory bulb.

**Stress-induced alteration of cell fate choice**

After proliferation and survival, selection of neuronal fate is the final phase that characterizes neurogenesis. Under normal circumstances, about 70-90% of newly born, surviving cells in the dentate gyrus express neuronal fate markers [12, 14]. Whether stress exposure during differentiation can alter this cell fate choice is currently unclear (see Table 5.3). Heine et al. [37], for example, found that injecting BrdU before three weeks of daily restraint stress did not lead to differences in the percentage of labeled cells that express NeuN. Several others have similarly found no difference in neuronal marker co-labeling with BrdU [19, 55], including one study that used in vitro hippocampal precursors allowed to differentiate in the presence of glucocorticoids [16]. In contrast to these studies, however, one detailed study by Wong and Herbert [18] does demonstrate a consistent inhibitory effect of GCs on neuronal fate choice. By injecting male rats with high levels of corticosterone over different time periods after a BrdU injection, they found that the percentage of new, BrdU-labeled cells expressing neuronal fate markers (DCX and NeuN) was decreased by corticosterone treatment over any seven day period during the first month. More recently, we have confirmed and extended these findings, showing both in vivo and in vitro that stress/GCs drive newly proliferative dentate cells away from neuronal fate towards an oligodendrocyte fate [34]. Though more work is needed to confirm these findings given the contrary past studies, they nevertheless suggest that neuronal fate choice can be influenced by GCs [20].

**Mechanism of stress-induced changes in adult neurogenesis**

Though it seems clear that the effect of stress on adult neurogenesis is mediated primarily by stress hormones (e.g. GCs), there are many competing potential molecular mechanisms for

how the GCs might alter precursor division and new cell survival/differentiation. Overall, the extra and intra-cellular cascades leading from increased GCs to inhibition of cell proliferation, survival and/or neuronal differentiation are not well characterized. It is not even certain whether GCs act directly on new cells, through neighboring cells in the niche, or both.

Direct effects of glucocorticoids on adult neurogenesis

When stress effects on adult hippocampal cell proliferation were first reported, work using labeling for GR combined with [$^3$H]dT autoradiography seemed to indicate that proliferating cells lacked receptors for GCs [56], meaning that GC effects on proliferation must occur through some other intermediary molecule/cell. More recently, however, evidence has emerged to suggest that a portion of recently proliferative cells do express GR [57]. Moreover, we show that GCs suppress proliferation in vitro in a GR-dependent manner, suggesting that the GR in neural precursors is not only present but also functionally active and relevant [47]. Nonetheless, other mechanisms might contribute, as well, to account for the full effect of stress on proliferation in vivo (see *Indirect Effects* below).

GCs may also act directly on newly born cells to influence survival and/or differentiation. In support of this mechanism, the percent of cells expressing GR in vivo is reported to increase to over 50% by 7 days after BrdU injection and to almost 100% by 4 weeks of age [57]. Therefore, during the time window in which newly born cells are sensitive to GC-induced suppression of survival and neuronal differentiation [18, 20], these cells have the molecular machinery necessary to respond directly to GCs. More research is necessary to determine if GCs do act directly on maturing cells in the adult hippocampus in vivo and, if so, what intracellular cascade(s) are involved.

Indirect effects of glucocorticoids on adult neurogenesis

Though some proliferating cells do express GR [57], work on contributing factors such as excitatory amino acids, serotonin and growth factors indicate a role for indirect mechanisms, as well.

Excitatory amino acids

In the adult hippocampus, levels of excitatory amino acids such a glutamate are known to rise prominently during stress [58], making EAAs one probable contributor to stress-induced suppression of adult hippocampal cell proliferation. Indeed, much as stress inhibits cell proliferation and ADX enhances it, administration of NMDA, a stimulator of glutamate receptors, leads to decreased BrdU labeling while blockade of the NMDA receptor leads to increased BrdU labeling [4, 28, 33]. More directly, a study by Cameron et al. [33] showed that systemic administration of an NMDA receptor antagonist, MK-801, before acute corticosterone injection blocked the normal corticosterone-associated suppression of [$^3$H]dT labeling. The NMDA-dependency of stress effects on cell proliferation likely extends to chronic stress effects as well—one study in male mice found that daily injection of agmatine, an NMDA-R antagonist, blocked the effect of 24 days of variable stress on hippocampal cell proliferation [46]. These studies strongly suggest that stress effects on proliferating cells are mediated by increases in the EAA glutamate and its actions on the NMDA receptor. Because only a small portion of dividing cells in the SGZ show NMDA-R expression [41], NMDA-R activation probably affects cell division in the adult hippocampus via its effects on the mature neuronal population. In addition, little work has been done on whether NMDA-receptor dependency of stress effects on adult neurogenesis extends beyond proliferation to effects on cell survival and differentiation.

Serotonin

A number of studies have shown that adult hippocampal neurogenesis is influenced by serotonergic input. In normal adult rodents, chronic treatment with selective serotonin uptake inhibitors such as fluoxetine lead to upregulation of SGZ proliferation [36, 59] while serotonin depletion leads to suppression of cell division in the adult rodent hippocampus [60]. The effect of serotonin seems to be limited to division of early progenitors, with little effect on cell survival or differentiation [36, 59]. In addition to regulating basal levels of cell proliferation, serotonin may play a role in the effects of GCs on cell proliferation. Twenty days of chronic, variable stress has been shown to result in lower $5HT_{1A}$ mRNA levels in the DG [61]. Moreover, acute stress-induced reductions in SGZ and SVZ cell proliferation have been shown to be reversed or blocked by treatment with SSRIs [36, 50, 62].

Growth factors

A number of growth factors are known to influence neurogenesis in the adult DG, including BDNF, FGF-2, EGF, IGF and TGF-α (for review see [14]). Research on manipulations that increase GC levels yet do not impair hippocampal neurogenesis have suggested that two of these factors may be involved in GC effects on neurogenesis: BDNF and FGF-2.

Voluntary exercise, enriched environment, and cognitive tasks all have been shown to lead to elevations in circulating GCs [63-66] and yet, paradoxically, to increases in hippocampal neurogenesis, primarily through increased cell survival [8, 26, 67, 68]. Interestingly, these manipulations also all lead to elevations of hippocampal BDNF [69-71]. Given that BDNF has been shown to promote cell survival of adult rodent progenitor cells [16], it seems likely that BDNF may be acting to block the effects of high GCs caused by exercise, enrichment and/or learning. FGF-2 may also influence stress effects on adult neurogenesis. Experiments using controllable and uncontrollable shock have shown that controllability is associated with both an increase in hippocampal FGF-2 and a block of stress-induced suppression of cell proliferation, despite normal elevations in plasma corticosterone [72, 73]. More research is needed to clarify the role of these and other growth factors in stress-related regulation of adult neurogenesis.

Intracellular mechanisms of glucocorticoids effects

Regardless of whether GCs act directly on NPCs or through other intermediary cells, there is still the question of what intracellular cascades are the final mediators of the NPC response to stress. The Akt/PI3K and MAPK/ERK pathways are both good candidate intracellular mediators of GC effects because both of these pathways play key roles in regulating NPC proliferation and in signaling from several extracellular factors that can influence GC effects on NPC proliferation (FGF-2, BDNF, IGF, etc.) [74-78]. Activation of Akt appears to be essential for FGF-2-mediated stimulation of NPC proliferation [74], suggesting that this pathway is an essential regulator of NPC mitosis. The MAPK/ERK pathway also regulates NPC proliferation and has been implicated in control of NPC proliferation in response to a number of stimuli such as hypoxia [76] and seizures [75]. GR activation may interact with either or both of these cascades to influence NPC proliferation but more work is needed to determine how/whether this occurs.

An overall picture of mechanism: putting the pieces together

How do all the parts of the stress effect on neurogenesis fit together? Given the direct effects of GCs on in vitro neuronal precursors, it seems clear that GCs could have some direct effect on cell proliferation. However, in vivo, the indirect mechanisms probably contribute, as well. Therefore, it is likely that those precursors that do express GR respond directly to elevated GCs by initiating genetic programs that slow the cell cycle, upregulating cell cycle inhibitors such as p21 and p27Kip1, among others. Simultaneously, in precursors without GR, and perhaps

also in some precursors with GR, elevated GCs may act through glutamatergic transmission, serotonergic transmission from the DRN and/or growth factors. The downstream cascades the lead from these extracellular signals to slowing of the cell cycle are not well characterized but could rely on changes in signaling cascades known to regulate stem cell division such as the Akt/PI3K and MAPK/ERK pathways. How stress effects new cell survival and differentiation is less well studied than the mechanism of proliferation effects. Given the higher percentage of cells expressing GR over time, though, it seems possible that this mechanism could rely more heavily on GR activation than proliferative effects do. However, BDNF may also play a large role, particularly in cell survival, as discussed above.

**Function of regulation of adult neurogenesis by stress and glucocorticoids**

The question of whether changes in adult hippocampal neurogenesis due to stress have functional or behavioral implications is largely unanswered. Indeed, the exact function of adult hippocampal neurogenesis is itself somewhat unsettled. Given that the hippocampus is important for many aspects of learning and memory [79] and that adult neurogenesis is enhanced by hippocampal-dependent [26, 53] but not by hippocampal-independent-tasks [80], some have hypothesized that, though small in absolute numbers, these newly born neurons have disproportionate influence on hippocampal function. In support of this hypothesis, electrophysiological studies have shown that newly born neurons are more excitable than mature granule neurons, having lower thresholds for LTP and greater response amplitudes than older neurons [81-83]. More direct evidence for the functional importance of adult hippocampal neurogenesis comes from studies where cell mitosis is reduced or eliminated experimentally. The DNA methylating agent, methylazomethanol acetate (MAM), inhibits mitosis and, when administered systemically, has been shown to impair memory in a trace conditioning task [84] and to block enrichment-induced improvement in object recognition memory [27]. Irradiation, a more localized method of inhibiting cell mitosis [85], has also been shown to cause hippocampal memory deficits [86]. A number of computational approaches have suggested essential roles for new neurons in hippocampal function despite their limited quantities, as well [87, 88]. In summary, while there are some contradictory findings, it seems at least possible that adult neurogenesis may contribute to hippocampal cognitive function in a behaviorally relevant manner.

Even if adult neurogenesis does play an important role in hippocampal-dependent cognition, whether stress-induced suppression of neurogenesis has any functional consequences is still unclear. Stress certainly does alter hippocampal function at the behavioral level, leading to impaired hippocampal-dependent learning and memory performance in a variety of tasks [89-91]. However, stress influences many aspects of hippocampal physiology and morphology other than adult neurogenesis (such as LTP [92] and CA3 dendritic branching [93]), making attribution of any single stress effect to neurogenic changes difficult. The change in the number of new neurons due to an acute episode of stress or GC exposure is transient (see cell proliferation and survival sections), suggesting that effects of acute stress on hippocampal function are likely minimal. Changes due to chronic stress, however, are longer-lasting and may have more relevance due to prolonged inhibition of proliferation combined with reduction of cell survival and inhibition of neuronal fate choice. Whether the scale of change observed is enough to have functional or behavioral relevance in vivo has not been well investigated. The finding that enrichment-induced gains in neurogenesis may be functionally relevant [27] is encouraging, but that finding has been questioned more recently [94]. Though there is some evidence pointing to a possible role of adult neurogenesis in normal hippocampal function, more research is needed to determine if stress-

induced loss of new neurons contributes to any of the known effects of stress on hippocampal function.

**Conclusion**

While there is a great deal of evidence pointing to a role for stress in regulation of adult neurogenesis, many questions remain unanswered. Exposure to high levels of GCs whether through stress or exogenous administration, inhibits cell proliferation, survival and possibly neuronal differentiation. The inhibition of proliferation is by far the best studied phenomenon. It appears to rely on a slowing of the cell cycle, but the molecular mechanism is largely unclear. Excitatory amino acids, serotonin and growth factors all seem to play a role but which of these factors acts directly on proliferative cells and what intracellular cascades trigger the slowing of progression through the cell cycle is uncertain. Less is known about stress effects on cell survival and differentiation, but the greater presence of GR as cells mature suggests that direct actions of GCs could play more of a role here than they could in proliferation. Finally, the functional consequences of stress-associated regulation of adult neurogenesis are largely undetermined. This will perhaps become clearer, though, as more work is done to determine the functional meaning of adult neurogenesis.

Future work on stress and adult neurogenesis should focus on better utilizing in vitro models of adult neurogenesis to clarify the cellular mechanism of stress-related suppression of neurogenesis. While the many in vivo studies investigating adult hippocampal neurogenesis have provided solid evidence for the existence of a stress effect and have given some hints at mechanism, by using neuronal progenitors derived from the adult brain, more detailed and controlled investigation can be done on what molecular cascades are activated by GCs, how other compounds such as EAAs and serotonin contribute to GC effects and what intracellular cascades and genetic programs are activated by exposure to stress hormones. The results of such investigations would provide not only a better understanding of stress-response biology, but would also contribute to a greater understanding of the regulation of plasticity in the adult brain.

## B. Stress and sexual reproduction

The subjective experience of stress leads to reproductive dysfunction in many species [95, 96] and may significantly influence the efficacy of assisted reproductive procedures in humans [97]. Stress effects on reproduction rely heavily on multi-level interactions between the hormonal stress response system, the hypothalamic-pituitary-adrenal (HPA) axis, and the hormonal reproductive system, the hypothalamic-pituitary-gondal (HPG) axis [95, 98].

Stress alters reproductive function at all levels of the HPG axis[95]. Centrally, activation of the HPA axis by stress leads to suppression of GnRH secretion through both elevations in hypothalamic corticotrophin-releasing hormone (CRH) and plasma glucocorticoids (GC)[99, 100]. Recently, a novel hypothalamic negative regulator of the HPG axis, gonadotropin-inhibitory hormone (GnIH), was discovered in the quail [101], along with homologous neuropeptides in rodents and primates[102, 103].

As a hypothalamic RFamide peptide that inhibits gonadotropin secretion[101], the presence of GnIH in the hypothalamus suggests another level of central HPG control that could be regulated by stress. The mammalian homologue of avian GnIH (RFRP) is expressed in neurons within the dorsomedial nucleus of the hypothalamus (DMH) with fibers extending both to the median eminence and in close proximity to GnRH neurons within the hypothalamus[102, 104]. RFRP

has been shown to suppress gonadotropin release and sex behavior in adult male rats as it does in avian species[104]. In birds and mammals, RFRP plays a functional role in suppression of HPG function [103-105]. RFRP is also altered by stress in breeding house sparrows [106], making this recently-identified neuropeptide [103] a likely key integrator of stress effects upon reproduction (Figure 1.1).

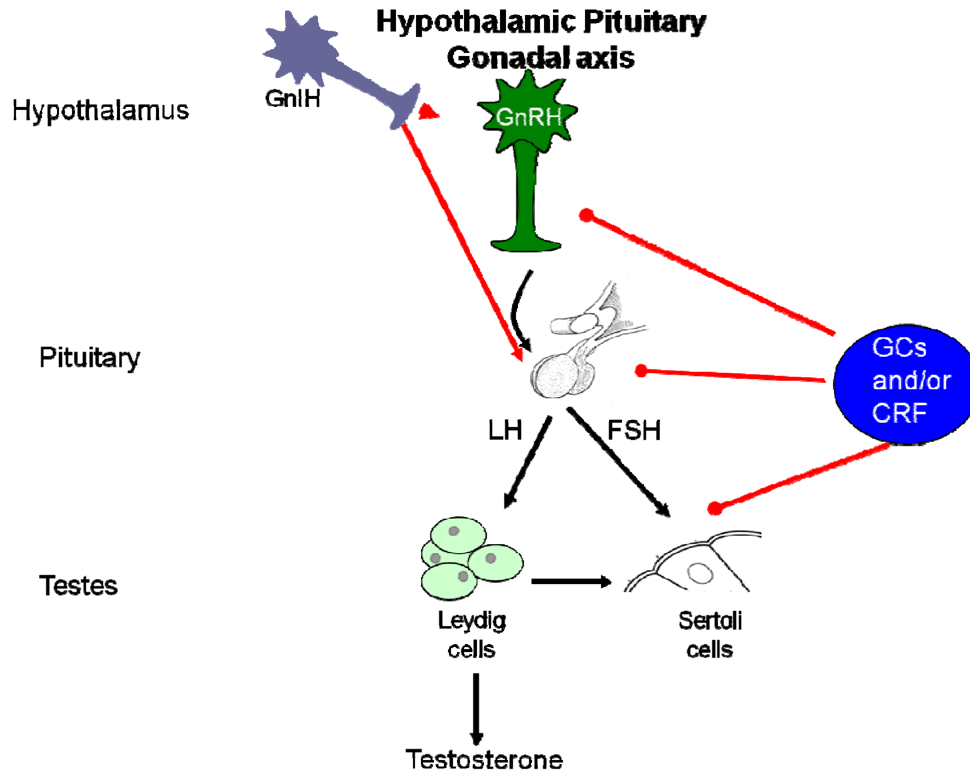



Figure 1.1: Hypothalamic pituitary gonadal axis. Glucocorticoids (GCs) and corticotrophin releasing factor (CRF) inhibit the HPG axis at all levels. GnIH also inhibits the HPG axis at the hypothalamic and pituitary levels (effects in the testes are also possible but less well investigated). GnRH = gonadotropin inhibitory hormone; LH = luteinizing hormone; FSH = follicle stimulating hormone.

