Title
The role of RFRP-3 in chronic stress induced reproductive dysfunction and astrocyte communication

Author
Geraghty, Anna Christine

Publication Date
2015

Peer reviewed|Thesis/dissertation

The role of RFRP-3 in chronic stress induced reproductive dysfunction and astrocyte communication

by

Anna Christine Geraghty

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Integrative Biology

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:
Dr. Daniela Kaufer, Chair
Dr. George E. Bentley
Dr. Lance J. Kriegsfeld

Spring 2015

Abstract

The role of RFRP-3 in chronic stress induced reproductive dysfunction and astrocyte communication

by

Anna Christine Geraghty

Doctor of Philosophy in Integrative Biology

University of California, Berkeley

Professor Daniela Kaufer, Chair

Though it is well established that chronic stress induces female reproductive dysfunction, whether stress negatively impacts fertility and fecundity when applied prior to mating and pregnancy has not been well explored. My dissertation has investigated the mechanism behind stress-induced infertility in female rodents, as well as the long-term effects of chronic stress on reproductive success. Using naturally cycling female rats, Chapter 2 looks at changes in reproductive hormones and behavior after chronic immobilization stress. I also examined the long-term repercussions of chronic stress, allowing animals recovery from the stressors and looking at later mating and pregnancy success. My research has focused primarily on the role of a reproductive inhibitory hormone, RFRP-3, a hypothalamic peptide modulated by high stress. However, in Chapter 3, I investigate a new mechanism for RFRP-3 outside of that role, in hippocampal astrocytes. I see that RFRP-3 may also mediate an effect on how astrocytes connect and communicate with each other within the hippocampus. This opens a new intriguing line of research for novel roles of RFRP-3 outside of reproduction. These studies show that chronic stress has long-term effects on pregnancy success, even post-stressor, that are mediated by RFRP3, and point to RFRP3 as a potential clinically-relevant single target for stress-induced infertility.

Table of Contents

List of Figures and Tables

Acknowledgements

Many people were absolutely critical to my success in graduate school, and I would not be here at all without them. I have to thank the entire Kaufer lab, both past and present, for their help in my graduate career, and my development as a scientist in general. I have been in the Kaufer lab almost as long as it has been in existence and it has been the most incredible experience of my life. I am grateful for it every day. Though everyone has helped in some capacity, whether in training techniques or just general advice, a few deserve special recognition. Liz Kirby, a former graduate student, trained me from start to finish during my undergraduate career and I know without a doubt that if she hadn't picked me as the student to work with her during her rotation in the Bentley lab, I would not be here today. She was the best mentor I could ask for in the lab, and I am a hardworking and critical scientist because of her. She also made me a better mentor in turn. I owe you so much more than you could ever know. Sam Sakhai, Aaron Friedman, Soo Young Kim and Jeremy Hamilton were always supportive and helpful, whether it was complaining about graduate school or celebrating it. Kereshmeh Taravosh-Lahn was the best bay-mate and best bitch I could have asked for in lab, and I will miss our procrastination and laughs (and the lab zoo). I am so thankful you came to our lab. Our shenanigans were the greatest and I will miss spending every day with you. Not the spiders though. I could leave those behind.

There are no words to describe my appreciation for my incredible committee. I was lucky to have them, and they gave me not only great advice, but always were a fun and supportive committee, responsive even when they didn't need to be, and always making every meeting entertaining. Lance Kriegsfeld was a fantastic mentor, and teacher. His help in experimental design and advice was critical to my success. George Bentley was always a happy and fun member to have, and I will always be grateful that he, with almost nothing to base it on, gave me a position in his lab when I was a lowly sophomore in college and started me on this amazing path. His course, Comparative Endocrinology, was my first ever upper-division class, and he was the reason I found my love for endocrinology and research. I will always be grateful for that. Daniela, of course, is the greatest mentor I could have ever dreamed of. She always saw success in me, and supported me even when the research was struggling and I no longer believed in myself. She always gave me an office to complain in, a place to learn and a work-environment that was fun and supportive. I never felt I couldn't come to her, even for personal reasons, and her support of David and me in our early days (well, in all our days) was just amazing. I will miss being in her lab.

Graduate school isn't an island…. it definitely takes a village to make it through this madness. And my village was pretty incredible. I am so lucky to not only keep the friends I had, but also find even more compatriots through these six years to have some of the greatest times in my life. My mom and dad were always supportive, even if they had no idea what it was I was doing. Rachel Martin, the best roommate ever, saw me through those crazy early years, when I had no idea what I was doing, let alone why I was in grad school. She is my sister, and I love her for all the wine and TV marathons we had when things got tough. Our weekly dinners are still some of my favorite nights. Though when I first met her I thought she was crazy, Jenny Hofmeister became one of my closest friends, a person whose shoulder I cried on and laughed with, who helped me throw some of the best costume parties in the world and who will always grab boba when I just want to get out of lab. Livy Wilz, Scott Brier, Nicole Beier, Molly and Ben Gowen, you crazy bitches are some of the best people I have ever met, and I am so glad we

watch football together, and drink way too much crappy beer and have all these amazing adventures. Thank you for the stupid movie nights, the complaining sessions, the great camping trips, the wine and forcing me to begrudgingly like running. As we all finish up I can't wait to continue figuring it all out with you guys. I almost certainly wouldn't have made it through these last two years without your support and friendship.

David. You were the best part of it all. Meeting you made grad school worth it, and even if I learned nothing, I am glad I found you. You supported me through everything, every irrational freak out, every time my paper was rejected, every time an experiment failed. You celebrated with me when suddenly things finally worked. You cook for me when I am in lab till 11, and just eat cheese with me when I have no interest in leaving the couch. We may travel the world or just stay lazy, and through all of it, I am just so glad I am with you. I cannot wait to marry you this summer and see where this next part of the journey goes. I am so glad we are doing it together. While everyone on this list made me a better scientist, you made me a better human.

Chapter 1
Introduction

A. Introduction

It is well accepted that stress, as measured by increased glucocorticoid secretion, leads to profound reproductive dysfunction. In times of stress, glucocorticoids activate many parts of the fight or flight response, mobilizing energy and enhancing survival, while inhibiting metabolic processes that are not necessary in the moment. This includes reproduction, an energetically costly procedure that is very finely regulated. In the short term, this is meant to be beneficial, so you do not waste precious energy needed for survival. However, long-term inhibition can lead to persistent reproductive dysfunction, even if no longer stressed. This is because glucocorticoids orchestrate a large complex inhibition of the entire reproductive axis. Stress and glucocorticoids exhibits both central and peripheral inhibition of the reproductive hormonal axis. While this has long been recognized as an issue, understanding the complex signaling mechanism behind this inhibition remains somewhat of a mystery. What makes this especially difficult are attempting to differentiate the many parts of both of these hormonal axes, and new neuropeptide discoveries in the last decade in the reproductive field have added even more complexity to an already complicated system. Glucocorticoids (GCs) and other hormones within the hypothalamic-pituitary-adrenal (HPA) axis (as well as contributors in the sympathetic system) can modulate the hypothalamic-pituitary-gonadal (HPG) axis at all levels- GCs can inhibit release of GnRH from the hypothalamus, inhibit gonadotropin synthesis and release in the pituitary, and inhibit testosterone synthesis and release from the gonads, while also influencing gametogenesis and sexual behavior. This chapter is not an exhaustive review of all the known literature however is aimed at giving a brief look at both the central and peripheral effects on stress and reproduction.

B. Reproductive Physiology: A primer

Reproduction in mammals is a complex and precisely regulated hormonal process that requires the coordination of both the central nervous system and the peripheral reproductive organs for successful procreation. Negative and positive feedback signals tightly regulate the reproductive hormonal axis, also known as the hypothalamo-pituitary-gonadal (HPG) axis to maintain homeostasis. Perturbations in the axis, such as those caused by stress, can therefore have profound effects on reproductive ability, as even small changes can have large effects downstream, or even stop the axis in its tracks. Much of this chapter is focused on research conducted in rodents, however there are many species and sex differences in reproductive research indicating that stress can exert a myriad of effects to inhibit reproduction.

Studying the reproductive system of males offers a simplistic view of the HPG axis to begin examining reproductive physiology. Gonadotropin-releasing hormone (GnRH) is secreted from the hypothalamus in pulsatile release, crossing the hypophysial portal system into the anterior pituitary. In the pituitary, GnRH stimulates the synthesis and release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH and FSH circulate systemically to trigger testosterone release from the testes and gametogenesis. In turn, testosterone (T), as well and LH and FSH, negatively feedback on the axis to keep it regulated[1–6].

Females, however, present a much more complicated picture when thinking about the HPG axis. Unlike males, females experience hormonal surges to trigger ovulation, a phenomenon where estrogen switches from exerting negative feedback on the axis to positive feedback[7–12]. In rats, ovulation occurs once every four days. Similarly to males, GnRH is released from the hypothalamus, and LH and FSH circulate to the ovaries to trigger the release of

estradiol (E2) and progesterone (P) as well as development of the ovum[6,12–14]. Estradiol from the ovaries tightly regulates the HPG axis, negatively feedbacking onto the hypothalamus to inhibit GnRH release. However, when the developing ovum is near completion, and ovulation is due to occur, there is a switch in the hormonal system. Increasing estradiol secretion from the developing follicle triggers a change from negative to positive feedback of estradiol on the hypothalamus, resulting in an increase of GnRH secretion and leading to a surge of LH secretion from the pituitary to trigger ovulation. However, it was unknown what triggered the switch between negative and positive feedback.

Research in the last decade has shed more light on that switch during ovulation while also revealing that the axis is not nearly as simple as it appeared. There are currently two different hypotheses on what triggers the switch between negative and positive feedback in the HPG axis. Earlier research found that the estrogen surge initiates synthesis of progesterone receptors (PRs) in the hypothalamus, indicating progesterone is just as critical for ovulation as the estrogen surge[15–17]. However, studies on ovariectomized (OVX) and andrenalectomized (ADX) rats indicate that this progesterone does not come from the ovaries or adrenals, areas typical for steroid synthesis and systemic release. OVX rats given a normal dose of E2 can still exhibit LH surges[18]. Additionally, this surge can be blocked by administering trilostane, which inhibits the enzyme 3β- hydroxysteroid dehydrogenase (3β-HSD), an enzyme critical for synthesis of progesterone[18]. This data suggests that not only is pre-LH surge progesterone necessary for a successful LH surge, it is progesterone synthesized outside of the ovary and adrenals that regulates the surge. Hypothalamic progesterone synthesis, also known as neuroprogesterone, is a likely location, particularly hypothalamic astrocytes, which have been found to be the main source of neuroprogesterone in the hypothalamus, as they have all steroidogenic enzyme necessary for synthesis, regulate releasing factors and possess both ERα and ERβ receptors, which enable them to respond to the estradiol peak that precedes the LH surge[18–23].

While GnRH is the main coordinator of the HPG axis, secreting from the hypothalamus through the hypophysial portal system to the anterior pituitary to stimulate release of LH, there are many factors upstream of GnRH that can impact this release, preventing successful ovulation. Two in particular, directly in the HPG axis, that have been recently discovered are kisspeptin (KISS1) and gonadotropin-inhibitory hormone (GnIH)[24–32]. These two neuropeptides have opposing effects- KISS1 stimulates GnRH release from the hypothalamus[33–38], while GnIH inhibits it[28,39–43]. A proposed mechanism of KISS1 action in the neuroprogesterone model of the LH surge states that the peak of estradiol also triggers an increase of progesterone receptors (PRs) on KISS1 neurons in the hypothalamus which respond to the neuroprogesterone secreted from the astrocytes (activated also by estradiol to trigger the neuroprogesterone synthesis, likely through membrane estrogen receptors, specifically mERα). Neuroprogesterone, binding to the PRs on the KISS neurons, trigger the release of KISS, which then activates the GnRH neurons, leading to increased secretion of GnRH to trigger the LH surge from the pituitary[22,44–46].

Another hypothesis for the switch between negative and positive feedback of E2 in the hypothalamus argues that progesterone is not the driving factor of ovulation, but it is a derivative of progesterone, allopregnanolone, that modulates GnRH release from the hypothalamus via increasing glutamate release. This in turn could act on NMDA receptors on the GnRH neurons, stimulating GnRH release[47–49]. While it is still not clear exactly what mechanism is truly responsible for positive feedback of E2 during ovulation, the many regulators of GnRH and GnRH itself are all impacted by glucocorticoids, and stress can lead to a disruption of homeostasis that results in both short-term reproductive dysfunction and long-term infertility.

Stress, whether it is psychological or physical, and the HPA axis can inhibit the reproductive axis at every level, from the hypothalamus down to the ovaries or testes. Due to how closely regulated the HPG axis is, even small interferences in the hormonal milieu responsible for successful breeding can cause major dysfunction in the system. The female ovulatory system discussed above, as well as GnRH afferents such as GnIH and KISS, exhibit many points at which stress can disrupt the axis and cause fertility issues. While acute stress inhibiting the reproductive axis is found to be adaptive, preventing animals from breeding when times are not optimal for raising young, chronic stress and long-term shutdown of the reproductive axis can lead to prolonged dysfunction and infertility. We will examine how stress impacts each part of the reproductive axis individually, and how this can add up to detrimental fertility issues.

C. Glucocorticoids and the hypothalamus

Stress and high glucocorticoids have a profound negative impact on the reproductive system, and within the hypothalamus both corticotropin-releasing hormone (CRH) and corticosterone (CORT) affect GnRH and its afferents to inhibit reproductive success. As discussed above, the timing of ovulation in female rodents is highly regulated by many factors influencing GnRH. Stress can impact each one of these factors, combining to effectively shut down the reproductive axis at the very beginning. A difficulty in studying the effects of stress on reproduction is accurately differentiating between the effects of CRH and the effects of glucocorticoids such as CORT directly on stress, as well as determining specifically which level of the HPG axis is being directly affected. While it is well accepted that there are central effects of CRH directly on GnRH, completely separating those effects from downstream peripheral effects of CORT on GnRH is complex. Many manipulations of either CORT or CRH have a feedback effect on the other, and you can't rule out completely the other hormone. However, this section will try to help differentiate the two to better understand how stress influences reproductive success.

Direct regulation of GnRH by glucocorticoids

All central influences on reproduction driven by stress converge on GnRH, and an inhibition of the GnRH signal from the hypothalamus can occur both directly and indirectly. Even without affecting the peptides, steroids and neurotransmitters that can influence the GnRH surge, stress can directly inhibit GnRH release. To regulate LH secretion from the pituitary, GnRH is released from the hypothalamus in pulses, and modulation of the pulsatile release of GnRH affects downstream gonadotropin release from the pituitary. Many types of stress have been found to affect the pulse generator of GnRH, and both CRH and CORT have been implicated in this mechanism of action. In rodents, the small size of the hypophysial portal system between the hypothalamus and the pituitary has made it has been difficult to truly say that GnRH release itself has been inhibited, and many studies use downstream LH release as a proxy for inhibition of GnRH. Recent studies using sheep, however, who have a much larger hypophysial portal system than rodents, have allowed researchers to truly show that glucocorticoids inhibit GnRH release. Studies found that prolonged corticosterone administration caused a drop in GnRH release in the portal system of ewes[50] supporting the long-standing theory that glucocorticoids' central actions function to inhibit GnRH release from the hypothalamus. Intracerebroventricular (ICV) administration of CRH has been found to inhibit LH pulses in rats[51] and this response can be blocked or reversed by administration of CRH

antagonists[51,52]. It appears that this suppression is mediated in part by both CRH receptor subtypes, CRH-R1 and CRH-R2, however which receptor is predominant depends on the type of stressor utilized[53–55]. CRH axon terminals directly interact with GnRH dendrites, and *in vitro* studies have shown that CRH can inhibit GnRH release. Other studies have also shown that infusing CRH into the medial preoptic area of females rats lead to a 60% decrease in GnRH release from the hypothalamus[56,57]. Release has also been inhibited in hypothalamic explants incubated with CORT and DEX in a dose-dependent manner[58].