# Chapter 2: Basolateral amygdala regulation of adult hippocampal neurogenesis and fear-related activation of newborn neurons.

From: Kirby, E.D., Friedman, A.R., Covarrubias, D., Ying, C., Grant, W.G., Goosens, K.A., Sapolsky, R.M., & Kaufer, D. (2011). Basolateral amygdala regulation of adult hippocampal neurogenesis and fear-related activation of newborn neurons. Molecular Psychiatry, In Press.

## A. Introduction

Emotion strongly modulates memory function in adult mammals, altering the strength and longevity of memories and sometimes leading to memory dysfunction. Notably, disordered emotional memory contributes to several affective disorders, including depression, anxiety and post-traumatic stress disorder. Emotion-associated modulation of memory appears to rely largely on interaction between the memory processes of the hippocampus and emotional input provided by the basolateral complex of the amygdala (BLA)[107-109]. Behaviorally, BLA activity supports memory for emotionally salient stimuli in rodents and humans[107, 110, 111] while at the neurophysiological level, BLA activity supports hippocampal long-term potentiation (LTP)[112-115].

Recent studies suggest a role for adult hippocampal neurogenesis in emotional memory function[57, 116-119]. In adult mammals, new hippocampal neurons arise from a resident population of neural stem cells located in the dentate gyrus[11, 120-122] and form a population of immature neurons that incorporate into existing networks within weeks of birth[16]. Ablation of adult neurogenesis by genetic knockout or irradiation impairs contextual fear memory[57, 116] and may also modulate the transfer of fear-related memories from the hippocampus to other neural structures for long-term storage[117]. The heightened plasticity of newly born neurons appears to be key, as acceleration of their maturation impairs fear learning[123]. The requirement of new neurons for fear memory suggests a clinical role for adult neurogenesis in several affective disorders[109, 124, 125]. However, the mechanisms by which adult neurogenesis responds to emotional stimuli to influence memory formation are not yet clear.

We investigated the adult neurogenic response to emotional input from the BLA and its potential role in fear memory. We predicted that BLA activity would promote adult neurogenesis and modulate activation of immature neurons in response to fear-associated context.

## B. Results

Bilateral BLA lesions suppressed hippocampal neurogenesis. Adult hippocampal neurogenesis is a multi-phase process regulated by the proliferation, differentiation, migration, and survival of new cells[122]. To assess the effect of loss of BLA input on the neurogenic process, we excitotoxically lesioned the BLA of adult male rats bilaterally (Figure 2.1)[126] and investigated cell proliferation as well as differentiation (Figure 2.2a). BLA lesion reduced the number of proliferation cell nuclear antigen (PCNA) positive cells by 58.2% compared to rats who received bilateral sham surgery (Figure 2.2b). BLA-lesion also led to a 62.5% reduction in the number of BrdU positive dentate gyrus cells 5-10 days after proliferative cells were labeled by BrdU injection (Figure 2.2c). These results indicate suppression of cell proliferation following BLA lesion, resulting in a persistent reduction in immature cells. BLA lesion did not affect cell fate, with approximately 85-90% of new cells expressing the neural marker doublecortin (DCX) and

less than 5% expressing the astrocytic marker S100β or the oligodendrocyte marker myelin basic protein (MBP) regardless of lesion (Figure 2.2d-e). These data indicate that BLA lesions cause a reduction in the pool of immature neurons and glia three weeks after lesion.

Unilateral BLA lesions suppressed hippocampal neurogenesis. We next investigated whether the suppression of adult hippocampal neurogenesis following BLA lesion is mediated by ipsilateral neural connections or by possible systemic changes (such as a change in circulating hormone levels). Because ipsilateral connections mediate the influence of BLA activity on hippocampal LTP[115, 127], we predicted that BLA lesion-induced suppression of adult hippocampal neurogenesis would similarly rely on ipsilateral neural connections and be hemisphere-specific. To investigate this hypothesis, BRDU and PCNA positive cells were quantified three weeks following unilateral BLA lesion (Figure 2.3a).

Unilateral BLA lesion suppressed the number of BrdU-labeled proliferative hippocampal cells by 49.6% ipsilateral to the lesion compared to contralateral (Figure 2.3b). Furthermore, we found that the number of proliferative PCNA positive cells was suppressed by 32.2% ipsilateral to BLA lesion and that proliferation levels contralateral to the BLA lesion were similar to those found in bilateral sham-operated rats and rats who received no surgery (Figure 2.3c). Together, these results imply that BLA-associated regulation of neurogenesis is hemisphere-specific, most likely suppressing cell proliferation through ipsilateral neural connections.

CeA lesions did not suppress hippocampal neurogenesis. We next investigated the anatomical specificity of BLA lesion-induced suppression of hippocampal neurogenesis. Previous studies show that while BLA lesions reduce hippocampal LTP, lesions of the central nucleus of the amygdala (CeA) do not[127]. We therefore predicted that CeA lesions would also have no effect adult hippocampal neurogenesis.

Three weeks after surgery to lesion either the BLA or the CeA (Figure 1 and Figure 2.3a), we found that while there were 26.8% fewer BrdU positive cells ipsilateral to BLA lesion compared to contralateral, there was no difference in the number of proliferative BrdU positive cells ipsilateral versus contralateral to CeA lesion (Figure 2.3e). We found a similar suppression in the number of proliferative PCNA positive cells in the dentate gyrus ipsilateral to BLA, but not CeA, lesion (Figure 2.3f). These results indicate that BLA lesions ipsilaterally suppress hippocampal neurogenesis while CeA lesions do not, suggesting that the BLA influence over hippocampal neurogenesis is specific to loss of BLA input, and does not result non-specifically from ipsilateral excitotoxic cell death or surgical damage *per se*.

Viral vector-mediated reduction of BLA activity suppressed adult hippocampal neurogenesis. We next determined whether suppression of neural activity in the BLA is sufficient to modulate neurogenesis. To suppress BLA neural activity, we ectopically expressed the outwardly rectifying potassium channel Kv1.1 or a GFP-only control from a herpes viral vector in BLA neurons. This transgene construct has been shown to reduce basal BLA neural firing[128, 129]. GFP-Kv1.1 overexpression reduced proliferative BrdU positive cell number by 36.5% and 30.5% (Figure 2.4) as compared to GFP-only viral vector infusion and sham-operated controls, respectively. These results indicate that temporary BLA basal inactivation is sufficient to suppress hippocampal neurogenesis.

Fear memory activates newly born neurons. To investigate the functional importance of BLA regulation of adult hippocampal neurogenesis, we examined how new neurons participate in BLA-dependent fear memory. Previous studies show that ablation of adult hippocampal neurogenesis causes impairments in BLA-dependent fear conditioning[57, 116, 117], suggesting a functional role for new neurons in fear-associated memory networks. Previous work also shows that new neurons may be particularly prone to integration into memory networks as they are preferentially activated by exposure to previously experienced contexts such as an enriched environment, a water maze task and fear conditioning[130-132].

To test the activation of newborn neurons by fear memory, we exposed sham-operated rats to a contextual fear conditioning task, which is dependent on both BLA and hippocampal activity[109, 110, 126], and then assessed expression of the immediate early gene (IEG) cFos in newly born neurons. cFos expression reflects neuronal activation and incorporation into hippocampal memory circuits[123, 130, 131]. cFos expression is also critical to hippocampal mediation of contextual fear conditioning specifically[133, 134].

Two weeks after labeling proliferative cells with BrdU, rats were exposed to a series of 10 unpredictable shocks in a conditioning chamber (Figure 2.5a). Re-exposure to the shock-associated context (the fear context) the next day led to greater freezing compared to exposure to a novel context (Figure 2.5b), indicating robust memory for the fear-associated environment. Forty-five minutes after re-exposure, the rats were perfused and assessed for BrdU co-labeling with cFos. BrdU positive cells at this point were 15-19 days old, an age characterized by preferential recruitment into memory networks and possibly enhanced importance for hippocampal memory function[118, 130]. Using DCX as a marker of immature neurons, we found that exposure to the fear context increased the proportion of BrdU cells co-expressing DCX and cFos compared to rats exposed to the novel context (Figure 2.5c and d). Exposure to fear context did not alter the total number of cFos-positive cells in the dentate gyrus (Figure 2.5e). These data suggest that new neurons are activated by fear-associated memory but not simply by exposure to a novel environment.

BLA lesions prevented activation of new neurons by fear memory. We next investigated whether BLA lesion could influence the activation of new neurons by fear memory. Unilaterally BLA lesioned rats showed greater freezing in the fear context than in the novel context, indicating robust fear memory despite the presence of lesion, as expected in the presence of one functional BLA (Figure 2.5f). We found that BLA lesion blocked the fear-context associated increase in BrdU/cFos/DCX co-expression ipsilaterally (Figure 2.5g). In fear context exposed rats, there were more cFos/DCX+ BrdU cells contralateral versus ipsilateral to lesion. The proportion of triple labeled cells contralateral to lesion did not differ from that in sham-operated rats. In rats exposed to the novel context, by contrast, BLA lesion did not affect the proportion of cFos/DCX-labeled BrdU cells. These results suggest that BLA lesion blocks the selective activation of two week old immature neurons by a context associated with an aversive stimulus.

We also found that there were significantly fewer BrdU/DCX positive cFos cells ipsilateral to the BLA lesion compared to contralateral in rats exposed to the fear but not the novel context (Supplementary Figure 2.1). These results suggest that, of cells activated by exposure to a fear context (i.e. expressing cFos), the percentage representing immature neurons is reduced by ipsilateral BLA lesion. The overall percentage of BrdU cells expressing DCX was not affected by lesion (Supplementary Figure 2.2).

## C. Discussion

We demonstrate for the first time that BLA activity regulates adult neurogenesis and potentially modifies the recruitment of new neurons into networks underlying emotional memory. Bilateral and unilateral BLA lesions suppressed hippocampal neurogenesis, suggesting that ipsilateral BLA input supports basal hippocampal neurogenesis. These results extend previous studies showing that BLA lesion or inactivation suppresses dentate gyrus LTP ipsilaterally[127], further suggesting a supporting role for the BLA in hippocampal function.

CeA lesions did not suppress hippocampal neurogenesis, suggesting that reduction in hippocampal neurogenesis is not attributable to generalized ipsilateral amygdala damage, and is a specific response to loss of BLA activity. Previous work on dentate gyrus-perforant path LTP similarly shows no effect of CeA lesions on LTP[127]. The dependence of hippocampal plasticity on BLA but not CeA activity is consistent with the behavioral function of the BLA, which is required for hippocampal-dependent contextual fear conditioning. The CeA, by contrast, participates in hippocampus-independent behaviors such as cued fear conditioning[111]. Together with previous studies, our results suggest a general model wherein BLA input promotes multiple forms of hippocampal plasticity, thereby supporting hippocampal memory.

We further demonstrated regulation of hippocampal neurogenesis by BLA activity using a viral vector-mediated overexpression of the voltage gated outwardly-rectifying potassium channel, Kv1.1. The transgene for Kv1.1 was packaged into a herpes simplex viral (HSV) vector, allowing for preferential infection of neurons[128]. In electrophysiological studies, Kv1.1 overexpression in the BLA via HSV vector causes reduced neural resting potential and decreases basal firing rates[128, 135]. We found that overexpression of Kv1.1 in the BLA ipsilaterally suppressed hippocampal neurogenesis, suggesting that baseline neural activity in the BLA supports hippocampal neurogenesis.

The BLA sends the majority of its ipsilateral input to the hippocampus through two indirect pathways, relayed through either the medial septum or the entorhinal cortex[115, 136]. Both of these pathways have been implicated in BLA support of adult hippocampal LTP and provide excitatory input to the dentate gyrus[115, 137]. While the medial septum provides input through cholinergic projections to the dentate gyrus granule neurons, the entorhinal cortex primarily sends glutamatergic projections[115, 137]. Importantly, both acetylcholine and glutamate increase proliferation of adult neural precursor cells[138-140], suggesting that input from either pathway could mediate BLA support of adult neurogenesis in the dentate gyrus. In addition, induction of dentate gyrus LTP stimulates hippocampal neurogenesis itself[27, 141], raising the possibility that suppressed LTP following BLA lesion could in part underlie the suppression of neurogenesis. Future studies will address the extent to which these two ipsilateral pathways contribute to the support of hippocampal neurogenesis.

New neurons, particularly those around two weeks old, influence emotional memory function[57, 118]. New neurons are also context-sensitive, integrating strongly into memory networks for contexts initially experienced around two weeks of age[130]. This preferential integration may rely on the heightened plasticity of immature neurons compared to their older counterparts in the dentate gyrus[130]. Indeed, acceleration of maturation beyond this plastic phase can interfere with both integration of new neurons into non-fear related hippocampal memory networks as well as behavioral performance in hippocampus-dependent emotional memory tasks[123].

We investigated whether new neurons in this highly plastic phase of their development were integrated into fear memory circuits using a contextual fear conditioning task two weeks

after labeling newborn cells. Contextual fear conditioning relies on both the BLA and the hippocampus[109, 110, 126] and appears to be partially mediated by adult hippocampal neurogenesis[57, 142]. We chose cFos expression as a marker of neuronal activation due to its role in memory for a fear-associated context[133, 134]. We found that exposure to a fear-associated context activated two week old neurons more than exposure to a novel context, an effect that was blocked by ipsilateral BLA lesion. These results suggest that immature neurons are integrated into neural networks for fear-associated memory, but that this integration is dependent on BLA input. However, it is also possible that the new neuron activation could reflect participation in fear extinction. Future studies will further explore this possibility.

One recent study using mice suggests that while fear conditioning activates newly born neurons[132], this activation occurs in older neurons than those examined in our study (six weeks versus 2 weeks) and in a smaller proportion of new cells. A separate study further suggested that there was no cFos co-expression in two-week old neurons following exposure to a water maze task in mice[123]. Rather, IEG expression occurred only in new neurons 4-6 weeks old. Given that new neurons mature faster and potentially play a more influential role in hippocampal function in rats than mice[143], these differences in activation and integration of new neurons likely relate to species differences in how new neurons function and mature.

Our results demonstrate a novel mechanism for the influence of emotion over hippocampal memory function. By modifying adult hippocampal neurogenesis and altering the incorporation of new neurons into emotional memory networks, BLA input could shape how emotional stimuli influence memory. Whether these two impairments are the result of two independent processes or contingent one upon the other (such as through selective suppression of proliferation of cells that would later respond BLA input) is not clear and will require more research. To our knowledge, our results represent the first report of regulation of adult hippocampal neurogenesis and new neuron activation by BLA activity and fear-associated network activation. Future studies will further address the behavioral relevance of this phenomenon for emotional memory function as well as the underlying molecular mechanisms for emotion regulation of hippocampal proliferative capacity.

## D. Methods

**Animals**. Adult male Sprague-Dawley rats (Charles River) were pair-housed on a 12h light/dark cycle. All animal procedures were approved by the UC Berkeley and MIT Animal Care and Use Committees.