Populations of GnRH neurons have been found to express glucocorticoid receptors (GRs) directly in both mice and rats[59,60], indicating a method for which glucocorticoids (GCs) can act to directly inhibit GnRH synthesis and release. Effects of glucocorticoids have also been found to inhibit GnRH transcription[61–63]. Studies *in vitro* utilize GnRH specific lines such as the GT1 cell line, which synthesize and release GnRH[64,65] have shown that the GT1 cells do possess glucocorticoid receptors[66], and that GCs can represses GnRH gene expression and release from these cells[67]. Studies *in vivo* have shown that chronic CORT treatment can inhibit GnRH expression, leading to a decrease in serum LH levels[68]. This however, had no effect on FSH levels, nor did it affect gonadotropin mRNA levels (although further data on that is examined in more detail in the next section).

The effect of peripheral circulating glucocorticoids on GnRH and the LH surge is highly variable and likely dependent on the stressor. Many acute stress studies have found that glucocorticoids exert a range of effects on the LH surge that range from complete inhibition, to little or no effect to a positive activator of LH release. It emphasizes the importance of comparing stress paradigms and understanding how different stressors trigger the HPA axis. However chronic stress has been shown to inhibit the LH surge and ovulation consistently in the literature, indicating that while short-term stressors may exert variable effects on reproduction, likely due to the type of stressor, long-term stress reliably causes reproductive dysfunction (more of the effect of GCs on the LH surge in the next section).

There is also an effect of the stress-activated sympathetic system response in GnRH inhibition, as noradrenaline (NA) has also been shown to inhibit LH pulses, as well as GABA[69–72]. This review is focused on the endocrine interactions between the HPA and the HPG axis, and the effects of glucocorticoids specifically, however there is an entire field that could be discussed once GCs activate the sympathetic system. Regulation of reproduction is a complex, finely tuned process and much still needs to be identified in terms of precisely how stress regulates the system, both via the endocrine response as well as the sympathetic response.

Indirect regulation of GnRH by glucocorticoids

Glucocorticoids may also exert an inhibitory influence on reproduction and GnRH output by influencing hormones upstream of GnRH. This also makes it difficult to clearly determine experimentally how stress inhibits reproduction. Two hormones in particular have been recently identified to lie upstream of GnRH and respond directly to stress, affecting GnRH synthesis and secretion from the hypothalamus. Kisspeptin (KISS1) and gonadotropin-inhibitory hormone (GnIH), mentioned earlier, have opposing effects on GnRH. KISS1 is an activator of GnRH and plays a critical role in the maintenance of the GnRH pulse generator in the hypothalamus as well as the estrogen surge responsible for ovulation. Stress has been found to affect KISS1 signaling in multiple ways, however there is still much more research to be done to more closely examine precisely how stress affects KISS1 signaling. Many different types of stressors, including LPS treatment, acute hypoglycemia, restraint stress and isolation, lead to downregulation of KISS1

and KISS1r in the mPOA population of kisspeptin neurons, as well as decreases in KISS1 expression in the arcuate nucleus, leading to downstream decreases in LH secretion[73–75]. Kisspeptin neurons have also been shown to express CRH-R and GR, indicating that they can respond directly to stressful stimuli[76]. These changes can transmit downstream into inhibition of the GnRH pulse generator, which has been shown to rely on kisspeptin input, and indirectly to downstream inhibition of LH secretion from the pituitary.

A subset of neurons in the hypothalamus expressing kisspeptin has been implicated in modulation of the GnRH pulse generator. These neurons also colocalizes two other neuropeptides, neurokinin B and dynorphin (this group of neurons is also called the KNDy subpopulation) and this subpopulation has been shown to strongly respond to steroid feedback and project directly to GnRH neurons[77–80]. This subgroup offers even more targets in which stress and glucocorticoids can indirectly inhibit the reproductive axis. Dynorphin, an endogenous opioid in the brain signals through the kappa-subtype of the opioid receptor (KOR) in the hypothalamus, and administering dynorphin to female rats has been shown to inhibit LH pulses from the pituitary[81]. Stress has been shown to increase dynorphin release[82] which could lead to downstream inhibition of LH[83].

Gonadotropin-inhibitory hormone (GnIH) is another hormone upstream of GnRH that is regulated by stress to inhibit reproduction. GnIH was originally discovered in birds[28] and a mammalian orthologue, RFamide-related peptide-3 (RFRP3) has since been identified in many species including rats, mice, hamsters and humans[24,25,84]. RFRP3 inhibits the GnRH pulse generator, decreasing GnRH release from the hypothalamus and leading to decreased gonadotropin secretion from the pituitary[39,43,85]. Both acute and chronic immobilization stress in male rats were found to increase RFRP3 mRNA and peptide levels in the hypothalamus and adrenalectomy blocked this effect, revealing that this is due to circulating glucocorticoid levels[86]. This increase in RFRP3 by glucocorticoids led to downstream inhibition of LH release from the pituitary. *In vitro* studies utilizing an RFRP3 specific cell line has shown that RFRP3 neurons express the glucocorticoid receptor (GR) and also possess two glucocorticoid response elements (GREs) at the RFRP promoter region, further evidence pointing to a direct regulation of RFRP3 neurons by glucocorticoids[87,88]. These neurons in mammals can directly inhibit GnRH pulses from the hypothalamus, a response enhanced by high glucocorticoids after stress.

These are only a small sample of the possible indirect mechanisms of glucocorticoids on GnRH secretion. Much is still not well understood about the mechanism of action of glucocorticoids on GnRH levels, especially since there have been a slew of novel discoveries of peptides upstream of GnRH that increases the complexity of the regulation of reproductive neural systems by glucocorticoids. The difficulty in measuring GnRH levels from rodents has also made it difficult to come to concrete conclusions about the mechanism of action of GCs on GnRH. However this is an incredibly interesting field of research that is only growing as more novel techniques are being utilized.

D. Glucocorticoids and the pituitary

While many of the stress effects on GnRH can lead to downstream pituitary dysfunction, glucocorticoids can also directly influence reproduction at the level of the pituitary. This inhibition can happen via many different mechanisms, including changing the sensitivity of the pituitary itself to changes in GnRH secretion, a decrease in synthesis of the gonadotropes LH and FSH, as well as decreasing the secretion of LH and FSH from the pituitary. However, glucocorticoid effects directly on the pituitary in secretion and synthesis are highly variable,

indicating that the type of stressor and duration is very important when discussing this. This variability may stem from only looking at a part of the inhibition, such as focusing on just LH secretion as the output, rather than examining the mechanisms of synthesis and responsiveness of the pituitary itself.

Synthesis of LH and FSH is a highly regulated process involving not just GnRH levels, but steroid gonadal hormones as well[89,90]. LH and FSH are glycoprotein heterodimers that consist of a common alpha-subunit (aGSU) and a specific β-subunit for either FSH or LH[91]. As rat and mice gondatrope cells express GRs[92], it is likely that GR transcriptional regulation influences the synthesis of these subunits, both the specific β-subunits as well as the common alpha subunit to also regulate expression levels. Breen et.al. found that both daily immobilization stress and corticosteroid administration led to reduced LH-β mRNA levels, as well as decreased LH release from the pituitary *in vivo*. They also showed *in vitro*, using a gonadotope cell line, LβT2 that CORT decreases GnRH-induced LHβ mRNA levels as well as identified regions of the LHβ gene that respond transcriptionally to GR regulation, finding decreasing promoter activity in these regions in response to both CORT and DEX treatments[93].

Acute versus chronic stress exposure is the largest variable that must be considered when thinking about gonadotropin release from the pituitary. In fact, many studies of acute stress have found an activation of the HPG axis rather than an inhibition. Various studies utilizing multiple types of acute stressors on gonadotropin synthesis and release found that acute stress stimulated the HPG axis, leading to increased levels of LH, prolactin (PRL) and FSH in the plasma[94,95]. Others have found though that acute administration of glucocorticoids can reduce the LH peak in females[96,97] and injection of CRH peripherally has been shown to inhibit ovulation and block the LH surge completely[98]. Baldwin and Sawyer found that an acute injection of dexamethasone (DEX), a synthesized glucocorticoid that acts with high affinity on GR and mimics the effects of CORT, early in the estrous cycle of rats can delay the onset of ovulation. Administration of LH though, in addition to DEX, can recover the ovulatory event, indicating that this delay of the estrous cycle is due to DEX inhibiting the LH surge necessary for ovulation, rather than a problem within the ovary[99]. Differences in stressors, duration, sample collection and timing of the experiment may explain the differences found in LH release from the pituitary after acute stress and so it is hard to make concrete conclusions about acute stress and pituitary function in reproduction.

Chronic immobilization stress, on the other hand, has been shown to reliably reduce LH levels in both males and females[100–107]. One such study found that chronic immobilization stress reduced plasma levels LH, and prolactin (PRL) with no change in FSH. However, pituitary levels of LH and FSH increased, showing that synthesis and secretion are not always matched up[106]. This is critical when comparing studies- not all studies look at both synthesis and secretion simultaneously, which may also explain differences in findings. There is some debate that timing of sample collection is also critical here- some studies see an initial stimulation of LH but an inhibitory response later. One group found that after the first day of a chronic immobilization both female rats exhibited an increase in LH plasma levels, however by the end of the chronic stress experiment had eventually decreased below control values, supporting the theory that the acute stimulation of the HPG axis is only transitory[108], although male rats in the same study showed a decrease in LH levels at all times.

Glucocorticoids can also change the responsiveness of the pituitary to GnRH secretion. This may occur via regulation of the GnRH receptor in the pituitary, however other studies have shown that this can also occur independently of changes in the GnRH receptor[109]. Baldwin

(1979) found that stress affects estrogen feedback onto the pituitary, making it less responsive to GnRH secretion[110]. GnRH binding to its receptor on the gonadotropes is necessary to induce synthesis of the aGSU, LHbeta and FSHbeta subunits, as well as stimulate the dimerization of the subunits for successful synthesis and release from the gonadotrope cells. GnRH receptors appear to be transcriptionally regulated by GR[111,112].

This section focused predominately on the influence CORT and GR has on LH synthesis and secretion, while not really going into the effects of CORT on FSH levels. This is due to the even higher variability in the FSH response in regards to stress. FSH secretion levels are rarely affected by stress, especially acute stress, showing little to no change in most studies looking at it. This likely is due to other regulatory signals on FSH beyond corticosterone. Many studies, in fact, show increases in FSH beta mRNA post-stress, both after immobilization stress and corticosterone or cortisol administration in many species in both acute and chronic studies, sometimes accompanied by decreases in LH beta or with no change in LH at all[93,109,113–115]. This also happens with little to no change in actual FSH secretion, so it is hard to draw conclusions on how stress regulates FSH levels.

Glucocorticoid inhibition of reproduction at the level of the pituitary is a very complicated and confusing field of research with no clear answers. The range of studies utilizing different species, different sexes, a variety of stressors as well as different lengths of time of the stressors themselves is likely part of the cause of the confusion, however even replicating research is difficult. Some studies show that acute stress increases the pituitary gonadotropins, some find decreases in gonadotropins. Some studies show changes in the synthesis of the gonadotropins, but no change in release and others find that release is altered with no change in synthesis. Much research is still to be done in this area to fully elucidate how glucocorticoids affect the pituitary directly in terms of reproduction, however all research does agree that GCs have many possible effects here.

E. Glucocorticoids and the gonads

The final step in the HPG axis, the gonads, is yet another area in which GCs can regulate the HPG axis. In the gonads, GCs can act to inhibit many critical steps to complete the reproductive process. Corticosterone can inhibit steroidogenesis, inhibiting the synthesis of testosterone (T), estrogen (E) and progesterone (P), as well as directly inhibiting the release of these steroids from the gonads. They can modulate the expression of the LH-receptor (LHR) on the gonads, changing how the gonads may respond to LH and leading to downstream effects on steroids. GCs can also regulate gametogenesis, the development of mature sperm and ovum, to inhibit reproduction at the levels of the gamete. These effects can all be completed in the absence of influences from the hypothalamus and pituitary, emphasizing how profoundly stress can influence reproduction.

Glucocorticoids effects in the testes

Much of the early work understanding how GCs influence testicular function took place in men with Cushing's syndrome[116]. Patients with Cushing's syndrome have high circulating GC levels, either due to some sort of disease resulting in high secretion of ACTH from the pituitary or exposure to high GCs for long periods of time due to drug or steroid use. Cushing's patients presented an interesting way to study testicular dysfunction in response to high GCs because these patients have low circulating testosterone levels, but normal levels of LH release from the

pituitary, indicating that the decrease in T levels is due to effects of GCs on the gonads directly and not a downstream effect of pituitary dysfunction[117].

Research has shown that GR is localized to many cells within the testes, including importantly the Leydig cells, which is where steroidogenesis occurs within the testes, as well as in the primary spermatocytes and the epididymis[117–119]. This indicates that GRs can regulate not only steroidogenesis and the release of T, but spermatogenesis as well, either through affecting the primary population of cells or affecting that last steps of maturation in the epididymis. Both acute and chronic stress experiments have shown that high GCs inhibits testosterone secretion, spermatogenesis and libido[120–124] as expected. This effect is due specifically to circulating GC levels in the blood and action via GR because ACTH treatment in adrenalectomized animals fails to replicate these findings[125]. Some studies show that this decreased testosterone release can occur either via downregulation of the LH receptor in Leydig cells[126] or through inhibition of the enzymes necessary for testosterone biosynthesis[121,122,124,125,127–129]. Overall, these changes result in decreased testosterone synthesis and release from the gonads.

Glucocorticoids may also impact spermatogenesis, as GRs are present on the primary spermatocytes as well as within the epididymis. High glucocorticoids have been found to induce testicular germ cell apoptosis[130,131] as well as Leydig cell apoptosis[132], which has a profound inhibitory influence on male reproductive abilities. Chronic stress has been shown to also decrease the number of spermatids within the testis[133], and in humans it has been shown that chronic stress leads to decreased sperm numbers, likely through a combination of the above responses[134]. Expression of GR in all these spermatogenic area indicates that glucocorticoids can act directly on the testes to regulate sperm production. Stress and high levels of GCs likely inhibit reproduction both indirectly and directly at the level of the gonads, with decreased secretion of LH from the pituitary decreasing testosterone release, and direct inhibition of testosterone synthesis and sperm production by GCs.

Glucocorticoid effects in the ovaries

The role of glucocorticoids within the ovary is somewhat more complicated than it is within the testes. Rather than a straight inhibitory role of GCs on ovarian function, some GC effects are actually beneficial to the ovaries and are necessary for maintenance of the follicular development pathway. During each cycle, many follicles are activated for development within the ovaries, however not all fully develop to maturity and it appears GCs are an active part in that selection process. This is necessary for normal ovarian function, but likely is very finely controlled, and high stress may tip the balance between a “good” level of GCs and a “maladaptive” level that leads to ovarian dysfunction.