**Stereotaxic surgery**. Excitotoxic lesions of the BLA or CeA or sham surgeries were performed as per[126]. 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. Coordinates for CeA infusion were: -2.2 mm A/P and +/-4.4 mm M/L relative to bregma; -7.0 mm from dura (1 min). Six to 8 hrs after surgery all rats received an additional injection of buprenorphine (0.05 mg/kg, s.c.). For viral vector infusions, virus was infused 0.2 µl/min for 10 min (2 µl total) at the same BLA coordinates. Viral vectors were prepared as per[128] and titers were $10^6$-$10^8$ infectious particles/ml.

**Bromodeoxyuridine injections.** 5-Bromo-2'-deoxyuridine (BrdU, Sigma) was dissolved in physiological saline and injected intraperitoneally for all experiments.

**Immunohistochemical staining**. Rats were anesthetized with Euthasol euthanasia solution and transcardially perfused with ice cold 4% paraformaldehyde in 0.1M PBS. Brains were post-fixed for 24 hrs at 4ºC, equilibrated in 30% sucrose in 0.1M PBS and then stored at -80ºC. Immunostaining was performed on a 1 in 6 series of free-floating 30 µm cryostat sections.

For PCNA and BrdU staining, sections were rinsed in 0.1M Tris-buffered saline (TBS) and pretreated for 10min with 0.3% $H_2O_2$ in TBS. For BrdU only, sections were incubated in 2N HCl at 37ºC for 30 min. All sections were incubated in blocking solution (1% normal donkey solution, 0.3% triton-X 100 in TBS) for 30 min followed by overnight incubation at 4ºC on rotation in primary antibody (mouse anti-PCNA, 1:500 in blocking, Abcam, Cambridge, MA; mouse anti-BrdU, 1:500, BD Biosciences, San Jose, CA). Sections were rinsed and transferred to secondary antibody (biotin anti-mouse, 1:500, Jackson ImmunoResearch, West Grove, PA) for 2 hrs at room temperature. Following rinsing, sections were incubated in ABC reagent (Vector, Burlingame, CA) and then developed with DAB (Vector). Sections were mounted on gelatin-coated slides, dehydrated in alcohol and coverslipped with permount mounting medium.

Triple immunohistochemistry was performed similarly. Primary antibodies were: goat anti-DCX (1:200, Santa Cruz Biotechnology), mouse anti-S100β (1:200, Abcam), rat anti-MBP (1:100, Abcam), rabbit anti-cfos (1:100; Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies were: Cy5 anti-goat, Cy3 anti-mouse, Cy3 anti-rat, biotin anti-rabbit (1:500; Jackson ImmunoResearch). For the cFos/BrdU/DCX triple stain, a tertiary incubation for 1 hr at room temperature was included with streptavidin-Alexa488 (1:1000 in TBS, Invitrogen, Carlsbad, CA) after secondary incubation. All sections were then incubated in 4% paraformaldehyde for 10 min, rinsed and incubated in primary antibody against BrdU as above (1:500, rat anti-BrdU, Abcam; mouse anti-BrdU, BD Biosciences). The next day, sections were rinsed and incubated in secondary antibody: FITC anti-rat; Cy3 anti-rat; biotin anti-rat (1:500, Jackson ImmunoResearch). For MBP/BrdU staining, a tertiary incubation for 1 hr at room temperature was added with streptavidin-Alexa488 (1:1000 in TBS, Invitrogen). Sections were mounted on gelatin-coated slides and coverslipped with DABCO anti-fading medium.

**BrdU, PCNA and cFos quantification**. BrdU, PCNA or cFos positive cells were counted in the dentate gyrus and subgranular zone using a 40x air objective (Zeiss). The area sampled was calculated using StereoInvestigator software (MicroBrightfield, Williston, VT) and used to calculate the number of positive cells per $m^2$.

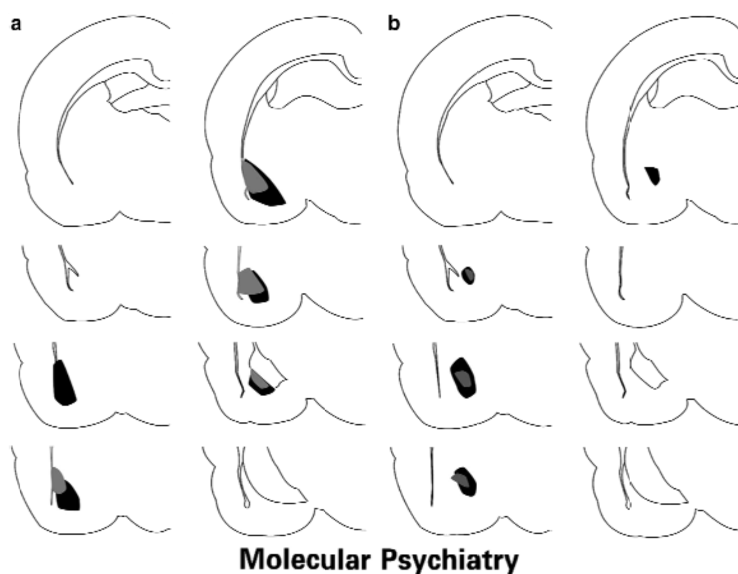
**Confocal analysis**. 25-50 BrdU or cFos positive cells were located in the dentate gyrus for each animal (or each hemisphere for unilateral lesion) and assessed in z-series of <1.0 um slices to determine if other markers (DCX, S100β, MBP, cFos, BrdU) were co-expressed. Confocal images were captured on a Zeiss 510 META/NLO confocal microscope with a 40x oil objective and adjusted for brightness and contrast using LSM Image Browser software.

**Lesion and viral vector assessment**. One series of sections was mounted and counterstained with cresyl violet for lesion assessment. If a lesion did not cover at least 75% of the area of interest without affecting surrounding areas, that rat was removed from analysis (Supplementary Figure 1-2). For virus expression, if GFP expression was not found in the BLA or if expression was focused outside the BLA, the rat was removed from analysis.

**Fear conditioning**. Two weeks after unilateral lesion of the BLA or sham surgery, rats received 4 daily injections of BrdU (100 mg/kg). Two weeks after the last BrdU injection, rats were exposed to fear conditioning. Fear conditioning chambers were 12 w x 10 l x 12 h inch boxes with an electrified grid floor inside a sound attenuating chamber (Coulbourn Instruments, Whitehall, PA). Chambers had two house lights and a house fan that were on at all times during testing and training. Rats were placed in the box and allowed 5 min to acclimate. They then received 10 unsignaled shocks with an intertrial interval of 10-120 s. Rats were left in the chamber for 3 min after the last shock and then returned to their home cage. Chambers were cleaned with 70% ethanol between trials. The next day, rats were placed back in the fear context or a novel context for 15 min without any shock delivery. The novel context was the fear conditioning chamber with the grid floor removed and laminated cork boarding covering three of the walls. Freezing behavior was tracked and analyzed with FreezeFrame software (Coulbourn). After context exposure, rats were returned to their home cages for 45 min and then perfused as in other studies. This time delay was chosen because it coincides with the elevation in IEG expression following context memory activation[144].

**Statistical analysis**. Unpaired t-tests were used to assess the effects of bilateral lesion on proliferation and cell differentiation, context exposure on freezing behavior, and context exposure on BrdU co-labeling of sham operated rats (unequal variances assumed). Paired t-tests were used to assess proliferation differences in the unilateral lesion study, with an unpaired t-test to compare control PCNA/BrdU levels to that contralateral to BLA lesion. Repeated measures analysis of variance (ANOVA) was used to compare the effect of unilateral BLA versus CeA lesion on cell proliferation as well as the effect of novel versus fear context exposure on BrdU co-labeling in unilaterally BLA lesioned rats. Bonferroni post-hoc tests were used to assess the effect of hemisphere within each lesion group in both cases. $p<0.05$ was considered significant for all studies.

## E. Figures

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doi:10.1038/mp.2011.71

**Figure 2.1**: Minimum (grey) and maximum (black) extent of excitotoxic BLA lesions. Brain images adapted from[145].

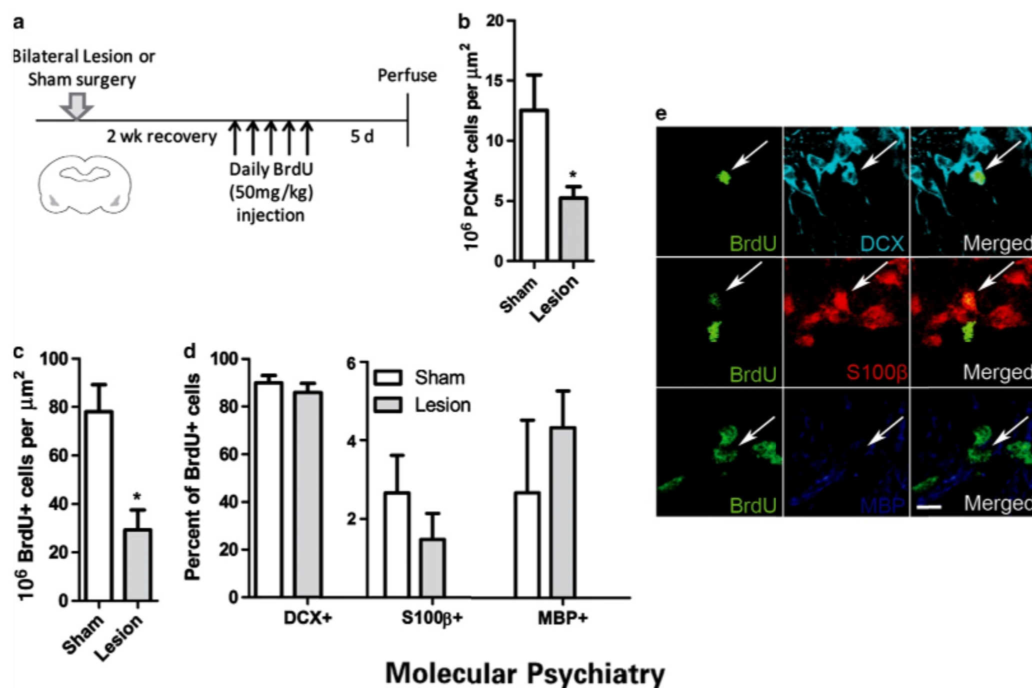


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**Figure 2.2:** Bilateral basolateral amygdala (BLA) lesions suppressed hippocampal neurogenesis. (**a**) Experimental timeline. (**b**) Bilaterally lesioned rats (n=5) had significantly fewer proliferation cell nuclear antigen (PCNA)-positive cells than sham-operated rats (n=6). $^{*}P<0.05$. (**c**) Bilaterally lesioned rats also had significantly fewer 5-bromo-2′-deoxyuridine (BrdU)-positive cells than sham-operated rats, representing a reduction in the number of 5- to 10-day-old cells. $^{*}P<0.05$. (**d**) BLA lesion did not affect the percent of BrdU-positive cells expressing one of three cell fate markers: doublecortin (DCX), S100β or myelin basic protein (MBP). (**e**) Representative confocal images showing colocalization of BrdU with DCX, S100β or MBP. Scale bar=10 μm.

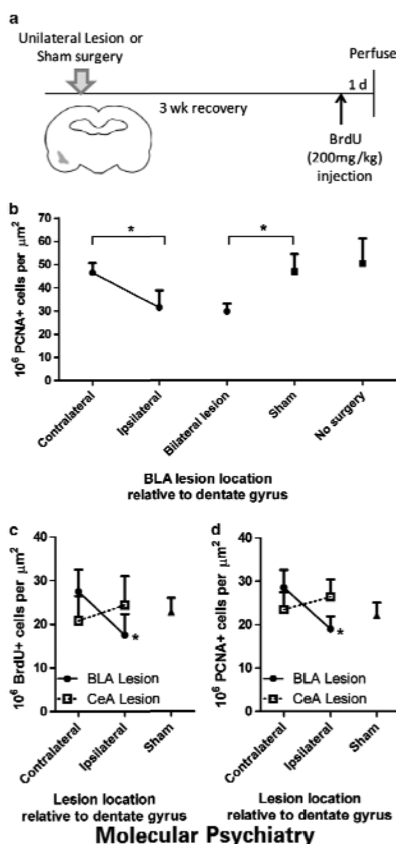


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**Figure 2.3:** Unilateral lesions of the basolateral amygdala (BLA), but not the central nucleus of the amygdala (CeA), ipsilaterally suppressed neurogenesis. (**a**) Experimental timeline. (**b**) There were significantly fewer dentate proliferation cell nuclear antigen (PCNA)-positive cells ipsilateral versus contralateral to unilateral BLA lesion (n=5). Similar suppression of PCNA-positive cells was found with bilateral BLA lesion (n=6) as ipsilateral to unilateral lesion. The number of PCNA-positive cells contralateral to lesion was similar to that found in the dentate gyrus of bilateral sham-operated animals (n=3) and no surgery rats (n=4). $^{*}P<0.05$. (**c**) In BLA (n=5), but not CeA, (n=6) lesioned rats, there were significantly fewer 5-bromo-2′-deoxyuridine (BrdU)-positive dentate cells ipsilateral versus contralateral to the lesion. Sham rats, n=6. $^{*}P<0.01$. (**d**) In BLA (n=5), but not CeA, (n=6) lesioned rats, there were significantly fewer PCNA-positive cells ipsilateral versus contralateral to the lesion. Sham rat, n=6. $^{*}P<0.01$

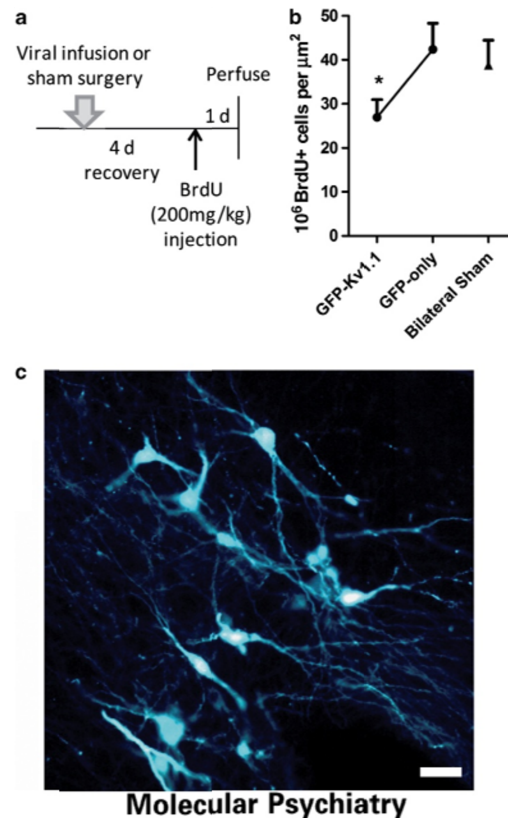


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**Figure 2.4:** Basolateral amygdala (BLA) silencing via potassium channel overexpression suppressed neurogenesis. (**a**) Experimental timeline. (**b**) In rats infused with GFP-Kv1.1 and GFP-only viral vectors to reduce BLA activity (n=8), there were significantly fewer 5-bromo-2′-deoxyuridine (BrdU)-positive dentate cells ipsilateral to GFP-Kv1.1 infusion. Sham rats, n=15. $^*P<0.05$. (**c**) Representative images of GFP expression in virus-infected BLA neurons. Scale bar=10 µm.

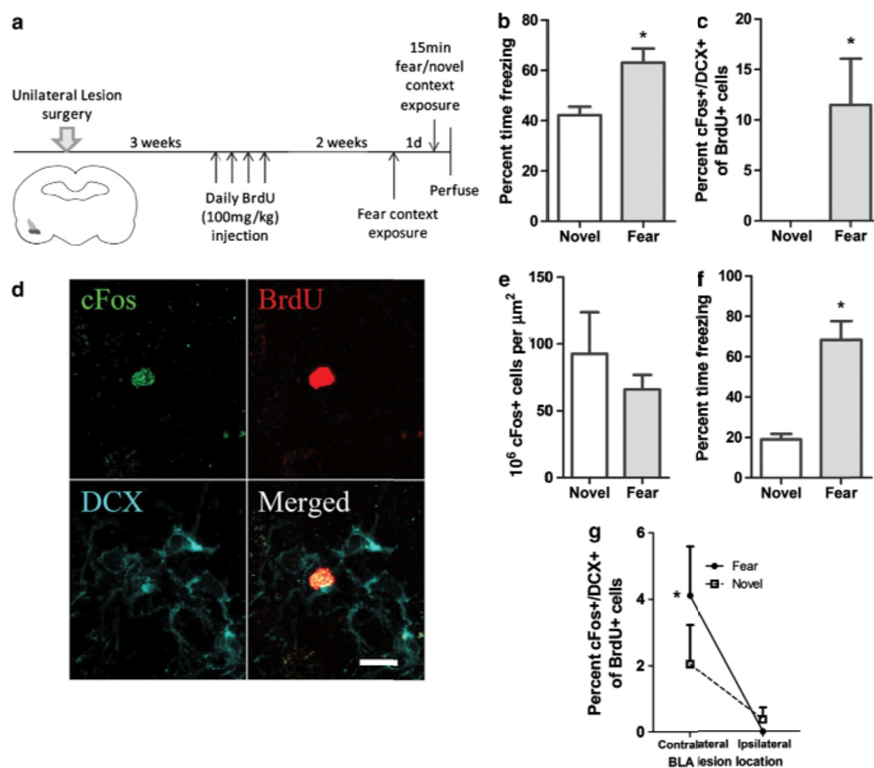


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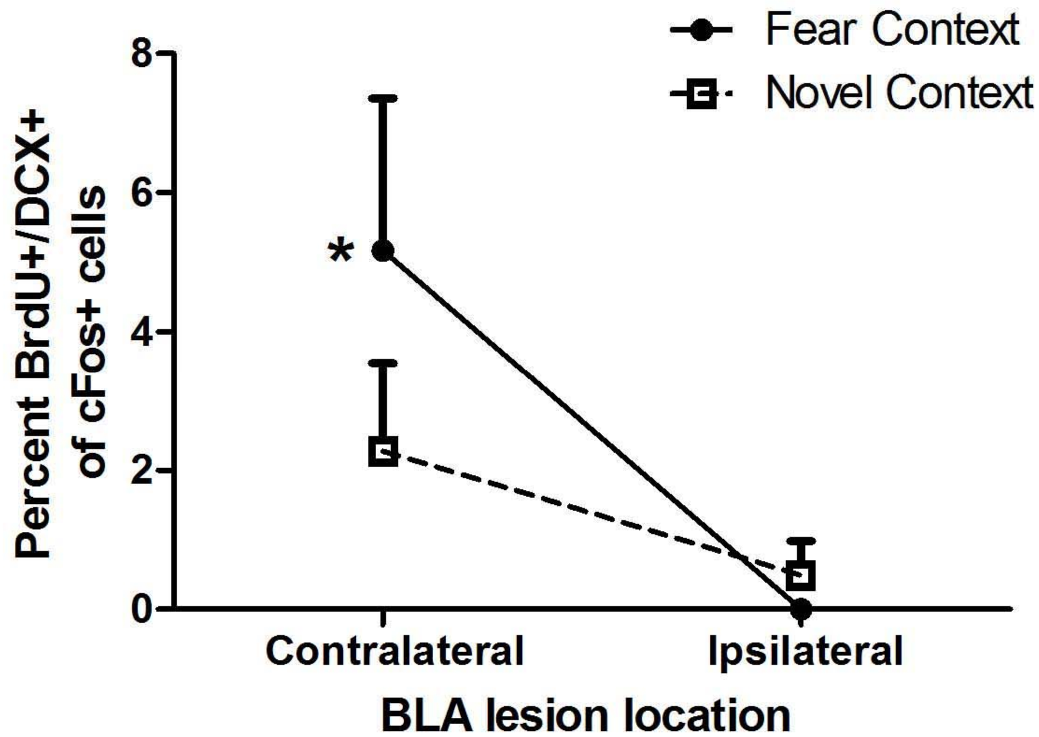
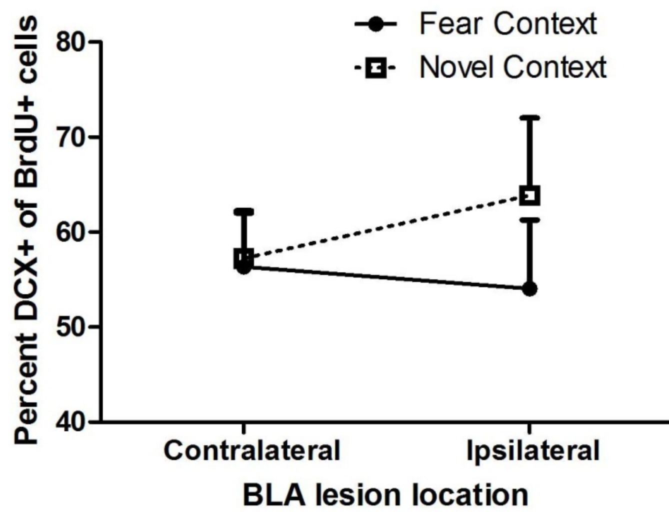
**Figure 2.5**: Unilateral basolateral amygdala (BLA) lesions blocked activation of immature neurons by exposure to a fear-associated context. (**a**) Experimental timeline. (**b**) Exposure to the fear context (n=7) resulted in significantly more freezing than exposure to the novel context in sham-operated rats (n=5). (**c**) Sham-operated rats exposed to the fear context had a greater proportion of 5-bromo-2′-deoxyuridine (BrdU)-positive cells that co-expressed doublecortin (DCX) and cFos. (**d**) Representative confocal image of a BrdU cell (red) expressing both DCX (blue) and cFos (green). Scale bar=10 µm. (**e**) Sham-operated rats exposed to the fear context had similar numbers of cFos+cells in the dentate gyrus as rats exposed to the novel context. (**f**) In unilaterally BLA-lesioned rats, exposure to the fear context (n=5) resulted in significantly more freezing than exposure to the novel context (n=6). (**g**) In rats exposed to the fear but not the novel context, there was a lower percentage of DCX/cFos labeled BrdU-positive cells ipsilateral versus contralateral to the lesion. $^{*}P<0.05$




**Supplementary Figure 2.1:** In rats exposed to the fear (n = 5) but not the novel context (n = 6), there was a significantly lower proportion of DCX/BrdU labeled cFos positive cells ipsilateral versus contralateral to the lesion.*p<0.01.




**Supplementary Figure 2.2:** Neither BLA lesion nor context exposure (n = 5 fear; n = 6 novel) affected the percent of BrdU positive cells that expressed DCX.

Chapter 3: Basolateral amygdala modification of stress-induced changes in adult hippocampal neurogenesis.

## A. Introduction

One of the most remarkable aspects of adult CNS plasticity is the birth, survival and differentiation of new neurons—all together a process known as neurogenesis—that occurs in the dentate gyrus (DG) of the hippocampus well into adulthood. A variety of factors can alter adult hippocampal neurogenesis[14], but perhaps the most powerful and ecologically relevant factor that influences all aspects of this process is exposure to stress hormones[1-3]. Chronic exposure to stress hormones, known as glucocorticoids (GCs), has been shown to suppress the birth of new neurons in the dentate gyrus[31, 41, 44], as well as reduce new neuron survival and neuronal differentiation[18-20, 25]. However, the link between high GCs and suppressed neurogenesis is not immutable. Voluntary wheel running, enriched environment, and cognitive tasks all have been shown to lead to elevations in circulating GCs [63-65] and yet, to increases in hippocampal neurogenesis[8, 26, 67, 68]. Therefore, it seems that other factors can modulate GC effects on proliferative cells in the adult DG.

The basolateral amygdala (BLA) modulates many hippocampal responses to acute stress/GC exposure. Lesions of the BLA can prevent the suppression of LTP[146], memory consolidation enhancements[147] and memory retrieval impairments[148, 149] that usually occur following acute GC exposure. We have previously shown (chapter 2) that BLA lesions can suppress hippocampal neurogenesis under baseline (no stress) conditions[150]. However, whether BLA lesions may interact with stress to prevent GC effects on adult hippocampal neurogenesis is not known.

## B. Results

BLA lesions block acute stress-induced increase in adult hippocampal neurogenesis.

To investigate whether stress effects on neurogenesis depends on BLA activity, we unilaterally lesioned the BLA in adult male rats using stereotaxic injection of the excitotoxin, N-methyl-d-aspartate (NMDA) before exposing them to an acute, 3 hour immobilization stress. At the end of immobilization, rats were injected with bromodeoxyuridine (BrdU) and then perfused 2 hours later (Figure 3.1a). Plasma samples taken at the beginning and end of immobilization confirmed a significant increase in circulating corticosterone due to the stressor (Figure 3.1b). In agreement with our previous studies[150], BLA lesion suppressed the number of proliferating cell nuclear antigen-positive (PCNA+) cells in the dentate gyrus ipsilateral to the lesion compared to contralateral (main effect of hemisphere, $P = 0.002$, Figure 3.1c). In addition, there was an interaction between stress and hemisphere ($P = 0.025$), suggesting that BLA lesion modifies stress effects on hippocampal cell proliferation. However, contrary to expectation, acute stress increased the number of proliferative dentate gyrus cells (sham stress versus sham no stress, $P = 0.086$, trend). Therefore, this initial data suggests that acute immobilization increases hippocampal neurogenesis and that BLA lesion partially blocks this increase ipsilaterally.

BLA lesions do not alter chronic stress effects on adult hippocampal neurogenesis.

In contrast to acute stress, chronic exposure to stress or stress hormones reliably suppresses adult hippocampal neurogenesis[18, 32, 122]. To investigate if BLA activity is also necessary for chronic stress-induced suppression of adult neurogenesis, we exposed unilaterally BLA lesioned or sham operated rats to two weeks of daily immobilization stress (three hrs/day), (Figure 3.2a).

Immobilization reliably induced elevations in plasma corticosterone throughout two weeks (Figure 3.2b). In sham operated rats, immobilization reduced the number of proliferative PCNA+ cells, as expected ($P = 0.001$; Figure 3.2c). BLA lesion also reduced the number of proliferative PCNA+ cells ipsilateral to the lesion compared to contralateral (main effect of hemisphere, $P = 0.020$). However, there was no interaction of stress with lesion ($P > 0.10$). No difference in the number of TUNEL+ apoptotic cells between groups was found ($P > 0.10$, main effects and interaction; Figure 3.2d). Together, these data suggest that chronic stress-induced suppression of new neuron proliferation in the adult dentate gyrus is not dependent on the BLA.

## C. Discussion

These findings suggest that the effect of stress on the hippocampus can depend on the BLA in the acute, but not chronic, timescale. Lesions of the BLA partially blocked acute-stress induced increases in hippocampal neurogenesis in the hemisphere ipsilateral to the BLA lesion. Interestingly, hippocampal neural precursor cells express the glucocorticoid receptor (GR) and can respond to stress hormones directly *in vitro*[57, 151]. These results therefore suggest that some kind of neural input from the BLA is necessary for stress hormones to alter neural precursor proliferation *in vivo*.

Our data suggest that acute immobilization may increase hippocampal cell proliferation. Previous work on the effects of acute stress on adult neurogenesis has produced conflicting results[152]. While a number of studies show that acute stress reduces hippocampal neurogenesis[4, 30, 32, 36], many studies also show no effect of acute stress[39-41, 153, 154]. In addition, Wolf et al.[151] recently suggested that acute exposure to low levels of GCs may even increase hippocampal neurogenesis in a GR-dependent manner. Indeed, low doses of CORT do increase levels of growth factors such as FGF2 in the hippocampus[155-157].

These findings are consistent with the inverse U model of GC effects on hippocampal function, wherein low and high GCs are detrimental while moderate levels are beneficial[158-160]. This model suggests that our stressor protocol may have led to moderate elevations in GCs in the neural precursor niche, enhancing hippocampal function and neurogenic potential. While plasma corticosterone increased substantially in response to stress, recent work suggests that GC concentration in the hippocampus can vary substantially from that in the plasma[161]. Future work will address GC levels in the hippocampus following acute stress. Future work will also address the GC-dose response relationship in relation to neurogenesis by using injections of varying doses of exogenous corticosterone and measuring neurogenic response.

We found that the acute-stress induced increase in hippocampal neurogenesis may be dependent on BLA activity. Unilateral BLA lesion seemed to at least partially prevent acute stress-induced increases in hippocampal proliferation. Previous work shows that the BLA modulates stress effects on hippocampal function in a number of ways. Extensive work from the McGaugh laboratory has shown that BLA activity is necessary for GC-induced enhancement of hippocampal memory consolidation as well as GC-induced impairment of hippocampal memory retrieval[108, 162, 163]. At the electrophysiological level, BLA lesions can also prevent stress-induced impairment of hippocampal long-term potentiation (LTP)[146, 148, 163, 164]. These effects likely rely on BLA activity interacting with GR stimulation in the hippocampus/BLA because temporary inactivation of the BLA can block the memory altering effects of direct hippocampal and/or BLA infusion of GR agonists[149, 165-167]. There are very few direct connections between the BLA and the hippocampus, making it likely that BLA activity effects hippocampal function through inputs

to the entorhinal cortex and medial septum, both of which provide direct excitatory input to the dentate gyrus[168-170].

In contrast to acute stress, the effects of chronic stress did not depend on BLA activity. Chronic stress suppressed hippocampal cell proliferation regardless of BLA lesion. Chronic stress or chronic exposure to stress hormones suppresses neurogenesis is a variety of species including mice, rats, tree shrews and monkeys[122, 152]. Most studies showing BLA mediation of stress effects on the hippocampus use a more acute model of stress or stress hormone exposure[109, 165, 171]. Long-term exposure to stress hormones likely impacts the hippocampus via different mechanisms and leads to different responses and adaptations than acute stress[172]. We therefore hypothesize that the BLA may not play as large a role in stress effects on the hippocampus over this longer time frame.

In summary, we show that acute but not chronic stress effects on adult hippocampal neurogenesis depend on BLA activity. We also show that acute stress can stimulate hippocampal neurogenesis, though the underlying mechanism is unclear. These findings provide insight into the mediation of hippocampal plasticity in response to environmental stress. In addition, given the potential role of stress and neurogenesis in anxiety and/or depression[173], such results may also shed light on the mechanisms of several debilitating brain disorders.

## D. Methods

**Animals**. Adult male Sprague-Dawley rats (Charles River) were pair-housed on a 12h light/dark cycle. All animal procedures were approved by the UC Berkeley and MIT Animal Care and Use Committees.

**Stereotaxic surgery**. BLA lesions were induced as per[150].

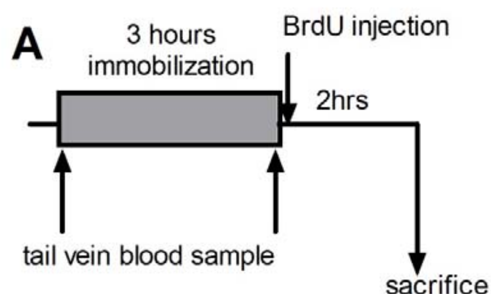
**Immobilization stress**. Rats were immobilized in Decapicone bags (Braintree scientific) and placed in individual cages in a fume hood for three hours. For the chronic study, immobilization occurred daily for 14 days.

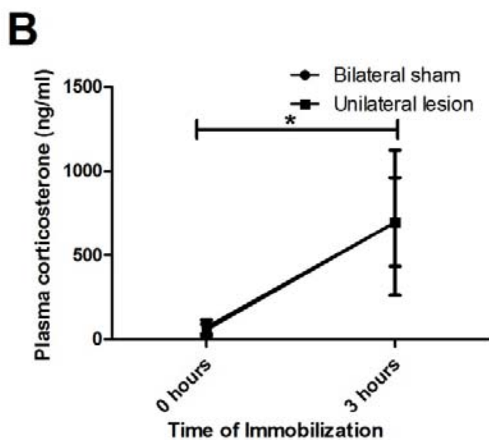
**Plasma hormone sampling**. Tail vein blood samples were obtained from all rats at the beginning and end of immobilization. For chronic stress, this procedure was repeated on days 1, 7 and 14. All blood samples were centrifuged at 2000g for 15 minutes and plasma was extracted and stored at -20°C until assayed. Corticosterone was measured using a Corticosterone EIA kit (Cayman Chemical).