A way in which the ovaries can control levels of GCs through follicular development during the female estrous or menstrual cycle is via expression of 11beta-hydroxysteroid dehydrogenase (11beta-HSD). Researchers have identified that many of the cells within the ovaries, including the follicles and corpus luteum, express 11beta-HSD1, 11beta-HSD2 and GR[135–139], indicating that there are possibly many regulatory effects of glucocorticoids on follicular development and ovarian function. Interestingly, the ovaries differentially regulate 11beta-HSD1 and HSD2 throughout the cycle. 11beta-HSD2, which inactivates GCs, is highly expressed in developing follicles in the ovary, while 11beta-HDS1, which activates GCs, is highly expressed in follicles that have been luteinized, meaning they have been activated by an LH surge and ready for an ovulatory event[135,140,141]. This indicates that the ovaries upregulate 11beta-HSD2 while developing in order to inactivate GCs present in the ovary while the follicles

are maturing in order to enhance development, but choose to activate circulating GCs once the follicle is released for ovulation. These activated and functional GCs may act as an anti-inflammatory response triggered by the rupturing of the ovarian surface epithelium during ovulation[142,143]. These two examples show how GCs are likely necessary for normal function of the ovaries, however their levels are tightly regulated via variability in expression of the 11beta-HSD1 and 2. These enzymes are actually manipulated via gonadotropin signals from the pituitary, with LH controlling expression of 11beta-HSD1 expression (thus activating GCs during ovulation). This regulation via gonadotropins provides a mechanism through which excess GCs could influence enzymatic regulation of GCs. As these two enzymes are so narrowly regulated during the ovarian cycle, stress and high GC secretion from the adrenals can easily dysregulate these signals and cause profound fertility problems in both ovarian function during ovulation and uterine function during fertilization, implantation and pregnancy.

In the ovaries, high amounts of GCs, surpassing the amount that is typically inactivated by 11beta-HSD2, can suppress LH function and inhibit estrogen release and synthesis[140,144,145]. Studies both *in vivo* and *in vitro* have shown that GCs can influence not only LH response, but also inhibit transcription of the enzymes necessary for steroid biosynthesis, critically inhibiting p450 aromatase, necessary for conversion of testosterone to estrogen. In rat granulosa cell cultures, FSH triggers the increase of aromatase activity, promoting estrogen synthesis for ovulation. Administration of both CORT and DEX inhibited this FSH-induced increase, however stimulated progesterone synthesis and did not inhibit pre-existing aromatase function. This indicates that GCs act to inhibit induction of aromatase activity specifically, not necessarily affecting granulosa cell function as a whole[146]. Glucocorticoid treatment was also found to decrease LH receptor in cultured granulosa cells[147], indicating that GCs can act directly on the ovarian cells to decrease FSH-stimulated functions, including aromatase activity and LH receptor binding.

Interestingly, GCs effects on oocyte maturation appears to be species dependent. Studies in humans and pig have shown that GCs can inhibit meiotic development in the oocytes[148,149], however studies in sheep and mice have shown no effect of GCs on final oocyte maturation[150,151]. However a recent study in mice showed that high levels of CRH in the serum of the ovaries due to restraint stress induced ovarian apoptosis, decreasing follicular development independent of GR. This increase of CRH was acting on thecal cells in the ovary, decreasing testosterone and estrogen levels and increasing progesterone, creating a hormonal imbalance between estrogen and progesterone that led to decreased oocyte success[152]. These differences are likely due to problems intrinsic to *in vitro* models that utilize only the ovarian granulosa cells. In addition, another *in vivo* study in mice utilizing predatory stress found that while high GCs did not affect oocyte maturation, blastocyst formation was significantly decreased in these mice, showing that GCs may have a stronger effect on embryo development or the oocyte potential for fertilization, rather than maturation of the oocytes in general[153]. The next section will explore GCs effects on pregnancy and fertilization more closely.

The role of glucocorticoid function in the ovary is incredibly complex and narrowly regulated. The actions of GCs are regulated via differential transcription of the two 11beta-HSD enzymes, transcription of which is controlled through gonadotropin release form the pituitary. Glucocorticoids are critical for maintenance of ovarian function, involved in functional apoptosis of follicles to maintain normal follicular development, as well as its anti-inflammatory role necessary for ovulation to occur. However, high stress can tip the scales from functional to dysfunctional, overwhelming the ability of 11beta-HSD to regulate GC levels and causing

ovarian problems ranging from a decreased responsiveness to LH levels, decreased synthesis of estrogen due to inhibition of aromatase release, and potentially inhibiting the final step of oocyte maturation.

F. Glucocorticoids, Implantation and Pregnancy Success

Even if an ovum can be successfully developed in times of stress, and the HPG axis still functional enough to trigger ovulation, GCs can still act to influence the uterus to prevent successful implantation and completion of pregnancy. Glucocorticoids typically act in opposition to estrogenic actions, and this becomes increasingly critical in implantation. For successful implantation of a blastocyst, progesterone and estrogen regulate uterine cell proliferation, and are necessary for the changes in both the blastocyst and uterine epithelium for successful adhesion. Glucocorticoids inhibit estradiol-stimulated uterine growth and decreases estrogen receptor concentrations in the uterus[154–157].

Pregnancy itself requires a delicate immune balance and regulation of the maternal immune cells in order for survival of both the fetus and the mother. It is suggested that the high levels of progesterone (P) released from the corpus luteum of the ovary after ovulation and sustained by the placenta throughout pregnancy help regulate the mothers immune system. There is some research indicating that membrane-bound progesterone receptors act to inhibit maternal T-cell during pregnancy[158,159]. This combines with a series of other downstream immune events that allows the maternal immune system to accept the foreign fetus and expression of progesterone and related progesterone factors such as progesterone-induced blocking factor (PIBF) continues to increase through pregnancy. High levels of Th1 cytokines in mice have been shown to be abortogenic, and progesterone during pregnancy binding to progesterone receptors have been shown to release PIBF, which in turn decreases natural killer (NK) cells in the uterus and induces Th2 cytokine development, changing the balance towards an anti-abortive immune response[158–162]. However studies using restraint stress in rodents has found that stress early in pregnancy leads to decreased embryo success, showing higher abortion rates in the mice and smaller litter sizes[163–165]. Wiebold et.al. found that this was due to decreases in corpora lutea, lower levels of serum progesterone and fewer implantation sites[163]. Administering a progesterone derivative, dydrogesterone, during stress reverses the abortive stress effect, and administering it before a stress event itself prevents the decreases in progesterone receptor and decrease in PIBF, preventing the stress effect even with lower P levels in the serum[164,165]. This has even been found to improve pregnancy success in women prone to frequent miscarriages[166,167].

Glucocorticoids play a significant role in pregnancy maintenance, opposing estrogen's ability to ready the uterus for implantation and inhibiting progesterone's anti-abortive immune response. While much of research into this focuses on stress during the pregnancy itself, there could be long-term effects of stress prior to the pregnancy that could affect pregnancy success as well, maybe via long-term inhibition of progesterone.

G. Conclusion: Stress and its many effects on reproductive ability

Physiologically, glucocorticoids exert many effects on surrounding cells and are necessary for life. Within normal ranges, GCs regulate homeostasis and are critical for our stress response. In times of stress, high levels of GCs shut down physiological processes not relevant for survival in that time, including reproduction. GCs and the HPA axis can act upon every level of the HPG axis, both directly and indirectly inhibiting gonadotropin release from the pituitary

and exerting direct effects on the gonads. Stress and high GCs decrease the release of GnRH from the hypothalamus, either by directly inhibiting GnRH pulses or inhibiting upstream regulators of GnRH release. This can lead to downstream decreases in LH release form the pituitary, however GCs can also directly inhibit the synthesis and release of gonadotropins from the anterior pituitary. The decrease of LH and sometimes FSH from the pituitary can decrease steroid release form the gonads, and circulating GCs can also act directly on the gonads to inhibit the transcription of enzymes necessary for gonadal steroid biosynthesis. There are sex and species differences in all these responses, and this review was only an introduction into the research on stress and reproduction. It is a complex and confusing field, however new techniques utilizing cell-specific knockdowns of GR and/or other peptides involved in this response can help clarify the more specific roles of GCs and reproductive dysfunction. This becomes increasingly important as we find that infertility rates continue to increase in humans, likely due to high anxiety in day-to-day lives. Understanding the molecular mechanisms behind how stress impairs fecundity and reproductive success, especially in females, is critical to helping improve fertility rates.

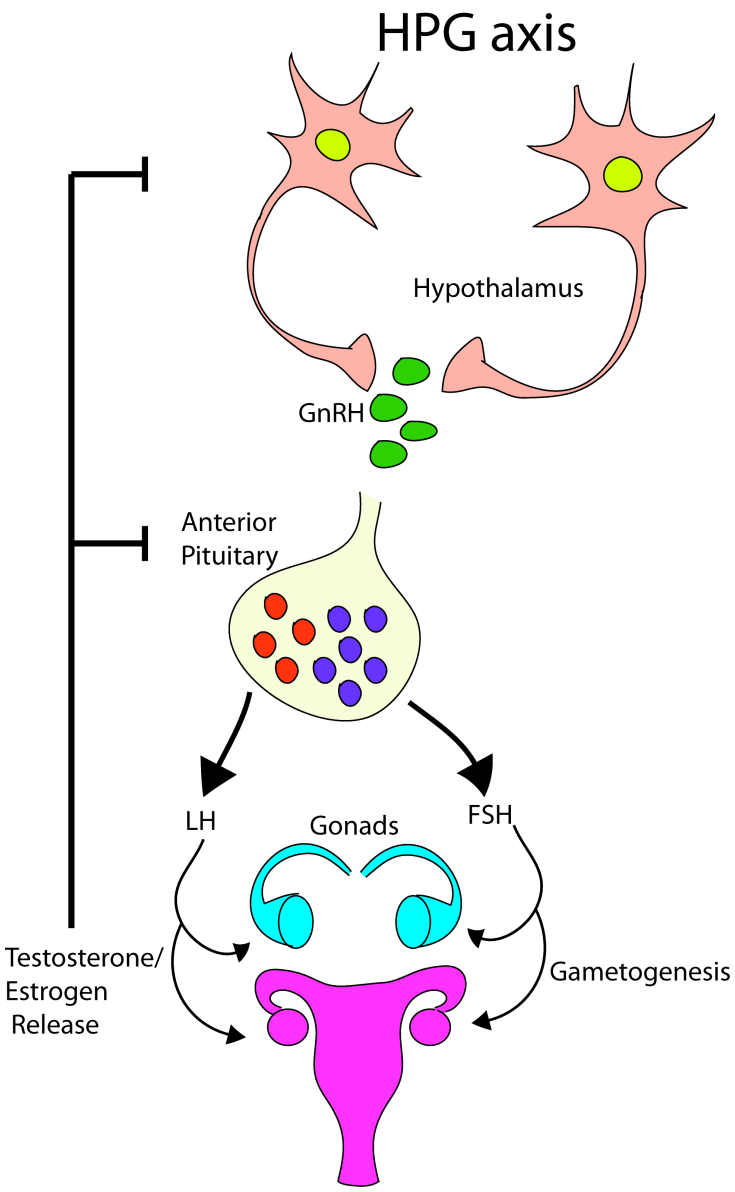

Figure 1: Summary of HPG axis. Hormonal control of the hypothalamic-pituitary-gonadal axis, or reproductive axis. GnRH is release from the hypothalamus, triggers release of LH and FSH from the pituitary, which travels systemically to release sex steroids and maintain gametogenesis in the gonads. Negative feedback regulates this axis.

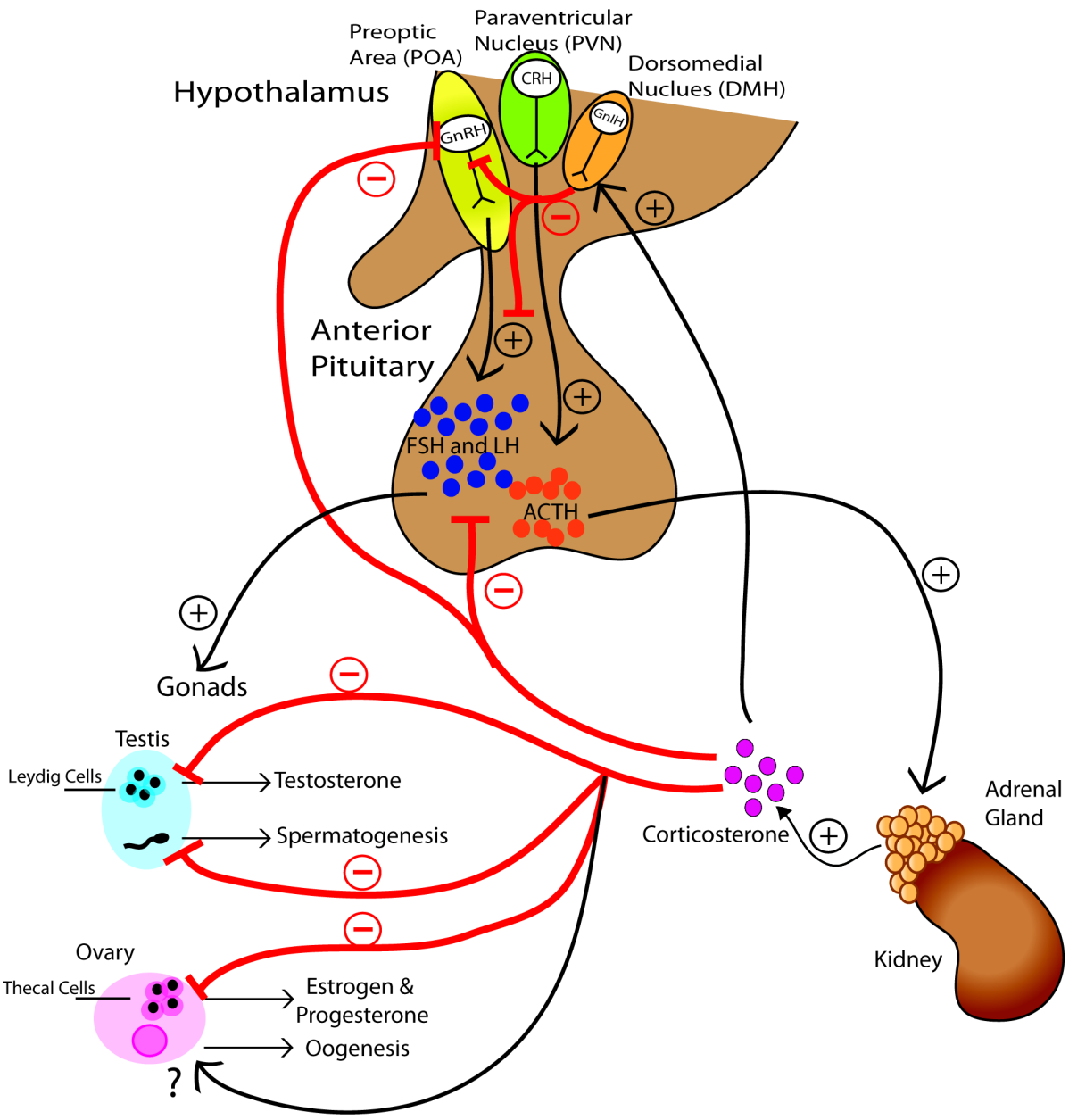

Figure 2. HPG and HPA axis interaction. The HPA axis inhibits HPG axis at all levels of the hypothalamus

Chapter 2:
Knockdown of hypothalamic RFRP3 prevents chronic stress induced infertility and embryo resorption

Knockdown of hypothalamic RFRP3 prevents chronic stress induced infertility and embryo resorption

From: Geraghty, A. C., Muroy, S. E., Zhao, S., Bentley, G. E., Kriegsfeld, L. J., & Kaufer, D. (2015). Knockdown of hypothalamic RFRP3 prevents chronic stress-induced infertility and embryo resorption. *eLife*, *4*. doi:10.7554/eLife.04316

Abstract: Whereas it is well established that chronic stress induces female reproductive dysfunction, whether stress negatively impacts fertility and fecundity when applied *prior* to mating and pregnancy has not been explored. Here we show that stress that concludes four days prior to mating results in persistent and marked reproductive dysfunction, with fewer successful copulation events, fewer pregnancies in those that successfully mated, and increased embryo resorption. Chronic stress exposure led to elevated expression of the hypothalamic inhibitory peptide, RFamide-related peptide-3 (RFRP3), in regularly cycling females. Remarkably, genetic silencing of RFRP3 during stress using an inducible-targeted shRNA completely alleviates stress-induced infertility in female rats, resulting in mating and pregnancy success rates indistinguishable from non-stress controls. We show that chronic stress has long-term effects on pregnancy success, even post-stressor, that are mediated by RFRP3. This points to RFRP3 as a potential clinically-relevant single target for stress-induced infertility.