**Bromodeoxyuridine injections and perfusion.** 5-Bromo-2'-deoxyuridine (BrdU, Sigma) was dissolved in physiological saline and injected intraperitoneally for all experiments. For the chronic stress study, BrdU was injected once per day, for 3 days (100 mg/kg) with chronic stress beginning the day after the last injection. Immediately after the end of stress, rats were anesthetized with Euthasol euthanasia solution and transcardially perfused with ice cold 0.1M PBS followed by 4% paraformaldehyde in 0.1M PBS. For the acute stress study, BrdU was injected at the end of three hours of immobilization. Rats were anesthetized two hours after BrdU injection. All brains were post-fixed for 24 hrs at 4ºC, equilibrated in 30% sucrose in 0.1M PBS and then stored at -80ºC.

**Immunohistochemistry and quantification**. Immunostaining for PCNA and BrdU was performed on a 1 in 6 series of free-floating 30 µm cryostat sections as per[150]. The number of PCNA+ or BrdU+ cells in the dentate gyrus were quantified as per[150]. TUNEL staining was performed on three hippocampal sections per rat as per manufacturer's instructions (In Situ Cell Death Detection Kit, POD, Roche). Quantification was performed similarly to that for BrdU and PCNA.

## E. Figures



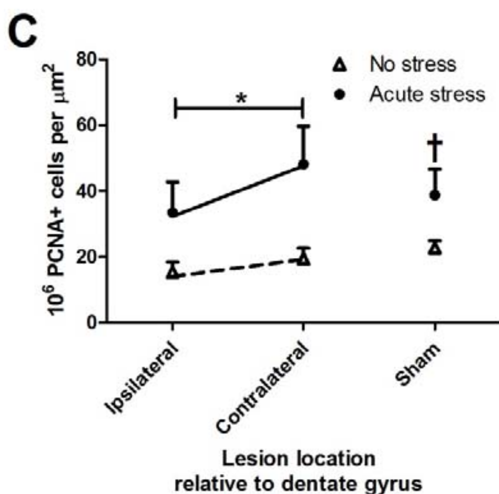




**Figure 3.1.** Acute stress effects on neurogenesis are blocked by BLA lesions. A) Timeline. B) Immobilization increased plasma corticosterone (average ± SEM) in sham-operated (n=5) and unilateral BLA-lesioned rats (n=5). C) Acute stress increased PCNA-positive cell count (average ± SEM) in the dentate gyrus (n=5/grp, †$p<0.10$). In unilaterally lesioned rats, BLA lesion blocked the stress-induced increase in PCNA+ cell number (n=5 stress, n=3 no stress, *$p<0.05$ main effect of hemisphere).

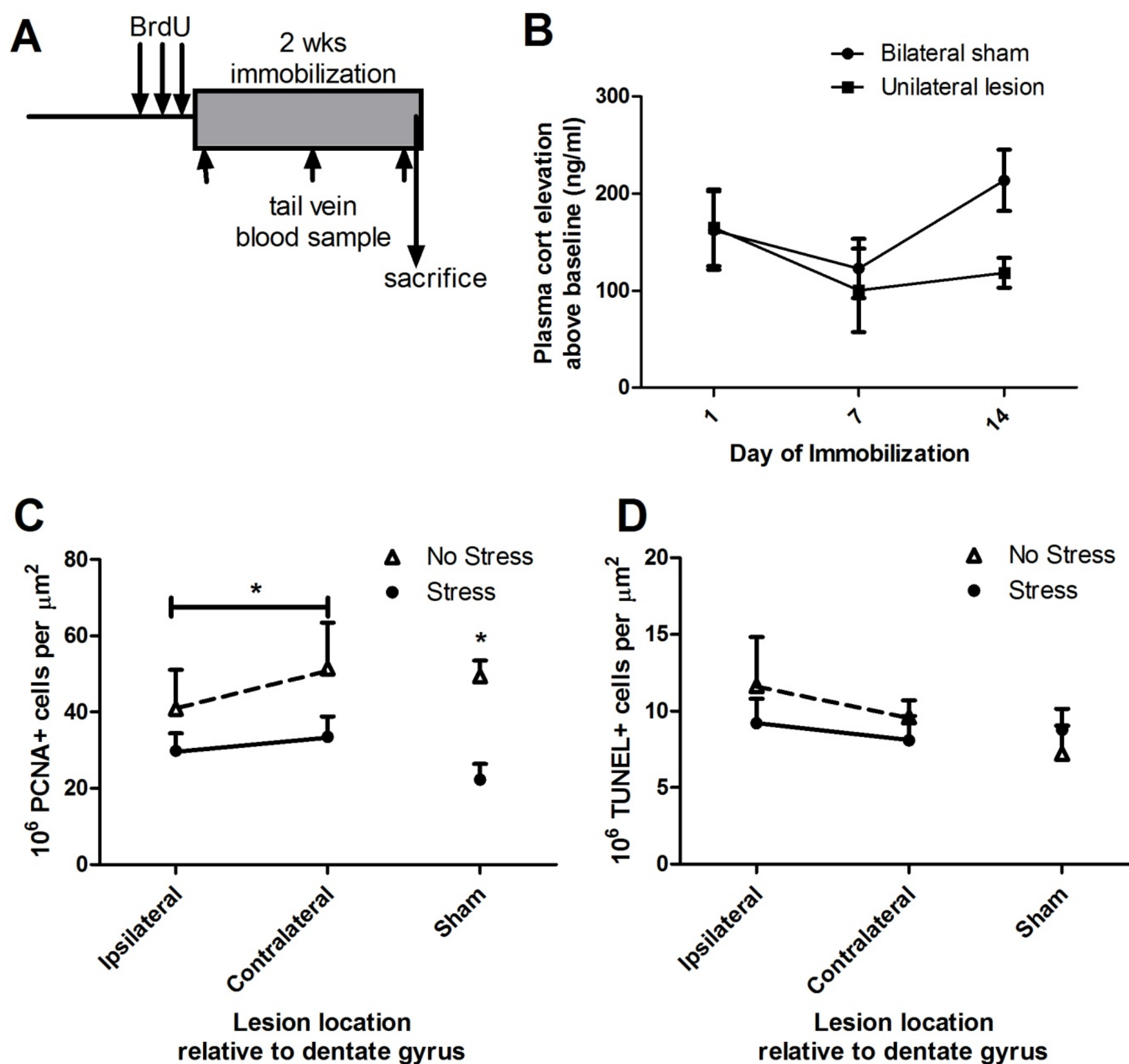



**Figure 3.2.** Chronic stress suppressed hippocampal cell proliferation regardless of BLA lesion. A) Timeline. B) Immobilization increased plasma corticosterone (average elevation above baseline ± SEM) throughout all 14 days of stress in both sham-operated (n=6) and unilateral BLA-lesioned rats (n=6). C) Chronic stress suppressed PCNA-positive cell number (average ± SEM) in the dentate gyrus of sham-operated rats (n=6/grp, *$p<0.05$). Unilateral BLA lesion also suppressed PCNA+ cell number (main effect of lesion, *$p<0.05$), but did not interact with stress (n=5 no stress, n=6 stress). D) Neither BLA lesion nor stress affected the number of TUNEL+ cells (average ± SEM) in the dentate gryus.

**Chapter 4:** Stress increases putative gonadotropin inhibitory hormone and decreases luteinizing hormone in male rats.

From: Kirby, E.D., Geraghty, A.C., Ubuka, T., Bentley, G.E., & Kaufer, D. (2009). Stress increases putative gonadotropin inhibitory hormone and decreases luteinizing hormone in male rats. *Proceedings of the National Academy of Sciences USA, 106,* 11324-11329.

## A. Introduction

Stress has acute and chronic effects on many aspects of vertebrate physiology and behavior. Reproduction is a key life-history component that is often adversely affected by physical and psychological stressors. The negative impact of stress occurs at several levels of the vertebrate hypothalamic-pituitary-gonadal (HPG) axis. Centrally, it leads to activation of the hypothalamic-pituitary-adrenal (HPA) axis, which in turn leads to suppression of HPG activity through inhibition of gonadotropin releasing hormone (GnRH) secretion [99, 100]. Downstream of GnRH, the functional effects of stress on reproduction can be seen with suppression of luteinizing hormone (LH) release from the pituitary [174, 175] and suppression of sexual behavior [176, 177]. The stress effect on HPG function appears to be mediated by the adrenal stress hormones glucocorticoids (GCs). In male mammals, systemic GC administration inhibits circulating gonadotropin levels [178], decreases seminal vesicle weight and results in fewer implantation sites and viable fetuses in female mates [179]. However, hypothalamic corticotropin releasing hormone (CRH) has also been strongly implicated in stress effects on reproduction [100, 180, 181].

Gonadotropin inhibitory hormone (GnIH) is a recently-discovered hypothalamic RFamide peptide that inhibits gonadotropin synthesis and secretion [101]. The inhibitory action of this neuropeptide raises the question of whether stress-mediated reproductive dysfunction could operate through the GnIH system. If so, this would provide a previously uninvestigated level of stress-regulated central HPG control. The mammalian ortholog of avian GnIH (RFamide-related peptide, RFRP) is expressed in neurons within the dorsomedial nucleus of the hypothalamus (DMH) of rats, with fibers extending both to the median eminence and to the preoptic area, making putative contact with GnRH neurons within the hypothalamus [102, 104]. Mammalian RFRP is produced as a full length precursor peptide that is cleaved into two active peptides *in vivo*, RFRP-1 and RFRP-3 [102]. The amino acid sequences of rat RFRP peptides are highly homologous to GnIH-precursor derived peptides in quail and white-crowned sparrow [102]. The receptor for RFRP (known as OT7T022) is also similar to the GnIH receptor in birds [150] and is found in the hypothalamus, pituitary and testes [103, 182]. In birds and mammals, RFRP plays a functional role in suppression of HPG function *in vivo* [103-105, 183]. When RFRP-3 is administered systemically to male rats, it suppresses both LH release and sexual behavior [104], much as stress does. Moreover, RFRP expression is altered by stress in breeding house sparrows [106]. Together, a body of data suggests that RFRP [103] may act as an influential mediator of stress effects upon mammalian reproduction.

The present experiments were designed to investigate whether stress could affect mammalian HPG function through increased expression of RFRP. Further, we wished to determine whether any observed effect is mediated by stress hormones such as GCs or CRH. We found that both acute and chronic stress elevate RFRP expression and lead to down-stream HPG dysfunction. We also found that RFRP cells express stress hormone-responsive receptors.

Additionally, adrenalectomy (ADX) prevents the stress-induced increase in RFRP expression. Thus our data provide strong evidence that RFRP expression and HPG function are directly influenced by stress-induced adrenal hormone release.

## B. Results

Acute stress increases hypothalamic RFRP expression

To determine whether RFRP could contribute to stress-induced suppression of the HPG axis, we investigated RFRP gene expression in response to acute stress in adult male rats (Fig. 1a). Three hours of immobilization stress led to an increase in RFRP mRNA and peptide in the hypothalamus when measured immediately after the immobilization ended (Fig. 4.1b-e). Using in situ hybridization with a probe that identifies the full-length rat RFRP precursor mRNA [102], we found a 2.3 (± 0.3 s.e.m.)-fold increase in RFRP mRNA levels in the DMH immediately after immobilization, relative to no stress controls. When measured twenty-four hours after the end of immobilization, RFRP mRNA levels in stressed animals did not differ from controls (Fig. 4.1b and d). A similar pattern was found for RFRP precursor peptide (Fig. 4.1c and e). Stressed rats showed a 1.4 (± 0.1 s.e.m.)-fold increase in number of RFRP-ir cells immediately after immobilization. RFRP in rats stressed then sacrificed 24-hours later did not differ from nonstressed control rats.

To investigate whether the stress-induced changes in RFRP expression might be important for HPG function, we used real-time reverse transcriptase PCR (RT-PCR) to determine hypothalamic RFRP mRNA transcript abundance, and correlated that with circulating LH levels. RFRP mRNA expression levels showed a significant negative correlation with plasma LH levels (Fig. 4.1f), a finding which is consistent with previous work showing that RFRP lowers circulating LH levels [104] either through reduced GnRH neuron activity or through reduced responsiveness of pituitary cells to hypophysial GnRH [184-187]. No effect of acute stress was found on RFRP (1.2 ± 0.1 fold change, $P = 0.33$) or OT7T022 (1.0 ± 0.1 fold change, $P = 0.82$) mRNA transcript expression immediately after stress as measured by real time RT-PCR in the testes. These findings indicate that acute immobilization stress leads to a temporary increase in hypothalamic RFRP expression, coincident with suppression of downstream HPG activity.

Chronic stress increases hypothalamic RFRP expression

Because chronic stress is also known to suppress reproductive function, with effects possibly extending beyond the termination of the stress [177, 188], we next investigated whether RFRP expression in the rodent DMH is also altered by chronic stress and whether those effects might be more prolonged or greater than acute stress effects. Adult male rats were immobilized for three hours per day for 14 consecutive days and sacrificed 24 hours after the last immobilization session (Fig. 4.2a). Chronic immobilization led to upregulation of RFRP mRNA and peptide expression in the hypothalamus up to 24 hours after the end of immobilization. Using real time RT-PCR, we found that chronic immobilization stress led to a 1.8 (± 0.1 s.e.m.)-fold increase in RFRP mRNA levels (Fig. 4.2b) compared to controls. Chronic stress also increased RFRP peptide levels. Immunohistochemistry revealed a 1.9 (± 0.1 s.e.m.)-fold increase in number of RFRP-ir cells in the DMH in stressed compared to control rats (Fig 3.2c). Stress did not alter RFRP mRNA levels in the testes. Similarly, there were no changes in RFRP receptor (OT7T022) expression in the hypothalamus, pituitary or testes (Fig. 4.2b). However, our

chronic stressor did reduce plasma LH concentrations, confirming that HPG function was suppressed in stressed rats (Fig. 4.2d).

Acute and chronic stress activate the HPA axis

To verify HPA axis activation during the immobilization stress protocol tail vein plasma samples were collected from acutely and chronically stressed animals. Acutely stressed animals showed a rapid and sustained rise in plasma corticosterone (Fig. 4.3a). Chronically stressed animals showed elevations of plasma corticosterone in response to restraint throughout all 14 days of restraint, indicating that they did not fully habituate to the stressor (Fig. 4.3b). These peak values of corticosterone are similar to those found in previous studies showing impaired HPG function following chronic or acute stress [189, 190].

Hypothalamic RFRP cells express stress hormone receptors

The stress paradigm reliably activated the HPA axis and stress effects on hypothalamic RFRP were observed. Thus, we investigated whether RFRP cells in the hypothalamus express receptors that would allow them to respond directly to the two major HPA-regulated hormones, corticotropin releasing hormone (CRH) and corticosterone (the major rat GC). Using confocal microscopy and double immunohistochemical labeling for CRH receptor-1 (CRH-R1) or glucocorticoid receptor (GR) with RFRP, we found that RFRP-positive cells co-express both of these stress-responsive receptors (Fig. 4.3c-e). Approximately 12.8 (± 1.1 s.e.m.) % of RFRP-positive cells were co-labeled with CRH-R1 while 53.1 (± 11.4 s.e.m) % were co-labeled with GR (Fig 4.3d-e).

Adrenalectomy prevents the stress-induced increase in RFRP expression

Because approximately half of hypothalamic RFRP cells expressed GR, we next investigated whether the stress-induced rise in RFRP was dependent on increased circulating GCs levels, by surgically removing the GC-secreting tissue – the adrenal glands. Male rats were bilaterally adrenalectomized (ADX) and allowed to recover for two weeks. Sham operated animals served as controls. Rats were exposed to daily immobilization stress for two weeks as above. The day after the end of stress, rats were sacrificed and RFRP mRNA expression was quantified. Corticosterone response to restraint on day 1 of stress verified that ADX effectively eliminated stress-related corticosterone release (Fig. 4.4a). In sham operated rats, chronic immobilization stress led to a significant increase in hypothalamic RFRP mRNA expression while in ADX rats, there was no change in RFRP expression following stress (Fig 4.4b), indicating that the stress-induced rise in RFRP expression is dependent on high circulating level of GCs.