A. Main Text:

High psychological stress inhibits reproductive function when both occur concomitantly [56,168–170]. From an evolutionary perspective, inhibition of reproductive function by acute stress may be adaptive, delaying reproduction in times of duress or resource scarcity [170,171]. Chronic stress, however can result in persistent, maladaptive sexual dysfunction and suppressed fertility [172]. Little is understood about the lasting effects of stress exposure. For example, after its cessation, can a prior, persistent stressor have long-term negative after-effects on reproductive health? In humans, a high-stress environment may be a significant barrier to sexual well-being and childbearing**.** In healthy couples under 30 years of age, 63-80% are unable to conceive within three months of attempting, and within one year of attempting pregnancy, 15% of couples remain unable to conceive [173]. A molecular framework to understand the long-term effects of stress on female reproduction, and its implications for human health, is currently lacking.

The present series of studies sought to answer two main questions: 1) Do stressful events negatively impact female reproductive function even following recovery of the stressor, and, if so, 2) are the deficits observed mediated by stress-induced elevation of the inhibitory neuropeptide, RFamide-related peptide-3 (RFRP3)? To our knowledge, no study to date has elucidated the molecular mechanisms of stress-induced infertility, nor has there been any investigation of long-lasting after-effects of pre-conception stress on reproductive success and pregnancy outcome. RFRP3, the mammalian ortholog of gonadotropin-inhibitory hormone (GnIH) first identified in Japanese quail (Tsusui et al., 2000), is common across mammals, including rats and mice [27,174,175], hamsters [24], non-human primates [26] and humans [25], and is a hypothalamic hormone that directly inhibits the firing of kisspeptin-sensitive gonadotropin-releasing hormone (GnRH) neurons in the hypothalamus in mice [42,176]. It also reduces downstream luteinizing hormone (LH) secretion in rats [177,178], mice [179] and hamsters [175]. There is some debate as to whether RFRP3 is a hypophysiotropic hormone [39,86,178,180] or only centrally

inhibits GnRH to elicit a response [181,182]. Regardless of its mechanism of action, RFRP3 decreases the synthesis and release of pituitary gonadotropins, LH and follicle stimulating hormone (FSH), in many species, including rats and mice [43,85,177–179,183,184]. In females, RFRP was shown to be regulated throughout the ovulatory cycle in rats and hamsters and it elicits a marked inhibitory effect on the preovulatory LH surge through inhibition of GnRH activation in rats [185]. In male rats, RFRP3 expression is elevated 24 hours after a chronic stressor, suggesting that RFRP3 may mediate enduring changes in reproductive function [86]. Levels of the glucocorticoid stress hormone (in rodents, corticosterone) may mediate this effect; RFRP3 neurons in the rat hypothalamus were shown to express glucocorticoid receptor (GR) (21), as well as RFRP-expressing neuronal cell line *in vitro* (29). Finally, the RFRP promoter region includes two glucocorticoid response elements (GRE)s, all together supporting the hypothesis that RFRP may be directly regulated by circulating glucocorticoid levels [87]. Together, these findings provide support for the notion that stress-induced increases in RFRP3 might have long-lasting negative impact on female reproductive functioning. Despite knowledge of RFRP's responsiveness to stress and its role in regulating reproductive axis activity, no study to date has established a causal link between RFRP and fertility in any species. We set out to test the potential role of RFRP expression in stress-induced infertility in females.

B. Results

In sum, we found that chronic stress led to elevated RFRP3 at all stages of the ovulatory cycle. This elevated level of expression persisted after a full cycle of recovery from stress, indicating that the impact of stress on RFRP3 lasts well beyond removal of the stressor. Stressed females exhibited fewer successful copulation events, fewer pregnancies in those that did successfully mate, and increased frequency of embryo resorption in the achieved pregnancies. These marked effects of stress on fertility were completely blocked by knockdown of RFRP3, even though RFRP3 function was restored following stress cessation. These findings indicate that stress has lingering negative consequences for female reproductive function that are mediated by a transient rise in RFRP3.

Female rats were subjected to an 18-day stress paradigm followed by quantification of hypothalamic markers of reproductive function either immediately after stress exposure or after one full estrous cycle (4 days) of recovery (Fig 2.1A). Serum levels of corticosterone (CORT) were measured on days 1, 4, 7, 11 and 18 of the 18 days immobilization stress paradigm, and on day 22, 4 days after the cessation of stressor. Baseline levels at the onset of stress exposure sessions were unchanged throughout the 18 days. However, CORT levels were significantly elevated in samples drawn on days 1, 4, 7 and 11 at the end of the three-hour stress exposure.

On day 22, after 4 days of recovery from the stressor, the stressed rats exhibited serum CORT concentrations indistinguishable from baseline values (Fig. 2.1B). Rats exhibit a 4-5 days long estrous cycle, with rising estrogen concentrations triggering a surge of luteinizing hormone (LH) to initiate ovulation, and estrogen and progesterone driving sexual receptivity on the night of proestrus [186]. Stress acutely inhibits the LH surge [106] and subsequent sexual receptivity and fertility [187,188]. However, it is unknown whether reproductive function continues to be negatively impacted even following recovery from stress (defined as exhibiting baseline levels of CORT after four days of no stress exposure). Rats were monitored daily by vaginal smear to determine whether estrous cyclicity was affected during application of the stressor and to allow separation

of animals into different cycle stages (diestrus, proestrus and estrus) at the termination of the stressor. Stress did not affect estrous cyclicity, with all animals exhibiting normal vaginal cytology throughout the stressor. At all estrous cycle stages, RFRP3 mRNA expression in the hypothalamus was significantly elevated both 0 and 4 days after the stressor was terminated (Fig 2.1C and D). Hypothalamic expression of the RFRP3 receptor, G-protein-coupled receptor-147 (GPR147), was also upregulated after stress during all stages of the cycle, and returned to baseline values after the cessation of stress (Fig. 2.1E and F). We did not find significant differences in either GnRH or kisspeptin (KISS1) mRNA expression post-stress in any stage of the cycle (Fig. 2.1G-J). However, hypothalamic samples were taken from whole hypothalamus, precluding the ability to differentiate between rostral and caudal kisspeptin cell populations, potentially masking subtle differences. Notably, the persistent increases in the expression of both RFRP3 and its receptor specifically in proestrus coincide with the cyclical onset of sexual receptivity, suggesting that RFRP3 provides a mechanistic basis for long-lasting suppression of reproductive behavior after stress.

To investigate whether the stress-induced increase in RFRP3 plays a causal role in prolonged sexual inhibition, we developed a conditional viral vector to knock down RFRP3 expression (tet-OFF lentivirus RFRP3 shRNA) *in vivo* during the strictly-defined time window of the chronic stressor. This lentiviral construct expresses RFRP3 shRNA from a constitutively active CMV promoter, driving both shRNA and blue fluorescent protein (BFP) marker expression. When exposed to doxycycline (DOX, via drinking water) (Fig. 2.2A), the tet-Off element is prevented from driving TRE-initiated transcription and both shRNA and BFP production cease (location and extent of viral infection can be seen in Fig. 2.2B and C). Stereotaxic infusion of RFRP3 shRNA lentivirus into the hypothalamus led to an 87% down-regulation of RFRP3 mRNA expression within 7 days relative to a control scrambled shRNA (Fig. 2.2D). Immunohistological labeling verified that the peptide level in the hypothalamus was similarly knocked down by 85% compared to scrambled control virus, measured 2 weeks following viral injection (Fig. 2.2E, representative images of RFRP labeling with either scramble or RFRP-shRNA virus and pre- and post-DOX administration Fig. 2.2F-I). Critically, administration of doxycycline in the drinking water restored RFRP3 mRNA to normal levels within 4 days (Fig 2.2D). This viral vector system permitted knocking down of RFRP3 expression during chronic stress, and restoration of RFRP3 during the later stages of copulation, mating and birthing, which may rely on RFRP3 function in unknown ways.

A second group of female rats received dorsomedial hypothalamic injections of either RFRP3 shRNA or a scrambled control shRNA lentivirus three weeks before the 18 days of immobilization. Estrous cycles were monitored for each rat with immobilization timed to coincide with the onset of estrus, leaving most rats in proestrus 4 days after the end of stress. All rats were administered DOX on the final day of stress so that restoration of RFRP3 expression coincided with the onset of proestrus after the 4-day recovery period. (Fig. 2.3A). After one full estrous cycle of recovery from stress, rats underwent a timed mating test on the night of proestrus and were monitored through gestation and birth, to assess the long-term effects of stress on reproductive success including successful copulation and pregnancy outcome.

Tail vein serum samples taken at the onset and end of stress sessions on days 1, 11 and 18 revealed that post-stress circulating levels of CORT were elevated on days 1, and 11 of the immobilization period (Fig. 2.3B). Moreover, RFRP3 knockdown during stress did not

significantly alter CORT response during stress, indicating an intact hormonal stress response (Fig. 2.3B). Stress exposure led to a profound decrease in total reproductive success in females that received the control virus: only 21% of stressed females became pregnant and carried to live birth, as compared to 78% of non-stressed females with control virus (Fig. 2.3C). 80% of the females that received the RFRP-shRNA virus became pregnant and carried to live birth (Fig 2.3C). The stress-induced decline in reproductive success resulted from a cumulative decrease in mating success (from 88 and 90% in non-stressed groups to 43% in the stress-scrambled group, Fig 2.3D) and pregnancy rates in the females that mated (from 87 and 89% in non-stressed groups to 50% in the stress-scrambled group, Fig 2.3E). Interestingly, knockdown of RFRP3 expression in the hypothalamus during stress exposure prevented the stress-induced suppression of reproduction, leading to 79% copulation success, 82% pregnancy success, and overall reproductive success to 64%, a rate statistically equivalent to control (non-stress) levels (78%, Fig 2.3C).

Exposure to acute stress on the evening of the third day of pregnancy was reported to lead to reduced litter size, via inhibition of implantation which occurs normally 5 days after mating [157]. Therefore, we next assessed whether pre-copulation stress exposure affects pregnancy outcome. Stressed females that received control-scrambled shRNA had significantly smaller litter sizes (Fig 2.3F, 12.77±0.91 vs. 7.667±2.60 pups/litter, $p<0.05$) with no difference in placental scars (Fig 2.3G, 13.0±0.91 vs.10.667±3.93, $p>0.05$). Embryo survival was analyzed in the females that were successfully impregnated by determining the ratio of placental scars (indicative of successful implantation) to the number of live pups in the litter. These were first pregnancies for all females, so the number of placental scars is indicative of implantation events during this pregnancy. Embryo survival in stressed females that received scrambled shRNA was 78.8±11.7% of fetuses, compared to 98.1±0.95% and 97.8±1.5% survival in the control scrambled and control RFRP-shRNA groups, respectively (Fig 2.3F, $p<0.05$). Most remarkably, RFRP3 shRNA administration suppressed stress-induced fetal resorption, showing a 93.4±3.2% fetal survival rate (Fig 2.3H). These results demonstrate that stress-induced increases in RFRP3 expression has long-term detrimental effects on female reproductive fitness that persist long after the stressor has been removed and CORT levels have returned to baseline. In addition, knocking-down RFRP3 expression during stress eliminated the stress-induced decrease in sexual motivation, decrease in pregnancy success and subsequent increase in embryo resorption.

We next examined plasma estradiol concentration in animals throughout the stress period in both scrambled and RFRP-shRNA groups. We found that animals with RFRP knocked down had significantly higher circulating estradiol in proestrus during the stress exposure than animals that received the scrambled virus (Fig 2.4A, $p<0.01$), indicating that RFRP knockdown reverses the stress-induced blockade of the E2 rise that occurs during proestrus. Examining animals more closely during proestrus, we found that the RFRP-shRNA animals that successfully mated after the stressor had significantly higher circulating estradiol in their proestrus periods over the course of the stressor than both scrambled groups (Fig 2.4B, $p<0.05$).

Finally, we investigated behavioral measures of female receptivity to test its potential contribution to the stress-induced reproductive deficits observed. Lordosis intensity is a rating (from 0-3) of the quality of all lordosis poses the female exhibits during the mating session, when 0 marks no lordosis and 3 is a fully mounted spinal flexion pose. In rats, a common index of relative sexual receptivity of a female in the presence of males, is the lordosis quotient (LQ), calculated by the number of times the female adopts a lordosis posture scored 2 or higher, divided by the number of times a male mounts her. All females included in the study exhibited

lordosis when introduced to a male (indicating that they were in the correct stage of their cycle). Lordosis intensity did not differ within groups (Fig 2.4C), but, a significant main effect of stress revealed that lordosis intensity was significantly suppressed by stress ($F(1, 61)=5.15$, $p=0.0268$). Furthermore, lordosis quotient measures revealed significantly lower ratio in the scrambled stress group compared to the non stressed groups that received scrambled or RFRP-shRNA (0.30±0.10 vs. 0.73±0.07 and 0.68±0.07), indicating that stress exposure decreased the relative sexual receptivity of the females (Fig 2.4D), congruent with the stress-induced drop in mating success we found. Interestingly, LQ ratios in stressed females that received RFRP-shRNA were not significantly different from controls ratios (0.53±0.10, Fig 2.4D), demonstrating that knock-down of RFRP reversed the stress-induced decrease in sexual receptivity, and congruent with the reversal of mating success found in this group.

C. Discussion

In humans, high anxiety and psychological stress can lead to long-term impaired fertility, ranging from reduced libido, delayed pregnancy success to the extreme of complete reproductive axis suppression as in the case of hypothalamic amenorrhea [168]. In our studies, selective knock-down of hypothalamic RFRP3 during stress exposure preserved all aspects of reproductive function that were otherwise reduced in stress-exposed animals. The stress-induced spike in RFRP3 initiates a long-lasting suppression of reproduction, well after removal of the stressor, perhaps via positive feedback that maintains elevated RFRP3 levels or engages downstream suppressive targets. These findings reveal a single molecular target that persistently underlies a range of different reproductive dysfunctions that may provide a novel translational framework for clinical study of human reproductive health.

The stress-induced rise in RFRP may be acting on neural circuits influencing mating and pregnancy, potentially independently of sex steroids. RFRP projects to multiple brain regions responsible for successful reproduction and mating behavior, including the medial preoptic area (mPOA) (where it is known to affect GnRH release) as well as the BNST, medial amygdala, anterior hypothalamus and arcuate nucleus [175]. Piekarski et. al. found that administering RFRP3 to hamsters reduced sexual motivation (as measured by percent of time spent with castrated vs. intact males) and vaginal scent marking without effect on lordosis behavior, similar to our present findings. Additionally, RFRP3 administration altered cellular activation in regions of the brain implicated in female sexual motivation, including the mPOA, medial amygdala, and BNST - all regions that receive RFRP projections. These effects were independent of gonadal steroids and kisspeptin cellular activation [189]. While we were unable to measure progesterone or prolactin in this study, it is possible that RFRP projections to the arcuate nucleus affect dopaminergic signaling required for prolactin release and maintenance of progesterone levels and pregnancy success. Future studies aimed at systematically examining each step in these processes is required to gain a full understanding of the neural circuits underlying the deleterious effects of stress on reproduction.

In humans, RFRP 1 and 3, and their cognate receptor are expressed in the hypothalamus (13). It is possible that manipulation of RFRP3 signaling in humans may relieve stress-related reproductive dysfunction, including decreased sex drive, impaired fertility, and increased miscarriages. Likewise, if similar mechanisms of stress-induced reproductive suppression are common across species, such strategies may be similarly relevant to species bred in captivity that

are susceptible to stress-induced infertility, in particular endangered species whose preservation depends on captive breeding programs.

D. Methods:

Experimental Subjects

Adult female Sprague-Dawley rats were triple-housed on a 12/12-hour light-dark cycle with lights on at 0700 hours and *ad libitum* food and water. For all studies, rats were acclimated for a week and then vaginal smears were obtained daily to verify normal cyclicity for 12 d before the studies commenced. Rats that did not cycle normally were removed from the study. For the chronic stress experiment, rats were immobilized daily from 9am-12pm (N=6 for each cycle time point) or left undisturbed in their home cages (N=6 for each cycle time point) until terminal samples were collected (stress paradigm described below). In the RFRP knockdown study, animals received stereotaxic injections of either RFRP-shRNA (N=30) or scrambled control (N=28), then allowed to recover for three weeks. After recovery, rats were exposed to the same stress paradigm as the previous experiment. After cessation of stress all animals were left undisturbed in their home cage for 4 days, and on the night of the 4th day observed during timed mating (see below). Rats that successfully mated were left in their home cages for the duration of gestation and then perfused within 24 h after parturition. (stress/shRNA, N=15, control/shRNA, N=15, stress/scrambled, N=14, control/scrambled, N=11). One cage of control/scrambled animals were removed from analysis due to fighting. All animal care and procedures were approved by the University of California–Berkeley Animal Care and Use Committee (Protocol R303-0313BC).

Immobilization Stress

Rats were immobilized in Decapicone bags (Braintree scientific) and placed in individual cages in a fume hood for 3 hours/day for 18 days. Blood samples were collected for corticosterone measurement on days 1, 4, 7, 11 and 18 at the onset of the stressor and again at the end of the 3 hours.

Plasma Corticosterone and E2 Hormone Sampling

All blood samples were collected from tail vein and centrifuged at 2000 *g* for 15 min. Plasma was extracted and stored at -20 °C until assayed. Corticosterone was measured using a Corticosterone EIA kit (Enzo Life Sciences) with individual samples used for analysis. Sample values below the detection level of the assay were included as the lowest detectible value. Samples were assayed in duplicate and groups were balanced across different plates. Inter-assay coefficients were <3% and intra-assay coefficients were <5%. Estradiol assays were measured by CalBiotech EIA (Spring Valley, CA) in singlet with individual samples used for analysis. Again, sample values below the detection level of the assay were included as the lowest detectible value. Inter-assay coefficients were <2% and intra-assay coefficients were <8%

Copulation Tests

Females verified to be in proestrus were paired with a novel male in a large rectangular cage under red light illumination during the lights off phase. The male was permitted to mate with the female for up to 2 ejaculations after which the male was removed from the cage. The interactions were videotaped and both male and female behaviors were blindly scored *post hoc*. Females that never exhibited lordosis posture during the test were removed from analysis. Females that exhibit

at least one lordosis posture but that did not allow the male to achieve intromission were termed an "unsuccessful maters" and after 15 min removed from the cage.

Scoring of Sexual Behaviors
All mating tests were videotaped in real-time for subsequent behavioral scoring. Videos were scored by two individuals blind to the experimental conditions. Behaviors of male and female animals were scored. The lordosis intensity was scored on a 4-point scale (0-3) as described by Hardy and DeBold [190] where 0 indicates no lordosis response and 3 indicates a pronounced spinal flexion, and averaged over the number of lordotic poses presented. The lordosis quotient (LQ) was determined as the number of lordosis responses (scores of 2 or 3) divided by the total number of mounts during the scored session. The number of proceptive behaviors was calculated as number of ear wiggles/minute during duration of test, as well as the number of darts and hops through duration of test. Males were scored for total number of mounts and intromissions.

Measurement of Placental Scars
Post-partum mothers were sacrificed 1-day post-partum. The abdominal cavity was opened and both uterine horns gently removed. Placental scars were identified as distinctive dark brown spots, counted and logged [191].

Virus Preparation
The viral vector pLenZs-tetOFF-BFP-shRNAmir-HygR was redesigned based on the backbone of pGIPZ vector originally from Open Biosystems to implement the new features and better single restriction enzyme cutting sites for molecular cloning. Briefly, PCR products for tetOFF and its response elements (TetOff Gene Expression System from Clontech), tagBFP (pTagBFP-H2B vector from Evrogen), and a hygromycin resistant gene (pSilencer-hygro vector from Ambion) were inserted in to the original pGIPZ vector to replace the unwanted components (e.g. original fluorescent protein and the puromycin resistant gene). The constructed vector map is shown in Fig. 2d. To construct the shRNA against RFRP, a 22 nucleotide-mer oligo against RFRP gene was designed using the online program maintained by Dr. Ravi Sachidanandam's Lab (http://katahdin.mssm.edu/siRNA/RNAi.cgi?type=shRNA). The oligo was inserted into the linearized pLenZs-tetOFF-BFP-shRNAmir-HygR vector using KpnI and EcoRI enzymes after adding enzyme arms on both sides of the oligo using PCR. Lentiviral particles were prepared by PEG-2000 purification of transfected Hek-293 cells and concentrated to titers of 10^9–10^{10} infectious particles per ml. The control virus was a non-silence vector commercially available from Open Biosystems, with similar GC content and BLASTed to verify non-specificity.

RFRP sequence: CACAGCAAAGAAGGTGACGGAA
Control sequence: CTCTCGCTTGGGCGAGAGTAAG

Stereotaxic Surgery
Stereotaxic microinjections of the RFRP-shRNA and scrambled control viral particles were injected in the hypothalamus as described previously [192]. Coordinates for viral injection into the dorsal medial hypothalamus were: -3.3mm anterior/posterior, ±0.5 mm medial/ lateral relative to bregma, -8.4mm relative to dura with skull level between bregma and lambda. Virus was infused at a rate of 0.2 ul/min for 5 min (1ul total). At 6 to 8 h after surgery, all rats received an injection of meloxicam (2 mg/kg, s.c.).

Immunohistochemical Staining for Virus Verification.

One series of free-floating sections were rinsed in 0.1M PBS then incubated in 0.3% H202 in PBS for 10 minutes. After rinsing, tissue was blocked with 2% normal donkey serum, 0.3% Triton-X 100 in PBS, then transferred into primary antibody against GnIH (PAC123/124, 1:5000 in PBS plus 0.3% Triton-100 [PBS-T]) and section were incubated in antibody overnight, on rotation, at 4°C. The next day, sections were rinsed in PBS and incubated in secondary for 1 hour at room temperature (Biotin donkey anti-rabbit 1:500, Jackson ImmunoResearch). Following rinsing, sections were incubated in ABC reagent (Vector) and then amplified by incubating in biotinylated tyramide for 30 min. Tertiary incubation for 1h at room temperature followed with streptavidin-Alexa594 (1:1000 in PBS, Jackson Immunoresearch). Following tertiary incubation, sections were incubated in an antibody against blue-fluorescent protein (anti-BFP; 1:5000, Abcam) on a rotating stage, overnight, at 4°C. The next day, sections were rinsed in PBS then incubated in secondary antibody for 2 hours at room temperature (donkey anti-rabbit cy5, Jackson Immunoresearch). After rinsing in PBS-T, slides were coverslipped using DABCO antifading medium and stored in the dark at 4°C.

Real-Time Reverse Transcriptase PCR

Real-time reverse transcriptase PCR was run on TRIzol-extracted RNA further purified with DNase (DNA-free, Ambion). Rat primers were designed using NCBI Primer BLAST software, which verifies specificity. The Ct values were determined using PCR miner [193] and normalized to the ribosomal reference gene, ribosomal protein L16P (RPLP). There were no significant differences in RPLP values across any groups. For all studies, two-step PCR was used, following the manufacturer's instructions for iScript cDNA synthesis kit (BioRad) and then the manufacturer's instructions for SsoAdvanced SYBR supermix (BioRad). Samples were run in a BioRad CFX96 real-time PCR system. After the PCR was complete, specificity of each primer pair was confirmed using melt curve analysis, and all samples run on a 2% ethidium bromide agarose gel with a 50bp DNA ladder (Invitrogen) to verify correct product size.

Primer sequences:

Primer	Forward	Reverse	Temp	Product Size
RPLP	ATCTACTCCGCCCTCATCCT	GCAGATGAGGCTTCCAATGT	55	159
RFRP	CCAAAGGTTTGGGAGAACAA	GGGTCATGGCATAGAGCAAT	55	110
GPR147	GGTCAGAACGGGAGTGATGT	AGGAAGATGAGCACGTAGGC	55	119
LHβ	GCAAAAGCCAGGTCAGGGATAG	AGGCCCACACCACACTTGG	55	92
FSHβ	TTCAGCTTTCCCCAGGAGAGATAG	ATCTTATGGTCTCGTACACCAGCT	55	305
TSHβ	TCGTTCTCTTTTCCGTGCTT	CGGTATTTCCACCGTTCTG	55	245

		T		
glycoprotein alpha subunit	CTATCAGTGTATGGGCTGTTG	CTTGTGGTAGTAACAAGTGC	55	199
KISS1	TGGCACCTGTGGTGAACCCTG	ATCAGGCGACTGCGGGTGGCA	61.4	202
GnRH	GCAGATCCCTAAGAGGTGAA	CCGCTGTTGTTCTGTTGACT	55	201

Statistical Analysis

In the chronic stress and reproductive success experiments, group differences in reproductive success, mating success and pregnancy success were examined using G-statistics and Fisher's exact tests on raw numbers, not percentages. Litter size, placental scar, embryo survival, lordosis quotient and intensity differences were assessed using two-way analysis of variance (ANOVA) followed by Bonferroni post-hoc tests. Differences in genes examined via RT-PCR were analyzed by a Kruskal-Wallis one-way ANOVA followed by Dunn's multiple comparison test for post-hoc analysis. Differences in corticosterone concentrations were subjected to repeated two-way ANOVAs followed by Bonferroni post-hoc test to determine statistical differences. *$p<0.05$, **$p<0.01$, ***$p<0.001$. Statistics were performed using R (for G-statistics and Fisher's exact test) and Prism software.

.

E. Figures:

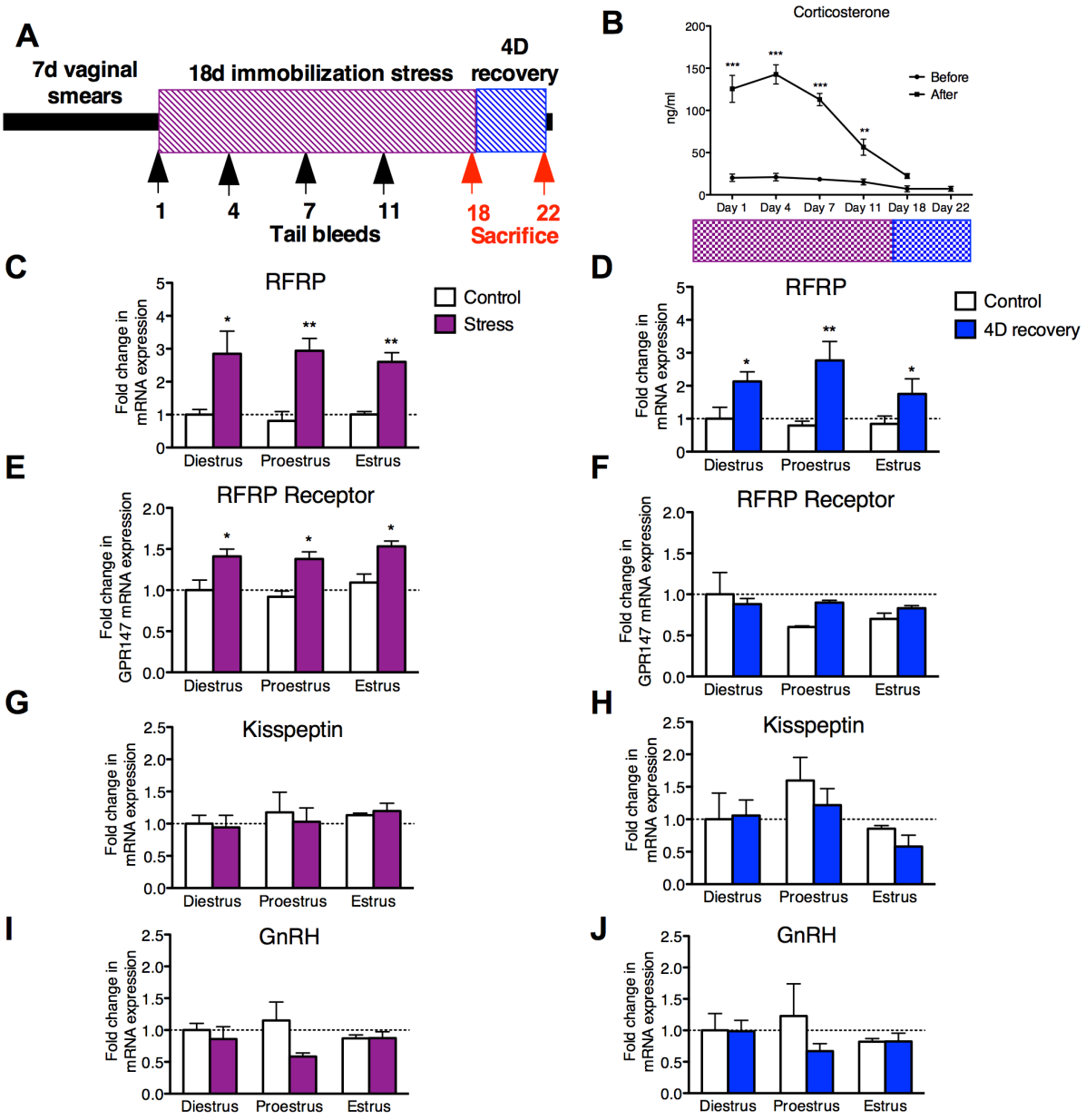

Fig. 2.1. 18 days chronic stress leads to an upregulation of RFRP mRNA that persists for at least one estrous cycle in the rat. (A) Experimental timeline. (B). Corticosterone was measured in serum samples from tail vein blood immediately before and after stress sessions on days 1, 4, 7, 11 and 18, and on day 22, 4 days post stress cessation (N=36/group in 1,4,7,11,18 timepoints, N=18/group on 22). (C, E, G, I) Gene expression changes in the hypothalamus immediately after stress and (D, F, H, J) 4 days after stress. mRNA levels of all (mean ± SEM, N≥6) were determined using qRT- PCR relative to the ribosomal reference gene RPLP at day 0 and 4 post stress cessation. Estrous cycle staging was determined by inspection of daily vaginal smears.

*p<0.05, **p<0.01, *** p<0.001. PCR statistics were done by a Kruskal-Wallis one-way ANOVA followed by Dunn's multiple comparison test for post hoc analysis, CORT statistics analyzed by a repeated two-way ANOVA.