**C. Discussion**

Here we show that both acute and chronic stress stimulate expression of rat RFRP (considered a GnIH) in the adult male rat hypothalamus while suppressing reproductive function. Our data also indicate that the stress-induced increase in RFRP expression is dependent on adrenal hormones. Acute and chronic immobilization stress led to increases in hypothalamic RFRP at both the mRNA and protein levels. Acute stress effects on RFRP expression largely dissipated within 24 hours of removal of the stressor, while chronic stress effects lasted longer. This latter result is consistent with previous work showing that the effects of stress are often greater the longer the stressor is applied [177, 188]. Stress and RFRP are involved in the suppression

of sex behavior in adult male rats [104, 177]. Moreover, administering RFRP to adult male rats suppresses plasma LH levels [104], as does exposure to stress [174, 175]. In the present study, we show a direct correlation between stress-induced increases in RFRP expression and reduction in plasma LH. In addition, we demonstrate that receptors for stress hormones exist on RFRP neurons, thus providing the neuroanatomical architecture for stress-mediated effects on the RFRP system. Taken together, these data suggest that RFRP plays an influential role in stress-induced effects on gonadotropin release and thus HPG function.

The functional consequences of stress on reproductive success are found in many species and appear to be a common feature of mammalian and avian systems. Stress has been linked to infertility in adult men [191], lower sperm motility in medical students [192], reduced proceptive sexual behavior [176] and fewer successful impregnations [179] by male rats, as well as lower hatchling survival in free-living European starlings [193]. In addition, the effects of malnutrition on reproduction may result from activation of the stress system [194, 195], suggesting that the importance of stress effects on the HPG may extend beyond traditional stressors. The HPA axis has also been implicated in aging and the increase in reproductive dysfunction with age [196].

The HPA axis interaction with the HPG axis occurs at multiple levels and appears to be conserved across many species. In response to stress, the hypothalamus releases CRH into the hypophysial portal system, after which anterior pituitary cells respond to CRH by releasing adrenocorticotropic hormone (ACTH). ACTH stimulates the adrenal medulla to release GCs into the bloodstream. The HPG axis runs in parallel to the HPA, with hypothalamic GnRH stimulating pituitary LH and FSH which in turn stimulate testosterone-secreting Leydig cells and sperm-producing Sertoli cells. Both GCs and CRH affect multiple levels of the HPG axis in several species (see supplementary figure 3.1, [95, 99, 197]). GC injections can suppress plasma LH in rats [100], rhesus monkeys [178] and humans [198] while CRH receptor antagonists can block the effects of stress on plasma LH [100, 181]. In vitro, GnRH neurons show changes in gene expression in response to GCs [199], but GnRH expression does not always change in response to a stressor that inhibits downstream reproductive function [174]. Indeed, our data did not show changes in GnRH expression, despite suppression of plasma LH secretion. These findings suggest that the stress effects on circulating gonadotrophin may act via suppression of GnRH release and/or via decreased pituitary sensitivity to GnRH. Previous studies have shown evidence for both of these mechanisms of stress-mediated suppression of HPG function [200, 201], and our present study provides strong evidence for a mechanistic basis for this response.

In avian and rodent models, GnIH/RFRP fibers extend to the median eminence and come in close proximity to GnRH cells [102-104, 202]. In addition, GnIH receptors are expressed on GnRH cells [202]. Together, these results suggest that GnIH/RFRP may regulate the HPG axis by influencing hypothalamic GnRH release and/or pituitary sensitivity to GnRH. Currently, there is evidence for GnIH/RFRP influencing hypothalamic GnRH neuron excitability/release as well as pituitary gonadotropin release in birds [103, 202] and mammals [102, 104, 186, 187, 203-205], suggesting that these mechanisms of regulating HPG function are conserved across species. For example, Rizwan et al.[184] found that systemic RFRP-3 administration in rats reduced LH response to GnRH (suggesting RFRP acts via pituitary mechanisms), while Ducret et al.[186] found that mouse GnRH neurons hyperpolarize in response to RFRP in vitro (suggesting that RFRP affects hypothalamic GnRH release). It is possible, then, that both pathways could play a role in the stress-reproduction interaction via GnIH/RFRP. Therefore, given the changes we found in RFRP in rats following acute and chronic stress, we propose that GnIH/RFRP acts as a common

contributory mechanism by which stress inhibits GnRH release and/or gonadotropin secretion in response to GnRH (see Figure 4.5).

In addition to being present in the hypothalamus, GnIH/RFRP is also found in the testes of rats and birds [103]. However, we found no change in RFRP expression in the testes of adult male rats following stress, indicating that the stress effect on RFRP expression is specifically a central, hypothalamic phenomenon. We also found no change in RFRP receptor expression at any level of the HPG axis. Therefore, it appears that stress largely acts on RFRP synthesis rather than altering the sensitivity of other cell types to RFRP via changes in RFRP receptor expression.

Within the hypothalamus, we found GR and CRH-R1 expressed in a large proportion of RFRP-expressing cells. Stress effects on the HPG have been linked to both GCs and CRH. Exogenous GC injection suppresses HPG activity and sexual behavior while CRH receptor blockers (including those specific to CRH-R1) can block stress effects on reproductive function [100, 179, 181]. Because our results indicated that a greater proportion of RFRP cells expressed GR than expressed CRH-R1, we investigated whether GCs might be the mediator of stress effects on RFRP expression by adrenalectomizing rats prior to stress. We found that ADX prevented the stress-induced increase in hypothalamic RFRP expression, indicating that RFRP changes depend on stress-induced elevations in GCs (see figure 3.5). Our results are therefore consistent with previous work showing that stress effects on HPG function are mediated by GCs [178, 179] and our data provide a mechanistic framework for those findings. In addition, a search of the rat RFRP promoter region reveals four potential glucocorticoid responsive elements (GREs) within 5000 base pairs (bp) upstream of the RFRP gene, the closest being approximately 1,240 bp upstream. While more work is needed to show definitively that GCs act directly on GR in hypothalamic RFRP cells, our findings of GC-dependent changes in RFRP, co-expression of RFRP and GR and the existence of potential GREs in the RFRP promoter strongly imply that stress-induced increases in circulating GCs stimulate GR in hypothalamic RFRP cells, causing increased transcription of the RFRP gene (see Figure 4.5).

Previous studies of HPA-HPG interactions have provided evidence for multiple, possibly overlapping mechanisms for stress effects on reproduction, including effects on dopaminergic, serotonergic and cholinergic transmission at multiple levels [99, 197, 206, 207], in addition to direct effects on the HPG axis [99, 197]. It seems possible that these mechanisms may have evolved in parallel to allow for HPG-HPA axis interaction in response to a variety of stimuli. The RFRP/HPA interaction that we have demonstrated here may therefore represent a pathway that provides a greater sensitivity to certain stressor stimuli than others. More research is necessary to determine whether different stressors activate different HPA-HPG interaction pathways, but such a phenomenon could help explain discrepant findings in the stress and reproduction literature.

In summary, our data indicate a novel mechanism by which HPA axis activation can influence reproductive function, namely through a GC-induced increase in RFRP signaling to GnRH neurons and/or the anterior pituitary. Moreover, given the common interaction of HPA and HPG axes across many species and the similarity of GnIH/RFRP actions in birds and mammals [103], the involvement of RFRP in the HPA-HPG interaction investigated here in rats is predicted to be pertinent to most vertebrates. HPA axis activation is implicated in a number of causes of infertility and reproductive dysfunction, including chronic stress and malnutrition [98, 194, 195] and may significantly influence the efficacy of assisted reproductive procedures in humans [95, 97]. In addition, chronic stress is of primary concern in captive breeding programs as well as in agricultural breeding programs [208]. The current findings, therefore, contribute a new key level of

understanding to the mechanism underlying one of the most common suppressors of HPG activity.

## D. Methods

Experimental subjects. Adult male Sprague-Dawley rats were pair-housed on a 12/12 light-dark cycle with lights on at 0700h and ad libitum food and water. For the acute stress study, animals were immobilized and sacrificed immediately after immobilization (n = 5) or 24 hours later (n = 4). Controls were left undisturbed in their home cages until perfused (n = 3). A separate group of rats was used for correlation of trunk blood LH and RFRP expression (n = 3 con, n = 4 str). For assessment of testes transcript expression by real-time RT-PCR following acute stress, n = 4 con and n = 4 str rats were used. For the chronic stress experiment, rats were immobilized daily (n = 6 for PCR, n = 4 for IHC) or left undisturbed in their home cages (*n* = 6 for PCR, n = 4 for IHC). Confocal analysis was performed on tissue from three control rats. For the ADX experiment, 14 animals received ADX (n = 6 stress, n = 8 no stress) and 16 received sham surgery (n = 8 stress, n = 8 no stress). All animal care and procedures were approved by the UC Berkeley Animal Care and Use Committee.

Immobilization stress. Rats were immobilized in Decapicone bags (Braintree scientific) and placed in individual cages in a fume hood for three hours. For the chronic study, immobilization occurred daily for 14 days.

Plasma hormone 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). Plasma LH was assessed by the National Hormone and Peptide Program RIA. For chronic stress experiments, tail bleed samples from two cagemates were pooled. For acute stress, individual samples were used for analysis. Sample values below the detection level of the assay were included as the lowest detectible value.

Adrenalectomy. Adult male rats underwent either bilateral removal of the adrenal glands or bilateral sham surgery. After one week of recovery in isolation, rats were pair-housed with their original cage mate. One week later, immobilization stress began as described above. ADX rats were maintained on water with 0.9% NaCl and 25 µg/ml corticosterone for the duration of the experiment. One ADX stress rat was removed from analysis because day 1 corticosterone response to restraint revealed that ADX was incomplete.

In situ hybridization and immunohistochemical staining for stress studies. Rats were transcardially perfused with 4% paraformaldehyde. Brains were post-fixed for 3-4 hours, equilibrated in 30% sucrose in 0.1M PBS and then stored at -80°C. In situ hybridization was performed similar to [102] with a DIG-labeled probe against full-length precursor rat RFRP. More detail is available in supplementary methods. For immunohistochemical labeling of RFRP peptide, sections were then rinsed with PBS, incubated in blocking solution (2% normal goat serum, 0.3% tritonX-100 in PBS) for one hour and then transferred into primary antibody against GnIH [102] (PAC123/124, 1:5000 in PBS plus 0.3% triton-100, PBS-T) overnight at 4°C. The next day, slides were rinsed with PBS-T and incubated in goat anti-rabbit Alexafluor 568 (1:500, Molecular Probes Inc.) for two hours at room temperature. After rinsing in PBS-T, slides were coverslipped using DABCO antifading medium and stored in the dark at 4°C.

Immunohistochemical staining for confocal analysis. Free-floating sections were rinsed in 0.1M PBS then incubated in tris buffer, pH 9 at 60°C for 20 minutes. After rinsing, tissue was blocked with 2% normal donkey serum, 0.3% Triton-X 100 in PBS. All antibodies were diluted in blocking solution. Primary (rabbit anti-RFRP with mouse anti-GR (1:200, Affinity

BioReagents) or goat anti-CRHR1 (1:200, Santa Cruz)) was applied overnight, on rotation, at 4°C. The next day, sections were rinsed in PBS and incubated in secondary for two hours at room temperature (Cy3 anti-mouse with FITC anti-rabbit or Cy5 anti-goat with Cy3 anti-rabbit, 1:500, Jackson ImmunoResearch). After rinsing in PBS, sections were mounted on gelatin-coated slides and coverslipped/stored as above.

Confocal analysis. Twenty-five RFRP-ir cells were located in the DMH for each animal and assessed in z-series of <1.0 um slices to determine if CRH-R1 or GR was co-labeled. Confocal images were captured on a Zeiss 510 META/NLO confocal microscope with a 40x oil objective and adjusted for brightness and contrast using LSM Image Browser software. The wavelengths used were 488 nm, 543 nm and 643 nm.

Real-time reverse transcriptase PCR. Rats were lightly anesthetized with isoflurane and rapidly decapitated before bilateral hypothalami, pituitary and testes were dissected and flash frozen in liquid nitrogen. Real-time reverse transcriptase PCR was run on Trizol-extracted RNA with primers for rat RFRP and OT7T022. Ct values were determined using PCR miner [209] and normalized to the reference gene, RPLP. Primer sequences were designed using Primer1 software and checked for specificity using BLAST. For all stress studies, manufacturer instructions for SYBR Green One-step PCR kit (BioRad) were followed. For the ADX study, 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. Primer sequences are in supplementary methods

Statistical analysis. Group differences in RFRP mRNA and peptide expression were detected using two tailed t-tests. For the acute stress experiment, no stress controls were found to not differ significantly from acute stress, 24hr delay rats in mRNA ($P = 0.444$) or peptide expression ($P = 0.604$) so they were combined for statistical comparison with acute stress, 0hr delay rats. The effect of immobilization time on plasma corticosterone in the acute stress experiment was assessed using a repeated measures analysis of variance (ANOVA). In the chronic stress experiment, the effect of immobilization on plasma corticosterone was tested using a 2x4 (time of immobilization by day) repeated measures (within day) ANOVA followed by paired t-tests to detect differences on each day from day 1 baseline. Stress effect on day 14 plasma LH was detected using a two sample t-test. In the ADX study, planned comparisons (one sample t-tests) were used to detect a stress effect in RFRP expression in sham and ADX rats. Similarly, planned comparisons (two sample paired t-tests) were used to detect a stress effect on plasma corticosterone in sham and ADX rats. $P < 0.05$ was considered significant.

**Supplementary methods**

In situ hybridization. 30 µm slide-mounted coronal brain sections were fixed with 4% paraformaldehyde for 30 minutes, rinsed with 0.1M PBS then incubated for 30 min at 37°C in 10 µg/ml Proteinase K in PBS. Slides were rinsed, fixed for another 30 min, rinsed again and then soaked in 0.2N HCl for 10 min. After rinsing with distilled water three times and once with 2x SSC in 50% formamide, slides were incubated overnight with 200 ng/ml probe in 50% Hybrimix solution/40% formamide/0.1% SDS at 50°C (sense 5’-
UAUUACCAGCUGAGAGGAAUCCCAAAAGGGGUAAAGGAAAGAAGUGUCACUUUU
CAAGAACUCAAAGAUUGGGGGGCAAAGAAAGAUAUUAAGAUGAGUCCAGCCCCU
GCCAACAAAGUGCCCCACUCAGCAGCCAACCUUCCCCUGAGGUUUGGGAGGAACA

UAGAAGACAGAAGAAGCCCCAGGGCACGGGCCAACAUGGAGGCAGGGACCAUGA
GCCAUUUUCCCAGCCUGCCCCAAAGGUUUGGGAGAACAACAGCCAGACGCAUCAC
CAAGACACUGGCUGGUUUGCCCCAGAAAUCCCUGCACUCCCUGGCCUCCAGUGAA
UUGCUCUAUGCCAUGACCCGCCAGCAUCAAGAAAUUCAGAGUCCUGGUCAAGAGC
AACCUAGGAAACGGGUGUUCAC-3'; antisense: 5'-
GUGAACACCCGUUUCCUAGGUUGCUCUUGACCAGGACUCUGAAUUUCUUGAUGCU
GGCGGGUCAUGGCAUAGAGCAAUUCACUGGAGGCCAGGGAGUGCAGGGAUUUCU
GGGGCAAACCAGCCAGUGUCUUGGUGAUGCGUCUGGCUGUUGUUCUCCCAAACCU
UUGGGGCAGGCUGGGAAAAUGGCUCAUGGUCCCUGCCUCCAUGUUGGCCCGUGCC
CUGGGGCUUCUUCUGUCUUCUAUGUUCCUCCCAAACCUCAGGGGAAGGUUGGCUG
CUGAGUGGGGCACUUUGUUGGCAGGGGCUGGACUCAUCUUAAUAUCUUUCUUUG
CCCCCCAAUCUUUGAGUUCUUGAAAAGUGACACUUCUUUCCUUUACCCCUUUUGG
GAUUCCUCUCAGCUGGUAAUA-3'). The next day, probe was washed off using 40% formamide/5x SSC then incubated in 1x SSC and 2x SSC twice each for 15 min at 50°C. After rinsing in PBS, 1.5% DIG blocking solution in PBS (Roche) was applied for 30 min followed by anti-DIG antibody for 1 hour (Roche, 1:1000 in blocking solution). Following rinses in 0.05% Tween in PBS and in alkaline phosphatase buffer, sections were incubated overnight at room temperature in NBT/BCIP (Roche, 1:50 in alkaline phosphatase buffer). The NBT/BCIP reaction was stopped the next day using 20mM EDTA in PBS. No staining was evident in sections incubated in sense probe (data not shown). For quantification, images of RFRP+ cells were taken throughout the DMH and integrated optical density with background subtracted was calculated using ImageJ and averaged per cell for each animal.