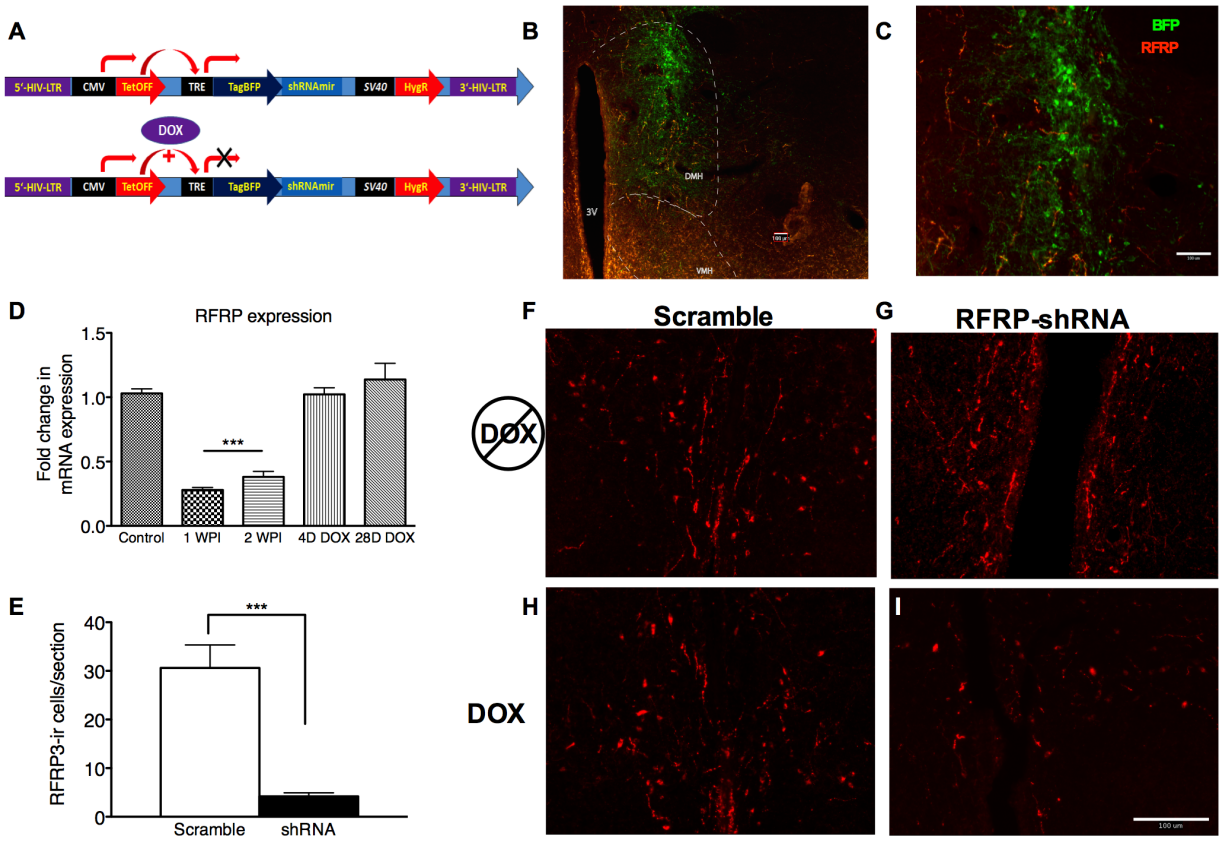

Fig 2.2. RFRP-shRNA successfully knocks down RFRP expression in the dorsal medial hypothalamus, and expression is recovered upon DOX induction. (A) Map of RFRP-shRNA viral plasmid. (B) Brain sectioned and stained with an anti-BFP antibody to label virus infection (green) and anti- RFRP two weeks post-injection (WPI) to show injection location and (C) spread. Scale bar indicates 100um.. (D) mRNA levels of RFRP following injection of RFRP-shRNA viral vector were determined using qRT-PCR (WPI= weeks post injection, mean ± SEM, N=4). (E) RFRP3-is cells/section counts in the DMH after two weeks post-injection with either scramble or RFRP-shRNA virus (F-I) Brain sectioned stained with anti-RFRP3 antibody two weeks post-injection with scramble or RFRP-shRNA virus and before and after DOX administration. Scale bar indicates 100um. *p<0.05, **p<0.01, *** p<0.001. Statistics were done by one-way analysis of variance (ANOVA) and Bonferroni post-hoc tests. For mRNA data, PCR statistics were done by a Kruskal-Wallis one-way ANOVA followed by Dunn's multiple comparison test for post hoc analysis and statistics for protein counts were a student's t-test.

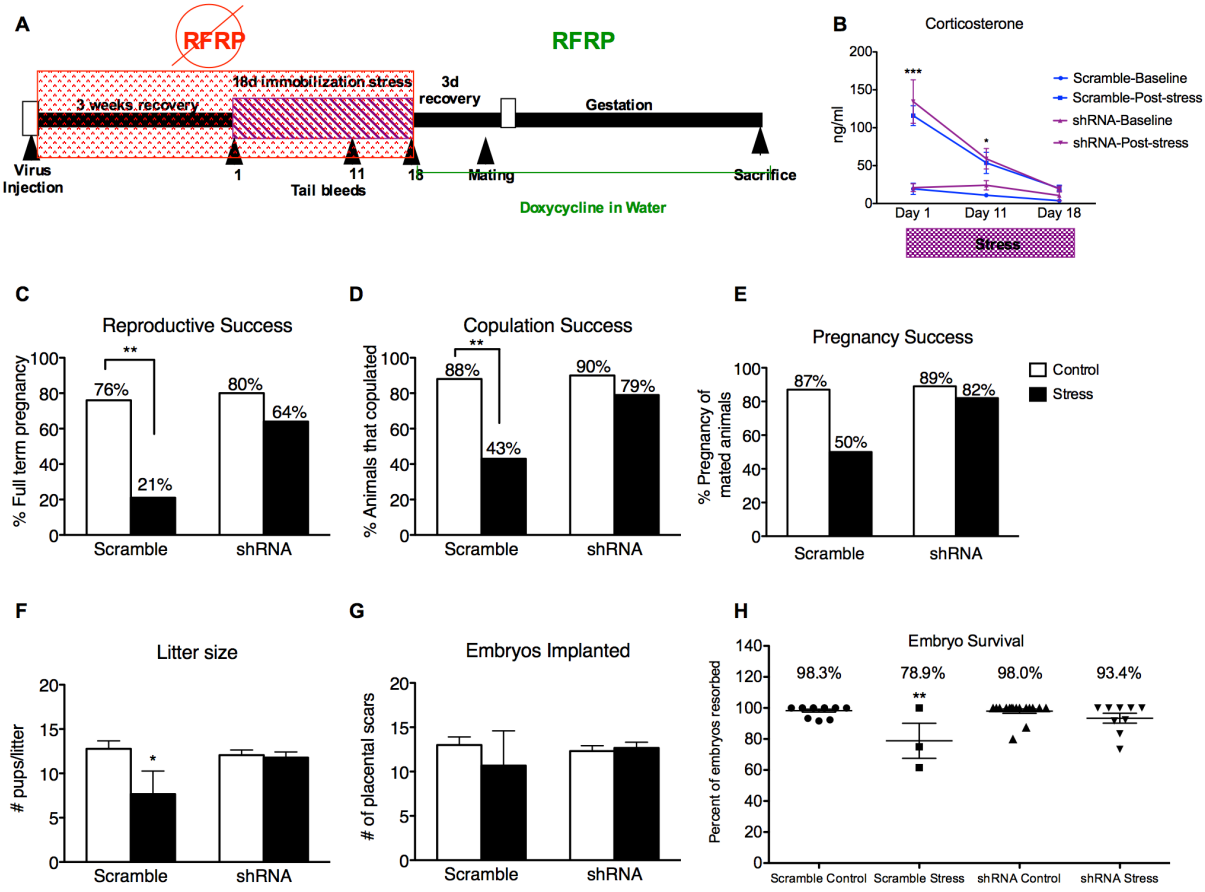

Fig. 2.3: Knocking down RFRP during stress completely prevents stress-induced reproductive dysfunction. (A) Experimental time line. (B) Corticosterone concentrations were measured in serum samples from tail vein blood immediately before and after stress sessions on days 1,11 and 18. (C) Total reproductive success was measured as percentage of females that successfully brought a litter to full term (Scramble/control N=17, Scramble/Stress N=14, shRNA/control N=20, shRNA/stress N=14, g-statistics: G=5.836, df=1 p=.016, fisher's exact test p=0.0031). Breaking down total reproductive success, (D) Copulation success was measured as percentage of females that exhibited lordosis and allowed a male to achieve intromission within 15 min (g-statistics: G=2.405, df=1 p=.028, fisher's exact test p=0.0062) and (E) Pregnancy success refers to the percentage of females that got pregnant out of the subgroup that successfully copulated (Scramble/control N=15, Scramble/Stress N=6, RFRP-shRNA/control N=18, RFRP-shRNA/stress N=11). (F) Litter sizes measured as number of pups born alive immediately after birth (dams-Scramble/control N=13, Scramble/Stress N=3, RFRP-shRNA/control N=16, RFRP-shRNA/stress N=9). (G) Embryos implanted measured as number of placental scars identified in the dam's uterine horns after birth. (H) Embryo resorption was calculated as the number of birthed pups divided by number of maternal placental scars and

shown as a percentage (indicative of initial implantation, mean ± SEM) *p<0.05, **p<0.01, *** p<0.001. Reproductive success statistics were done by G-statistics tests followed by Fisher's Exact test, statistics for litter size, placental scars and embryo resorption were done by a two-way analysis of variance (ANOVA) followed by Bonferroni post hoc tests and CORT statistics analyzed by a repeated two-way ANOVA.

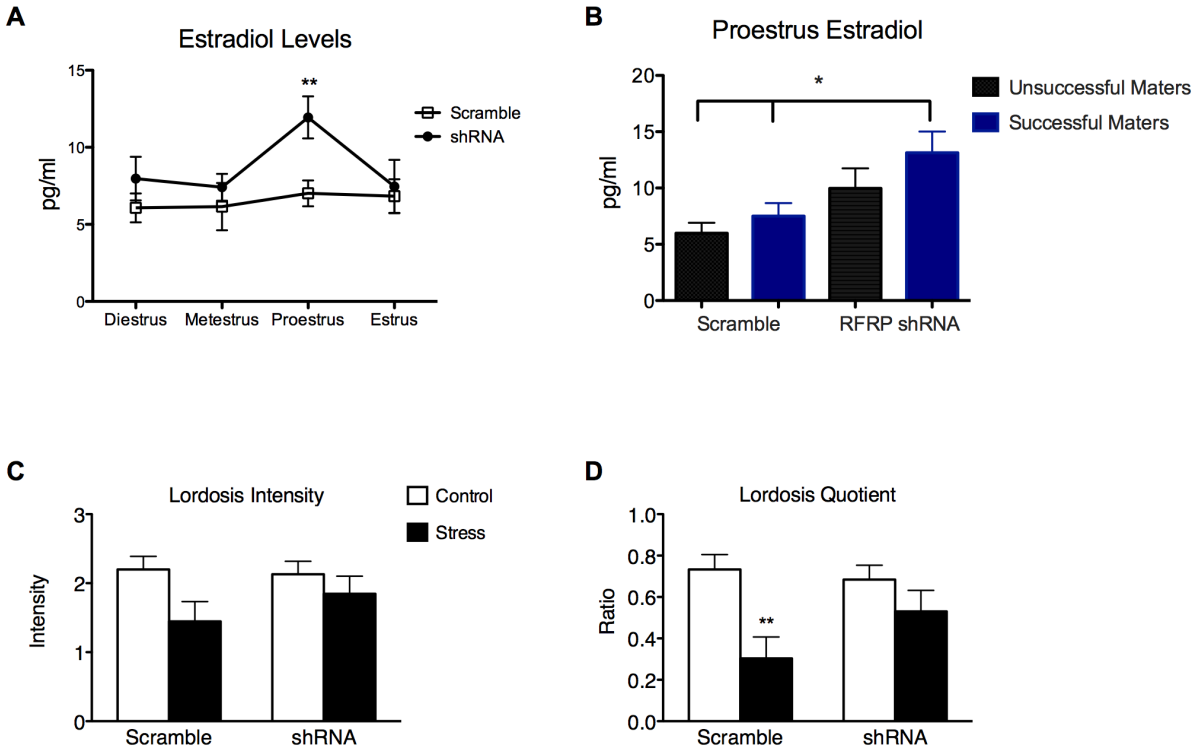

Fig 2.4 RFRP-shRNA animals had increased plasma estradiol on proestrus during stress and RFRP-shRNA animals that mated had higher circulating estradiol than scrambled animals. (A) Estradiol levels as measured from tail bleed samples over the cycles of all stressed rats with either scrambled or RFRP-shRNA virus (n= 14). (B) Within proestrus, estradiol measurements in were separated by mating success and virus (n=10, 21, 11, 25 samples for each successive group). (C) Lordosis intensity, or quality of the lordosis pose, scored between 0-3 as published in [190] . We found a significant main effect of stress ($F(1, 61)=5.15$, $p=0.0268$) however no significant differences within groups. (D) Lordosis quotient was calculated as the ratio of male mounts to female lordosis poses of a score of 2 or 3. We found a significant main effect of stress ($F(1, 61)=11.66$, $p=0.0011$), as well a significant decrease in the scramble stress group. p<0.05, **p<0.01, *** p<0.001. Estradiol, lordosis intensity and quotient statistics were a two-way analysis of variance (ANOVA) followed by Bonferroni post hoc tests.

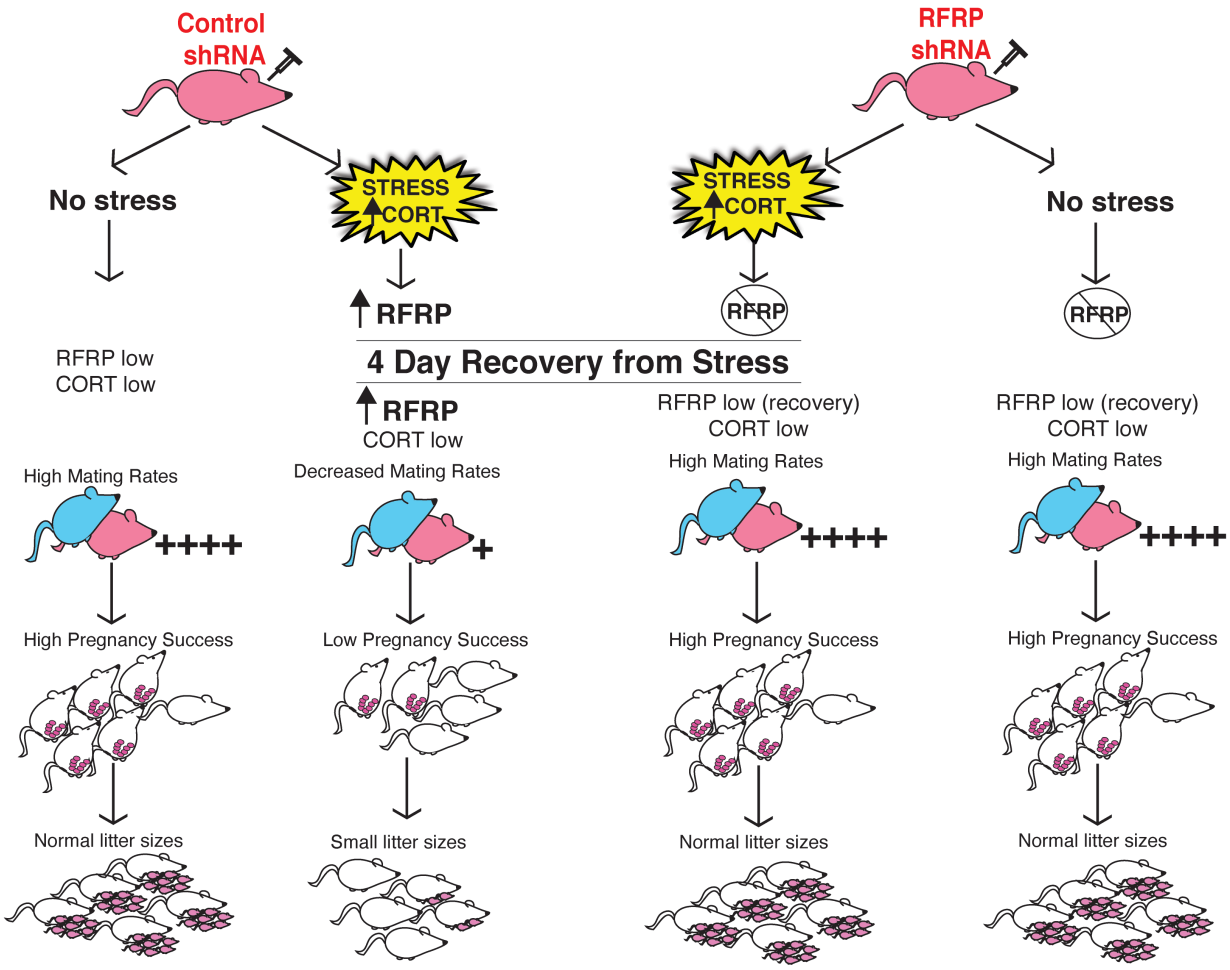

Fig. 2.5: Schematic illustration of experiments. Female rats (in pink at top) were injected with either an inducible RFRP-shRNA or a scrambled control virus. Each group was furthered separated into a no stress control or subjected to 18 days of immobilization stress. Stressed females exhibited fewer successful copulation events, fewer pregnancies in those that did successfully mate, and increased frequency of embryo resorption. These marked effects of stress on fertility were completely blocked by knockdown of RFRP3.