Primer sequences.

RFRP: fwd: CCAAAGGTTTGGGAGAACAA; rev: GGGTCATGGCATAGAGCAAT

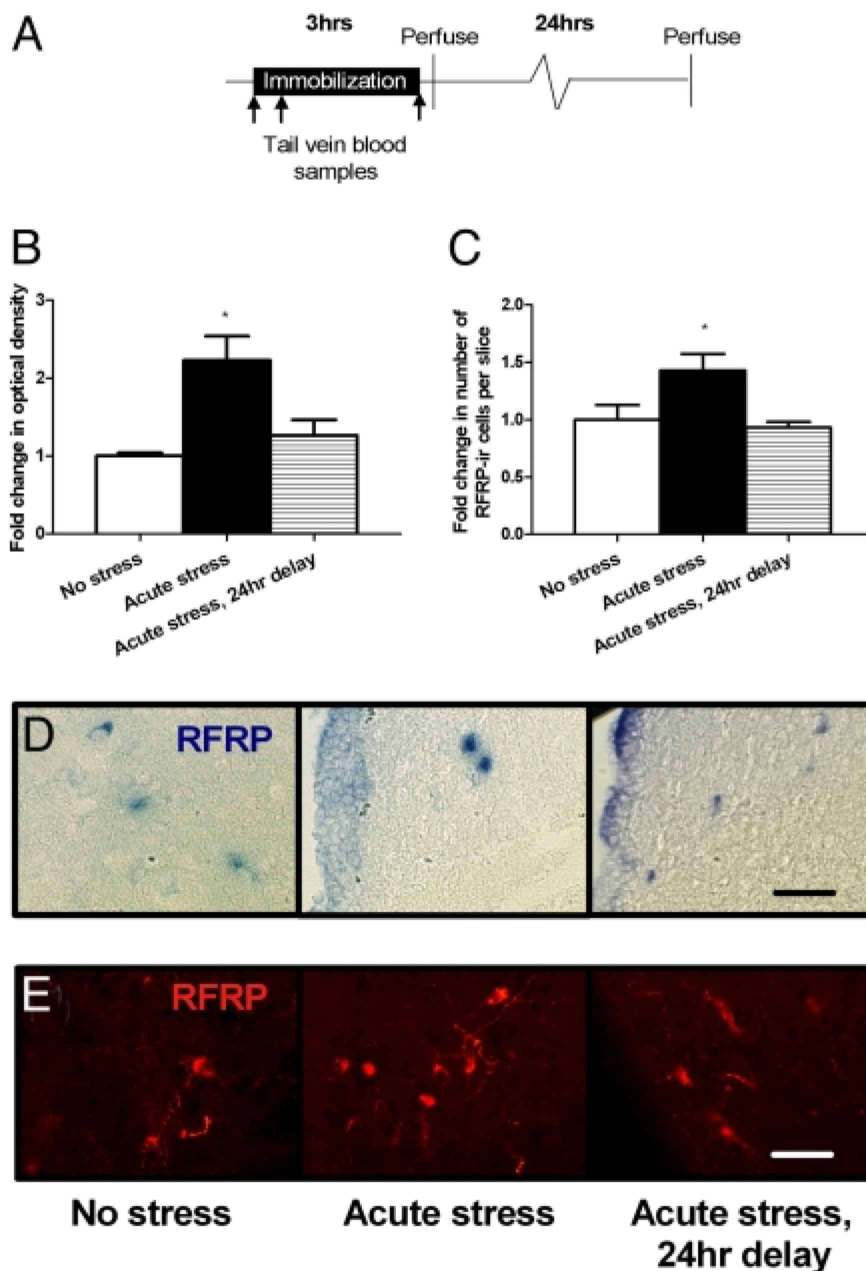
OT7T022: fwd: GGTCAGAACGGGAGTGATGT; rev: AGGAAGATGAGCACGTAGGC

GnRH: fwd: GCAGATCCCTAAGAGGTGAA; rev: CCGCTGTTGTTCTGTTGACT

RPLP (reference gene): fwd: ATCTACTCCGCCCTCATCCT; rev: GCAGATGAGGCTTCCAATGT

Equipment and settings. In situ hybridization and immunohistochemistry pictures for the acute stress study were captured using an Zeiss AxioPlan Imahger A.1 microscope with 40x air objective and a Zeiss AxioCam MRc5 camera. Images were equalized for brightness and contrast using Zeiss LSM Image Browser software. Immunohistochemistry images for the chronic stress study were captured using a Zeiss AxioVert 200M microscope with 40x air objective and Photometrics CoolSnap HQ camera. Images were initially acquired with Metamorph software and were re-colored with LSM Image Browser software.

**E. Figures**



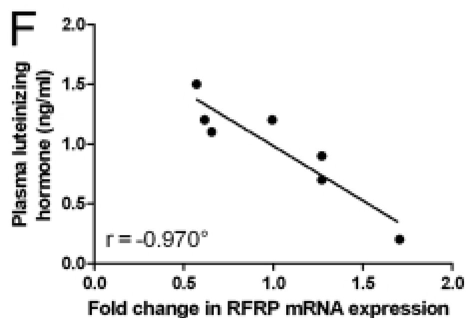




**Figure 4.1** Acute stress effects on RFRP expression. (A) Experimental time line. (B) Stressed rats showed higher RFRP mRNA expression levels (mean ± SEM) than controls immediately after stress (*P* = 0.002). (C) Stressed rats showed more RFRP peptide positive cells (mean ± SEM) than controls immediately after stress (*P* = 0.006). (D) Representative images of RFRP mRNA positive cells in the DMH. (E) Representative images of RFRP-ir cells in the DMH. (F) There was a significant negative correlation between fold change in RFRP mRNA and plasma-luteinizing hormone ($r = -0.920$, $P = 0.003$). *$P < 0.05$ compared with no-stress and acute stress, 24-hour-delay controls combined. °$P < 0.05$ correlation. Scale bar, 40 μM.

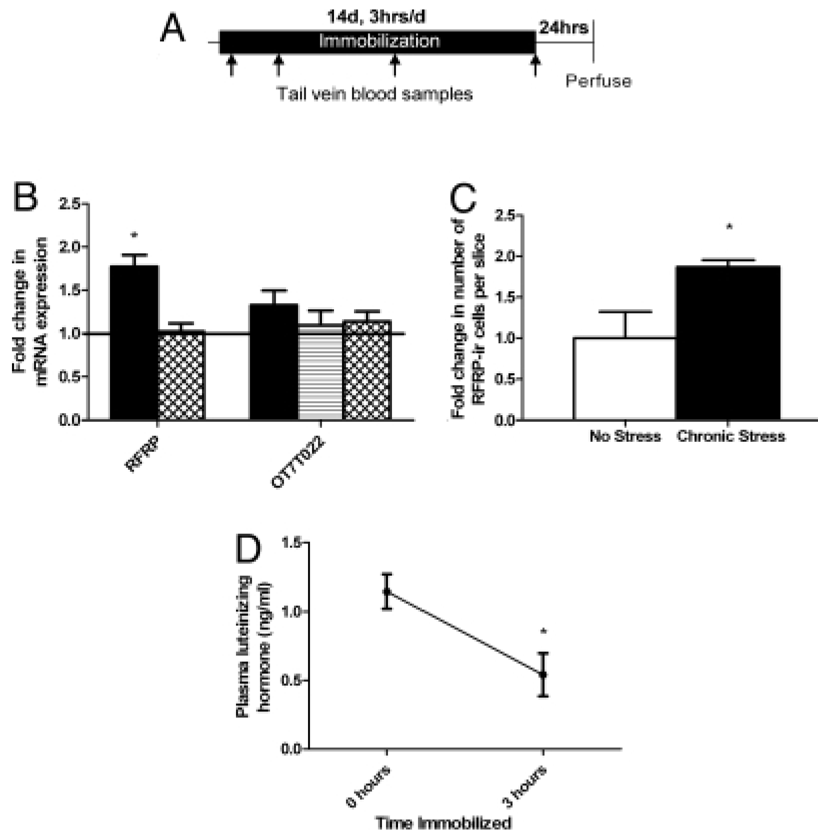
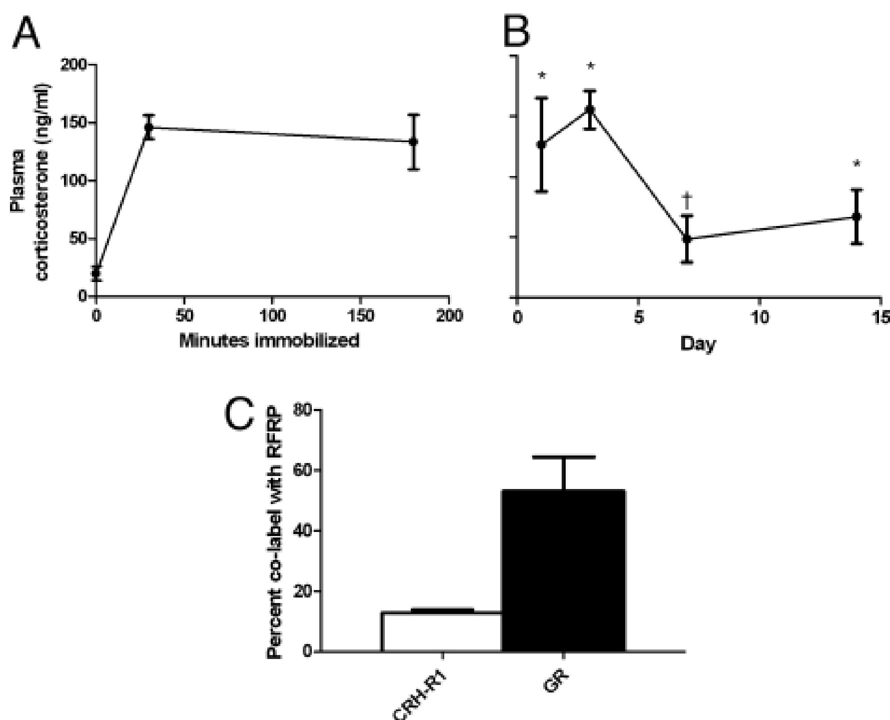
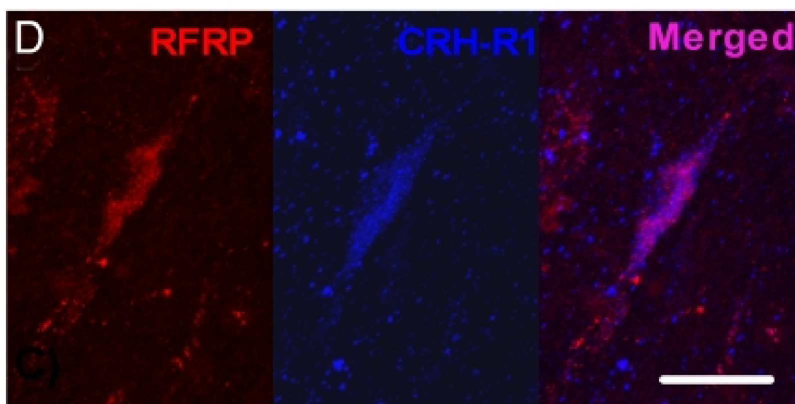



**Figure 4.2:** Chronic stress effects on RFRP expression. (A) Experimental time line. (B) Gene expression changes in the hypothalamus (solid bars), pituitary (lined bar), and testes (cross-hatched bars) after chronic immobilization, normalized to no-stress control levels. Immobilization led to an increase in hypothalamic RFRP mRNA expression (mean ± SEM, P = 0.007). No change was seen in hypothalamic, pituitary, or testicular RFRP receptor (OT7T022) or testicular RFRP expression (all P > 0.10). (C) Chronic immobilization led to an increase in hypothalamic RFRP-ir cell number in the DMH (mean ± SEM, P = 0.041). (D) Immobilization decreased luteinizing hormone (LH) on the last day of the stressor. Plasma LH levels after stress were significantly lower than those at baseline on day 14 (P = 0.023). *P < 0.05 no stress versus chronic stress or effect of time immobilized within day, as appropriate. †P < 0.10 effect of time immobilized within day. Scale bar, 40 μM.



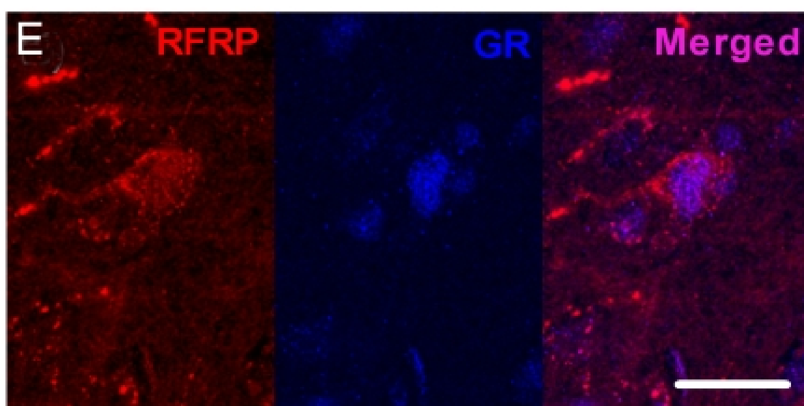




**Figure 4.3:** HPA responsivity of RFRP cells. (A) Three hours of immobilization led to a rapid increase in plasma corticosterone (mean ± SEM; P = 0.001). (B) Poststress corticosterone difference from baseline. Daily immobilization led to an increase in plasma corticosterone (P < 0.001 effect of time immobilized within day; P < 0.001 time by day interaction). Immobilization led to a significant increase in corticosterone (mean difference from day 1 baseline) on days 1 (P = 0.022), 3 (P < 0.001), and 14 (P = 0.030). The increase on day 7 was not significant (P = 0.054). (C) Percentage of RFRP cells co-expressing CRH-R1 and GR. (D) An RFRP-ir cell (red, first panel) co-expressing CRH-R1 (blue, second panel) in the adult rat DMH. (E) An RFRP-ir cell (red, first panel) co-expressing GR (blue, second panel). **P* < 0.05 effect of immobilization over baseline day 1. †P < 0.10 effect of time immobilized over baseline day 1. Scale bar, 20 μM.

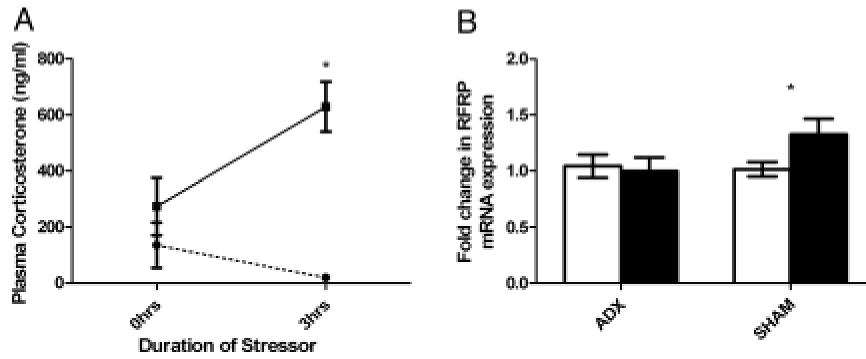



**Figure 4.4:** Adrenalectomy prevents stress-induced increase in hypothalamic RFRP expression. (A) Stress led to a significant increase in plasma corticosterone in sham-operated solid line, (P = 0.001) but not ADX rats dashed line, (P = 0.891). (B) Two weeks of daily immobilization led to a significant increase in RFRP mRNA expression in the hypothalamus of sham operated (P = 0.050) but not ADX (P = 0.794) rats. Open bar = control; closed bar = stress. * P < 0.05 stress versus no stress or 0 hours versus 3 hours of stress as appropriate.