Chapter 3
A novel role for RFRP3 in astrocyte connectivity and communication

A novel role for RFRP3 in astrocyte connectivity and communication

Abstract

RF-amide related peptide-3 (RFRP3) was originally identified in mammals as a negative regulator of the hormonal reproductive axis, the hypothalamic-pituitary-gonadal (HPG) axis. RFRP3 is a hypothalamic neuropeptide known for inhibiting gonadotropin-releasing hormone (GnRH) release from the hypothalamus and decreasing gonadotropin synthesis and release from the pituitary. Recently, however, we identified RFRP3 expression in hippocampal astrocytes and found that white matter astrocytes express both the peptide and its receptor, GPR147. This expression is specific to hippocampal astrocytes, as *in vivo* immunohistochemical staining and PCR analysis has found low expression in the cortex and hypothalamus astrocyte populations. We have found that high RFRP treatment to hippocampal astrocyte cultures decreases expression of connexin 43, a connexin specific to astrocytes and important in gap channel connectivity between astrocytes. RNA-seq analysis comparing astrocyte cultures with either RFRP expression knocked down or normal expression, has found further changes to astrocyte connexins, including decrease in connexin 30, another astrocyte-specific connexin, as well as calcium and potassium channels. Scrape-loading assays in hippocampal astrocytes revealed that 24 hour RFRP treatment decreases spread of dye in confluent cells, showing that RFRP decreases connectivity between astrocytes. These results indicate that RFRP3 may have a novel role in astrocytes, influencing calcium signals and communication between astrocytes.

A. Introduction

Astrocytes are the most common cell type in the brain, however research into their function only grew more popular in the past 20 years. Originally believed to simply be the "glue" of the brain, providing only structural support, astrocytes have since been identified to have numerous important functions, such as promoting synaptic development and function, regulating the blood-brain barrier, and they also play a critical role in extracellular potassium buffering and neurotransmitter reuptake[194–196]. However, an underappreciated field within the study of astrocytes is the heterogeneity of astrocytes themselves. Though Ramon y Cajal identified the vast array of structural differences in astrocyte populations throughout the brain as early as 1897[197], and others at that time and later identified specifically two different populations of astrocytes within brain regions- fibrous white matter astrocytes and protoplasmic grey matter astrocytes, differing significantly in structure[198–200], few studies investigated their functional differences, focusing instead on their phenotypic differences. Newer research, though, supports the idea that these two populations are not simply phenotypically different, but functionally different as well. Matthias et.al. identified that these two populations of astrocytes expressed not only differing levels of GFAP-eGFP expression, but that one population had outwardly rectifying K^+ channels and express AMPA receptors and no glutamate transporters, while the other cell type had inwardly rectifying K^+ channels and functional glutamate transporters[201]. Others have also seen this electrical difference[202–204]. More recently, some have suggested that not only are these different populations of astrocytes communicating differently with their neuronal counterparts, but they may also be communicating to each other differently[205–208].

Communication in astrocytes relies predominately on gap junction connections between large networks of cells. Gap junctions allow transfer of ions and other signaling molecules between astrocytes, and are composed of connexins, a family of transmembrane proteins. While there are many connexin proteins in the family, only a few are specifically expressed in astrocytes- connexin (Cx) 43 predominately and Cx30 and 26 to a smaller degree[209–211]. This connections also regulate potassium buffering and cellular signaling, both astrocyte-to-astrocyte and astrocyte to other cell types, such as oligodendrocytes[212]. As it becomes increasingly clear how critical astrocytes are to normal brain physiology, as indicated by the evidence of the role in astrocytes in many neurodegenerative disorders, normal connexin expression and communication is absolutely critical.

RFRP3 was recently discovered in avian and mammalian systems as a hypothalamic neuropeptide responsible for inducing massive inhibition of the reproductive axis[28,84,174,175]. It was originally identified within a small population of neurons in the dorsal medial hypothalamus (DMH) of rodents. However, RFRP3 neurons extend processes throughout the brains, including the medial preoptic area (mPOA) (where it is known to affect GnRH release) as well as the BNST, medial amygdala, anterior hypothalamus and arcuate nucleus[175], so it is hypothesized that it has many other functions other than just reproductive inhibition.

We discovered that in addition to expression in the DMH of rodents, RFRP3 is also highly expressed in astrocyte cells, specifically within white matter tracts of the hippocampus. We set out to test the function of RFRP3 expression in astrocytes. Within hippocampal astrocytes, we found that RFRP regulates connexin expression, as well as calcium and potassium channels, indicating a role for RFRP in astrocyte communication and potassium buffering. These findings show a novel role not yet identified for RFRP3, opening a new field of study for RFRP3 research, as well as potentially identifying a new marker specific for fibrous white matter astrocytes.

B. Methods

Animal protocol

Adult and juvenile male and female Sprague-Dawley rats (Charles River) were pair-housed on a 12 hr light dark cycle with lights on at 07:00 hr. All animal procedures were approved by the UC Berkeley Animal Care and Use Committees (Protocol R303-0313BC).

Astrocyte cell cultures

Primary astrocyte cultures were prepared from P1–2 day old Sprague Dawley rat pup hippocampi using the method described by McCarthy and Vellis[213]. Briefly, hippocampi were dissected in ice-cold media, chopped and digested using papain from papaya latex extract (Sigma, St. Louis, MO) in HBSS (Life Technologies, Carlsbad, CA) for 20 min at 37°C. Papain was inactivated using 10% horse serum, cells were centrifuged for 1 min at 350×g and resuspended in HBSS and triturated by passing through serological and flame-polished pipettes of progressively smaller bores. Cells were then plated in DMEM (Life Technologies) supplemented with 10% fetal bovine serum (Axenia BioLogix, Dixon, CA) and 1% Penicillin/Streptomycin (Life Technologies) at a density of 3 × 106 in T75 flasks. After reaching confluency, flasks were shaken on an orbital shaker at 225 rpm for 2 hr at 37°C. Cells were then

washed 5× with warm PBS to remove suspended microglia. Astrocytes were then trypsinized and re-plated in 100 mm dishes.

Virus Preparation
Virus prepared as described previously[214]. In short, lentiviral particles were prepared by PEG-2000 purification of transfected Hek-293 cells and concentrated to titers of 10^9–10^{10} infectious particles per ml. The control virus was a non-silence vector commercially available from Open Biosystems, with similar GC content and BLASTed to verify non-specificity.

RFRP sequence: CACAGCAAAGAAGGTGACGGAA
Control sequence: CTCTCGCTTGGGCGAGAGTAAG

Immunohistochemical Staining.

One series of free-floating sections were rinsed in 0.1M PBS then incubated in 0.3% H202 in PBS for 10 minutes. After rinsing, tissue was blocked with 2% normal donkey serum, 0.3% Triton-X 100 in PBS, then transferred into primary antibody against RFRP3 (PAC123/124, 1:5000 in PBS plus 0.3% Triton-100 [PBS-T]) and section were incubated in antibody overnight, on rotation, at 4°C. The next day, sections were rinsed in PBS and incubated in secondary for 1 hour at room temperature (Biotin donkey anti-rabbit 1:500, Jackson ImmunoResearch). Following rinsing, sections were incubated in ABC reagent (Vector) and then amplified by incubating in biotinylated tyramide for 30 min. Tertiary incubation for 1h at room temperature followed with streptavidin-Alexa594 (1:1000 in PBS, Jackson Immunoresearch). Following tertiary incubation, sections were incubated in an antibody against GFAP and NeuN (Millpore, Billerica, MA) to measure astrocytes and neurons, respectively, on a rotating stage, overnight, at 4°C. The next day, sections were rinsed in PBS then incubated in secondary antibody for 2 hours at room temperature (donkey anti-chicken cy5 (GFAP), donkey anti-mouse AF488 (NeuN), Jackson Immunoresearch). After rinsing in PBS-T, slides were coverslipped using DABCO antifading medium and stored in the dark at 4°C.

Real-Time Reverse Transcriptase PCR
Real-time reverse transcriptase PCR was run on TRIzol-extracted RNA further purified with DNase (DNA-free, Ambion). Rat primers were designed using NCBI Primer BLAST software, which verifies specificity. The Ct values were determined using PCR miner [193] and normalized to the ribosomal reference gene, ribosomal protein L16P (RPLP). There were no significant differences in RPLP values across any groups. For all studies, two-step PCR was used, following the manufacturer's instructions for iScript cDNA synthesis kit (BioRad) and then the manufacturer's instructions for SsoAdvanced SYBR supermix (BioRad). Samples were run in a BioRad CFX96 real-time PCR system. After the PCR was complete, specificity of each primer pair was confirmed using melt curve analysis, and all samples run on a 2% ethidium bromide agarose gel with a 50bp DNA ladder (Invitrogen) to verify correct product size.

Scrape-loading and dye transfer assay

In vitro confluent astrocytes on 8mm coverslips were washed thoroughly with 1xPBS and loaded with 0.5mg/ml Lucifer Yellow fluorescent dye in PBS. Three discreet scrape lines were cut with

a scalpel blade on each coverslip (2 coverslips for each group), and then incubated at room temperature for 5 minutes. Coverslips were then washed 6 times with PBS and fixed with 4% PFA and imaged on an inverted fluorescent scope. To estimate gap junction permeability, one image was taken at both sides of each slice, for a total of 6 images per coverslip (12 per group). The spread of dye was measured by tracing the fluorescent cell areas, and measuring distance across using metamorph software. Distances were averaged together and compared between treatment groups.

RNAseq analysis

In vitro hippocampal astrocyte cultures were plated and grown to 50-60% confluency. Plates were then treated with either RFRP shRNA or a scramble virus control and allowed 7 days for infection. Once infected, cells were treated with puromycin (2ug/ml) to select for viral infected cells. Once selection was complete, one group was treated with 10uM RFRP3 for 24 hours. All cells were then trizol'd and processed for RNA as explained in the RT-PCR section. Samples with high RNA purity (n=3/group) were then indexed using the Illumina TruSeq RNA Library Preparation Kit v2 (Illumina, San Diego, CA). Sequencing was run by the UC Berkeley Sequencing facility, and all samples were run single-end, multiplexed with 3 samples per lane.

The reads from the untreated sample were aligned using TopHat 2 to the rat genome with the Ensembl 78 rat annotation. Cufflinks was run on the resulting alignments to assemble novel transcripts. The reads in each of the nine samples were then aligned to that transcriptome and the expression in each sample was then quantified using eXpress. The effective read counts for each transcript as called by eXpress were then analyzed using DESeq2 using the negative binomial likelihood-ratio test to detect differences in mean between any of the three conditions. Transcripts with p-value consistent with a false discovery rate of less than 5% were called as differentially expressed.

Statistical analysis

Intensity differences were measured by one way analysis of variance (ANOVA) with a bonferroni post hoc test to measure within group differences. Differences in genes examined via RT-PCR were analyzed by a Kruskal-Wallis one-way ANOVA followed by Dunn's multiple comparison test for post-hoc analysis. Differences in distance as measured by scrape loading were determined by a 2 way ANOVA with bonferroni post hoc tests. *$p<0.05$, **$p<0.01$, ***$p<0.001$. Statistics were performed using Prism software.

C. Results

RFRP3 specifically labels fibrous white matter astrocytes in the hippocampus

Immunohistochemical labeling for RFRP3 in *in vivo* coronal brain sections shows high intensity labeling within the hippocampus. A 10x whole brain scan of a slice containing hippocampus shows visually how powerful the labeling is (fig 3.1A). RFRP3 is labeled in red, GFAP, a marker for fibrous astrocytes is in green, and NeuN, a label for neurons is in blue. Yellow labeling

indicates a co-label of RFRP3 with GFAP, and you can see much higher intensity of colabeling in the hippocampus than in any other area of the brain. A close-up view of the hippocampus at 20x shows this much more clearly (fig 3.1B-E). Again, RFRP3 is labeled in red (fig. 3.1C), GFAP is in green (fig. 3.1D), and NeuN is in blue (fig. 3.1E). The merged image of all three (fig. 3.1B) shows that RFRP3 is co-localized only with GFAP, not NeuN, and that this co-localization occurs in the white matter tracts of the hippocampus. Example pictures of the cortex (fig. 3.1F) show no labeling of RFRP3, with few GFAP cells at all. Within the hypothalamus (fig. 3.1G), there appear to be small populations of labeled astrocytes around the dorsal medial region, but nowhere near the same amount of cells as within the hippocampus. Integrated intensity measurements of RFRP3 show a highly significant increase of RFRP3 labeling within the hippocampus as compared to the cortex and hypothalamus (fig. 3.1H, $p<0.001$), supporting our visual confirmation that this is specific to fibrous astrocytes in the hippocampus. Integrated intensity measurements comparing juveniles to adults, as well as adult males to adult females, show that there are no differences in any group (fig. 3.1I, $p=0.1859$, $F=2.40$). This is important, as it shows us that there is no developmental or sex differences in RFRP3 expression, it is just constant. PCR analysis of *in vitro* populations of hippocampal, cortical and hypothalamic astrocytes show a similar trend as the *in vivo* intensity measurements- an increased expression within the hippocampal cells, as compared to the other two regions (fig. 3.1J, $p<0.05$). These data show that both *in vivo* and *in vitro*, RFRP3 is highly expressed in fibrous white matter hippocampal astrocytes, and that this is a specific to the hippocampus, with little staining or RNA expression in the cortex and hypothalamus, two other regions with high levels of astrocytes.

Treatment with RFRP3 for 24 hours leads to decrease in astrocyte connectivity and Cx43 expression

To determine whether a transcriptional decrease in Cx43 mRNA as shown in the RNAseq data leads to a functional decreases in connectivity between cells, we ran a Lucifer Yellow scrape loading assay to determine spread of dye in hippocampal astrocyte cells *in vitro*. Confluent astrocytes were treated with RFRP3 for 5 minutes, 1 hour, 6 hours, 18 hours and 24 hours (or with vehicle, n=2 coverslips/group). Cells were then washed of RFRP and media, and three disparate slices were made onto the coverslips. The cells were incubated with Lucifer Yellow dye for five minutes and then fixed and imaged. Representative images of RFRP treated cells and vehicle treated cells are shown in Fig. 3.2A&B. Distance, as measured by spread of dye through the astrocytes was calculated. Cells treated with RFRP3 for 24 hours showed a significant decrease in spread of dye as compared to 24-hour treatment with vehicle ($p<0.01$). No other time point showed significant changes (fig 3.2C). We also verified that cells from the same astrocyte cell culture experiment also experienced changes in connexins. Confluent astrocytes treated with RFRP3 for 24 hours showed a significant decrease in Cx43 mRNA levels (fig 3.2D,$p<0.001$). These two pieces of data replicate the same changes we see in connexin levels in the RNAseq data, and show that this decrease in Cx43 leads to a functional decrease in connectivity of the cells, as measured by dye spread. This could indicate that RFRP3 may also cause decrease in gap junction communication, changing astrocyte communication in networks.