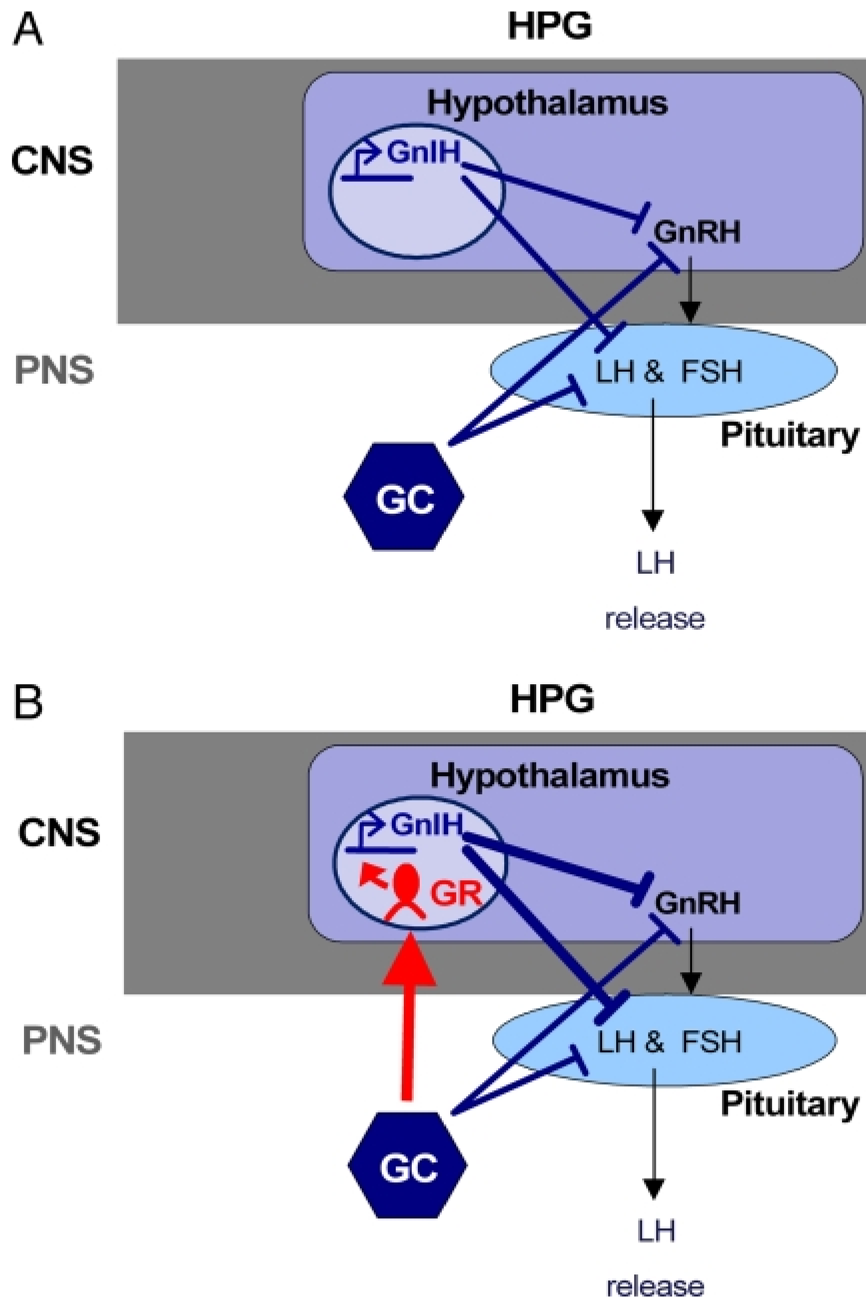
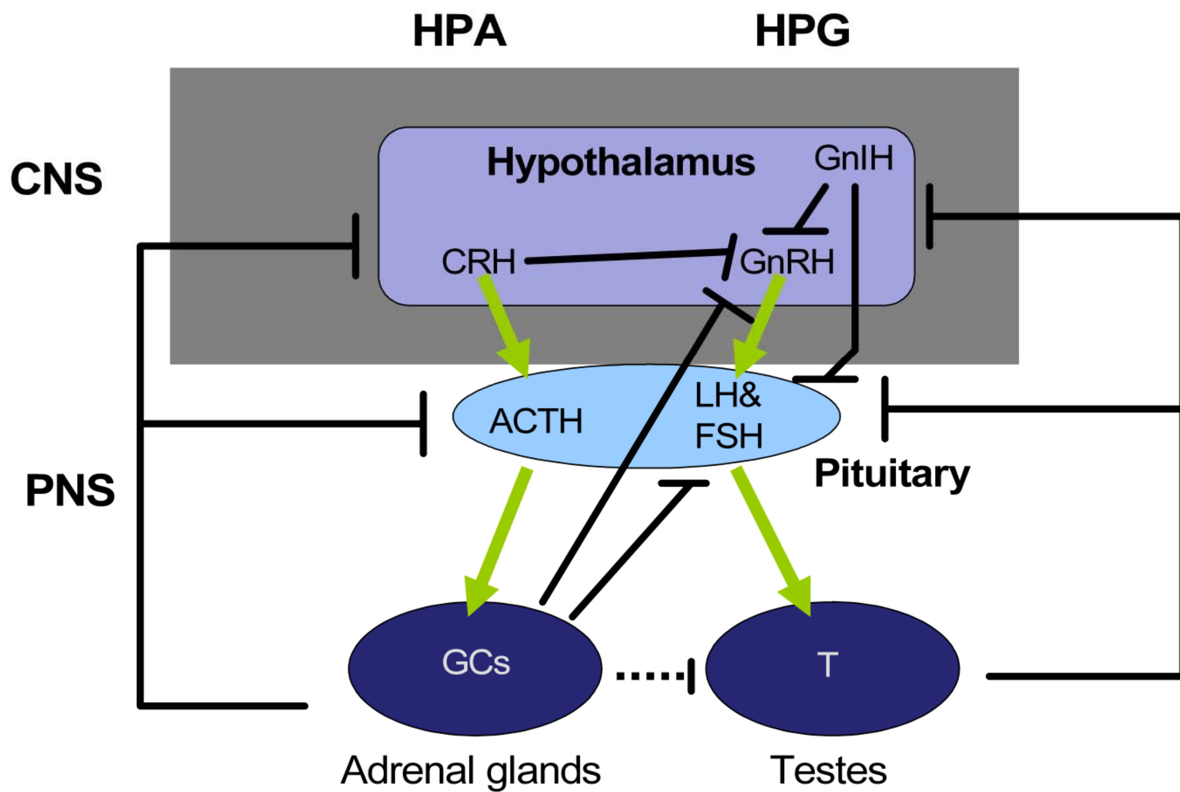



**Figure 4.5:** Model of how stress may affect the HPG axis through GnIH. (A) Previously, it was known that both GCs and GnIH inhibited the HPG axis independently. These interactions are shown in blue. (B) We propose that GCs released in response to stress act on GnIH via GR to increase the inhibitory actions of GnIH on GnRH secretion and/or pituitary sensitivity, resulting in decreased LH release. Our proposed pathway of GC–GnIH interaction is represented in red, joining the previously established independent effects of GCs and GnIH on reproduction. Arrows represent stimulation; Ts represent inhibition. CNS, central nervous system; PNS, peripheral nervous system.




**Supplementary Figure 4.1:** Multilevel interaction of HPA and the HPG axes, including GnIH contribution and negative feedback within each axis. Black Ts represent inhibition; dotted lines with T represent moderate inhibition; green arrows represent stimulation.

# Chapter 5: Discussion

## A. Stress and adult neurogenesis

Adult neurogenesis is highly responsive to environmental input. The preceding studies all confirm this environmental sensitivity, demonstrating reliance of new neuron proliferation on emotion information from the BLA. Our findings suggest that adult hippocampal neurogenesis is dependent on neural input from the BLA for maintenance of basal proliferation rates in a hemisphere-specific manner. They further demonstrate a role for newly born neurons in mediation of fear memory and BLA influence over stress and fear effects on neurogenesis.

Previous studies demonstrate several potential roles for newborn hippocampal neurons in mediating behavior. Knockdown models using both genetics and irradiation show dependence of spatial memory function on the population adult-born neurons in the hippocampus[119, 123, 210-212]. A role for new neurons in BLA-dependent contextual fear conditioning has also been described in several knockdown studies[57, 116, 117]. More recently, the first genetic model for enhanced neurogenesis has further demonstrated a role for new neurons in fear memory, showing better discrimination between similar contexts in fear conditioning when survival of newborn neurons is enhanced[213]. New neurons may be particularly important for this kind of fine discrimination; a recent genetic knockdown study showed spatial memory deficits only when small spatial discriminations were required[211]. We show activation of newborn neurons during a fear conditioning task, further supporting the hypothesis that these immature cells contribute to contextual fear memory. We also show that this activation is dependent on the BLA, consistent with the BLA-dependence of fear memory behavior. Further research is needed to better understand exactly what aspects of hippocampal memory function rely on neurogenesis.

New neurons could contribute to improved memory function in several ways. From about one week of age to four weeks, these immature neurons appear to be highly plastic, participating in hippocampal circuitry yet having lower thresholds for LTP than mature cells [81, 214]. By three months of age, the new neurons have matured into granule neurons indistinguishable from older resident granule cells, receiving synaptic normal input and sending projections to CA3 [16, 17, 30]. During the plastic, immature stage, these new neurons may be particularly apt to participate in new memory circuits. Previous studies show both preferential activation in response to previously encountered environments[130, 215] as well as dependence of several kinds of hippocampal memory on immature neurons in specific phases of their development[82, 117, 118]. In the present studies, activation of neurons during this plastic period was specifically targeted and the findings suggested integration of these immature cells into a new contextual fear memory. Future studies could further probe this sensitive period, determining when new neurons are most likely to be recruited into new fear memory circuits.

We show that the BLA can regulate both hippocampal neurogenesis and activity of those neurons in response to a behavioral task. The mechanism(s) by which the BLA influences the hippocampus are not clear, however. Several studies, including the present ones, have suggested that the BLA exerts influence over hippocampal plasticity through ipsilateral neural connections. Unilateral lesions of the BLA lead to impairment of hippocampal LTP in the dentate gyrus ipsilateral to lesion but not contralateral [114, but see: 216]. The BLA provides ipsilateral neural input to the dentate gyrus of the hippocampus through two pathways, relaying in either the medial septum or the entorhinal cortex. The medial septum receives glutamatergic inputs from the BLA and then in turn sends acetylcholingeric projections through the fimbria fornix to the

dentate gyrus [112]. Inactivation of the medial septum blocks the enhancing effects of BLA stimulation on hippocampal LTP, suggesting that this is the pathway responsible for BLA modulation of hippocampal plasticity. The locus coeruleus has also been implicated in this pathway, receiving cholinergic input from the BLA and sending noradrenergic projections to the medial septum as well as to the hippocampus directly [115]. Other studies have shown a role for the BLA-entorhinal cortex pathway as well, though, so it remains possible that both ipsilateral pathways play a role in BLA facilitation of hippocampal LTP [101].

Excitatory input, like that from the BLA, has been shown to increase neurogenesis [141]. This facilitation of neurogenesis may occur through activity-driven growth factor secretion. Neural activity in the hippocampus elevates several growth factors such as BDNF, IGF and FGF which are known to stimulate neurogenesis [141, 217, 218]. Interestingly, acute stress can stimulate both hippocampal excitation and growth factor secretion[58, 155, 156]. In agreement with this work, we found that acute stress increased hippocampal neurogenesis in a BLA-dependent fashion. Other studies of neurogenesis report the opposite, finding a reduction of proliferation following acute stress[30-32]. Some early studies also report negative regulation of neurogenesis by excitatory stimuli[33]. However, more recent work suggests a supportive role for excitatory activity[27, 140, 218, 219] and even for acute stress[151, 220, 221] in hippocampal cell proliferation. It is also worth noting that a number of studies show no effect of acute stress on adult neurogenesis[39, 153, 154]. This disagreement in the literature could stem from several sources, including inadvertent chronic stressors in the housing environment, different handling procedures, and strain/species differences. Future studies will address the reliability of stress-induced increase in hippocampal neurogenesis, as well as its mechanism. Currently, we predict that the enhancement of neurogenesis following acute stress in dependent on an activity increase that is mediated by the BLA.

Chronic stress, in contrast to acute stress, is a more reliable suppressor of adult neurogenesis[38, 41]. We found that chronic stress suppressed adult neurogenesis independent of the BLA. Most previous literature showing BLA-dependence of stress effects have relied on acute stress models. Therefore, it seems quite probable that chronic stress relies on a different mechanism (such as direct effects of prolonged stress hormone exposure on progenitors) to alter hippocampal neurogenesis than acute stress relies on.

When combined, the present data support an excitation-neurogenesis coupling model for regulation of new neuron proliferation in the adult hippocampus. Under basal conditions, the BLA provides glutamatergic and cholinergic excitatory input to the dentate gyrus, helping maintain the neurogenic niche and ensuring a supply of highly plastic new neurons via stimulation either of the progenitors themselves or of surrounding cells. The molecular mediators of this excitatory drive are unknown but likely involve one or many of the excitation-sensitive growth factors known to support adult neurogenesis. When the BLA is lesioned or silenced, this excitatory input is lost and the neurogenic niche no longer supports the same level of proliferation. Conversely, when acute stress provides excitatory input to the hippocampus, proliferation is stimulated. This acute stress excitation can be prevented by lesioning the BLA, suggesting that the excitatory drive of acute stress may come from the BLA.

One prominent question that remains from these data lies in the populations of cells affected by BLA activity during the proliferative and immature stages of neural development. Is there decreased fear memory-induced activation of new neurons following BLA lesion because 1) the population of cells that would respond to BLA input are selectively inhibited from proliferating or 2) because of the new neurons that survive into the plastic phase, fewer activate

without BLA excitatory input? In other words, is the loss in proliferative pool in any way cell-specific or is it simply a stochastic process determined by ambient growth factors, for example. Future studies will be required to address this complicated question.

### B. Stress and sexual reproduction

The reproductive system is acutely sensitive to a variety of environmental stimuli. Stress specifically negatively regulates all levels of the reproductive HPG axis, causing suppression of GnRH release from the hypothalamus, LH release from the anterior pituitary and testosterone release from the gonads. We show that in addition to these effects, stress-induced enhancement of GnIH expression may contribute another layer of stress regulation on the HPG.

The multi-level and cross-species effects of stress on reproductive function strongly suggest an evolutionary advantage to reproductive shutdown. Investment in sexual reproduction, from spermatogenesis to behavior, is energy intensive. It may be that sustained stress, and the consequent sustained elevation in GCs, are reliably indicative of environmental circumstances not conducive to successful procreation. Notably, the present studies dealt with male rodents which invest far less energy into reproduction than females. One might expect, then, that stress-related regulation of the reproductive axis in females would be even more prominent than that in males. Future studies will address this area of research, possibly leading to better understanding of reproductive dysfunction in this more vulnerable population.

### C. Summary

In summary, the brain is exquisitely sensitive to the environment it inhabits. From the external stimuli, through the electrical inputs to the molecules acting on individual cells, the brain can respond to a variety of inputs quickly and meaningfully. In some cases, that response is highly adaptive, supporting survival and future reproductive success. However, sometimes the response can become pathological, turning maladaptive and detrimental to survival and reproduction. The present experiments address several basic mechanisms by which the brain can respond to input, for better or for worse. Future work will expand on these findings, probing the mechanisms and significance of these findings.

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