D. Discussion

Astrocyte heterogeneity is a fascinating and important new line of research in the glia field. While it is well accepted that neurons are highly heterogeneous, and there has been much evidence that astrocytes have many phenotypic differences, little is understood how those phenotypically different sub-populations of astrocytes are functionally and developmentally different. It is becoming increasingly apparent, however, that these different populations have different electrical and gap junctional coupling. Two hippocampal populations of astrocytes, also show distinct differences in glutamate responsivity, a mechanism for their differing gap junction coupling. These populations are referred to as GluR (for possession of AMPA glutamate receptors) and GluT (for possession of glutamate transporters) and they differ profoundly in gap junction coupling[201,208]. When examined in GFAP-eGFP mice, GluT astrocytes are easily identified by their high intensity staining of GFAP, with robust processes. They express high levels of glutamate transporter genes, have inwardly rectifying K^+ channels, and have strong gap junction connectivity. GluR cells, on the other head show little expression of glutamate transporter genes, have high levels of AMPA receptors and outwardly-rectifying K^+ channels, and show little to no gap junction connectivity[201,208].

RFRP3 regulates connexin expression, decreasing Cx43, the predominant connexin in astrocytes. It also regulates inwardly-rectifying potassium channels, as well as metabotropic glutamate channels, as shown in the RNAseq data, revealing a potential role in not just connectivity, but cellular buffering as well. Interesting, the RNAseq data, as well as the scrape loading data, suggests that RFRP is expressed in these GluT astrocyte cells specifically, rather than the GluR cells. As more research investigates the differences between different subpopulations of astrocytes, RFRP3 could become an interesting molecule to focus on. That RFRP3 is only expressed in white matter astrocytes in the hippocampus is a completely new discovery and one that could be very exciting. Few markers exist that perfectly separate these groups of astrocytes, and the identification of one that only labels these GluT cells could prove to be very helpful. Much more research needs to be done, however, to better understand RFRP3's role in astrocytes.

Astrocytes also play a critical role in disease, as more and more research is revealing[215,216]. A better understanding of astrocyte heterogeneity is becoming increasingly needed, as these populations likely elicit very different effects in the brain. Especially within the hippocampus, which was originally believed to be a single uniform population of astrocytes[217]. As is becoming clearer, the hippocampus as two distinct populations of cells that differ greatly in how they communicate. Understanding those different cells, and how to correctly identify them and the role they may be contributing to disease, is important. RFRP3 and its novel role in these astrocytes could reveal more information into functional differences between these populations.

E. Figures

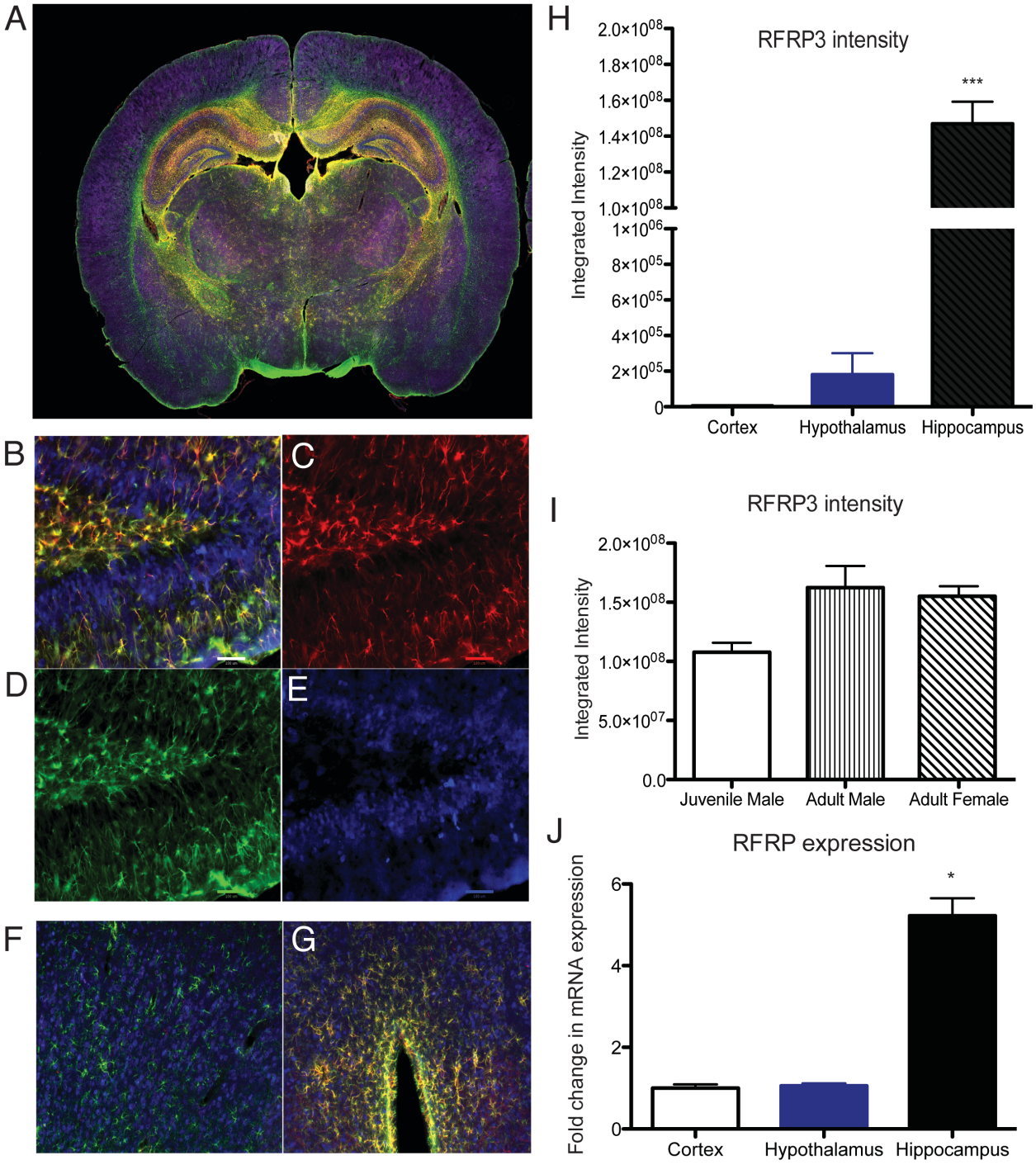

Fig 3.1. RFRP3 expression in hippocampal astrocytes. (A). 10x scan slide of RFRP3 (red), GFAP (green) and NeuN (blue) label. (B-E). Hippocamapal brain sections sliced and stained with RFRP3 (red, C), GFAP (green, D) and NeuN (blue, E). (F) representative image of cortex. Blue is NeuN and green is GFAP. (G) Representative image of hypothalamus. Red is RFRP3, green is GFAP and blue is NeuN. (H) Integrated intensity measurements of RFRP3 in cortex,

hypothalamus and hippocampus. (I) Integrated intensity measurements of RFRP3 by age and sex. (J) PCR analysis of mRNA levels of RFRP in *in vitro* cortical, hypothalamic, and hippocampal astrocyte cultures. Scale bar indicates 100um. *p<0.05, **p<0.01, *** p<0.001. Statistics were done by one-way analysis of variance (ANOVA) and Bonferroni post-hoc tests. For mRNA data, PCR statistics were done by a Kruskal-Wallis one-way ANOVA followed by Dunn's multiple comparison test for post hoc analysis.

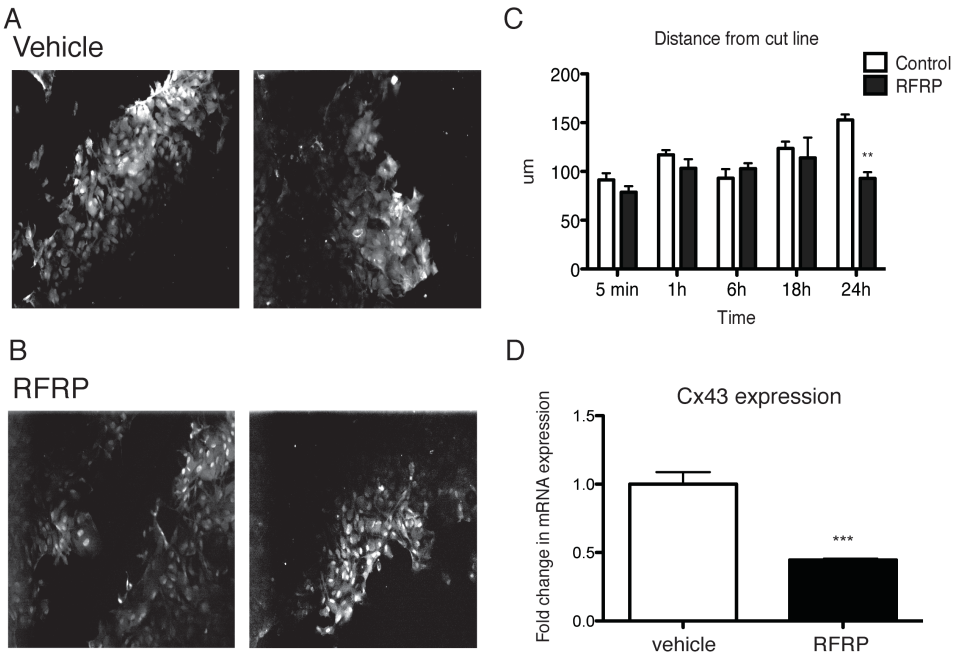

Figure 3.2. RFRP treatment decreases connectivity in *in vitro* hippocampal astrocyte cultures. (A) Representative images of vehicle and (B) RFRP treatment in dye spread in astrocytes. (C) Changes in distance measured as um from the scrape line. (D) PCR expression changes in *in vitro* hippocampal astrocytes of connexin 43 compared between vehicle and 24 hour RFRP treatment. Scale bar indicates 100um. *p<0.05, **p<0.01, *** p<0.001. Statistics were done by one-way analysis of variance (ANOVA) and Bonferroni post-hoc tests. For mRNA data, PCR statistics were done by a Kruskal-Wallis one-way ANOVA followed by Dunn's multiple comparison test for post hoc analysis.

Chapter 4
Discussion

Discussion

A. Stress and RFRP3/GnIH

Stress regulates all parts of the reproductive axis, inhibiting reproductive success at every level. RFRP3 effectively functions similarly to stress in its inhibition of reproduction. Understanding how stress regulates RFRP3 could be a critical step in better understanding the mechanism of stress-induced infertility. While it has been shown that stress has a myriad of effects on reproduction, it could be that RFRP is the actual integrator of all this information, and stress signals directly to RFRP itself to inhibit reproduction, rather than influencing things directly. My dissertation examined the ways in which stress regulates RFRP3 signaling, and how this change in signaling may impact reproductive success.

Our lab previously showed in males that both acute and chronic stress upregulates RFRP3 mRNA levels, as measured by PCR, and RFRP3 peptide levels. This increase in turn led to downstream inhibition of the HPG axis, as shown by decreases in LH levels measured in the serum of stressed males. When animals were adrenalectomized before the stressor, removing circulating glucocorticoids (GCs) from the blood and preventing a stress-induced increase in CORT, these effects on RFRP3 levels were blocked. This indicates that this rise in RFRP3 during stress is indeed due to the increase in glucocorticoid levels from stress. Immunohistochemical staining revealed that RFRP3 neurons in the hypothalamus express high levels of the glucocorticoid receptor (GR), which binds GCs in times of stress[86]. This shows that GCs have a direct mechanism for interacting with the RFRP3 neurons. Indeed, other studies have shown that the RFRP3 sequence contains at least 2 glucocorticoid response elements (GREs), the promoters responsible for triggering GCs effects via the GR receptor[87,218]. This evidence shows that stress can, and does, directly increase RFRP3 levels and respond strongly to the influence of stress.

All the work done previously focused in a male model, where it is simpler to measure changes in the reproductive system without the confusion of the estrous cycle and constantly changing hormones. Females, however, are a much more interesting model to investigate reproductive problems with, though, because female reproduction is typically the limiting step- in most species, females bear the young, and if a female cannot reproduce, no young will be born that year. Research has shown that females are frequently an ignored model in research however[219], as many researchers fear that the circulating sex steroids during estrous cycles introduce too much variability into the research. Moreover, those that do study females, tend to ovariectomized (OVX) the animals and regulate hormone levels that way, as a mechanism for decreasing variability. In reproduction, however, that technique is clearly limiting. You cannot measure things like pregnancy and litter success in OVX'd animals.

My thesis focused on examining the regulation of RFRP3 in chronic stress and it's downstream effect on reproductive success in naturally cycling female Sprague-Dawley rats. Cycle times were monitored and controlled throughout all experiments by vaginal smearing. This allowed us to gain a full picture of the reproductive dysfunction experienced by the animals, with an intact stress response and estrous cycle. We first identified that, like in males, females increase RFRP3 mRNA levels after 18 days chronic immobilization stress. This increase happens in all stages of the estrous cycle. If you allow the animals 4 days of recovery from stress (the length of the estrous cycle), increased RFRP mRNA levels are maintained. This increase

persists despite the fact that CORT levels are back to baseline within 24 hours after the stressor ceases. This is a very interesting connection, as some studies claim that stress levels, as measured by CORT, do not in fact correlate to rates of pregnancy success[220,221], despite the frequent connections attributed to stress and fertility. What this data may show, instead, is that while CORT levels are brought down back to baseline through negative feedback within a day, there could be downstream effects of stress that do in fact correlate to fertility problems that are not measured when simply looking at CORT levels. RFRP3, maintained for at least one estrous cycle likely still elicits a strong inhibitory effect onto the reproductive axis, long after CORT levels are returned to normal.

Next, reproductive success was measured post-stress, in terms of mating success rates and pregnancy success rates, with no hormonal manipulations. This gives us an idea of how 18 days of chronic stress may influence reproductive success in general. We found that stress significantly decreased reproductive success by 50%, a cumulative decrease of mating success and pregnancy success. These data show that not only does 18 days of stress increase RFRP mRNA levels, but also leads to a profound increase in reproductive distress, all while CORT levels are low.

To see whether high RFRP3 post-stress played a role in the mechanism of stress-induced reproductive dysfunction, we used an inducible RFRP3 shRNA injected directly into the DMH of rats. Utilizing an inducible virus allowed us to specifically eliminate the stress-induced rise of RFRP, and return RFRP3 levels back to normal. What we found was that removing RFRP3 during the stressor completely prevented all stress deficits in reproductive. Animals mated and got pregnant in numbers similar to controls, with no apparent deficits. Interestingly, stress induced higher levels of embryo resorption than controls during pregnancy, which was also prevented by RFRP3 knockdown. This happened after implantation already occurred, as placental scar counting showed no differences between any groups. Vaginal smearing also indicated that few rats actually lost cyclicity during the stressor. This indicates that maybe RFRP is functional in neural networks not critical in ovulation or implantation, but in actual maintenance of pregnancy, independently of sex steroids. Others have found data to support this conclusion[189]. Interestingly, RFRP3 fibers project to the arcuate nucleus, an area responsible for control of prolactin release via dopaminergic signaling[175,222,223]. Dopamine-releasing neurons are responsible for inhibition of prolactin signaling, and correct regulation of dopamine inhibition on prolactin is critical for normal pregnancy maintenance[224]. RFRP3, via its projections to the arcuate nucleus, could be affecting this network, maybe through activation of these dopaminergic neurons at the critical early stage of pregnancy when prolactin is absolutely necessary for maintenance of the fetuses.

Another possibility of the role of RFRP3 in pregnancy maintenance could be how in relation to progesterone secretion. In humans, Oishi et.al. found that RFRP reduced gonadotropin response in the gonads, as well as inhibited progesterone secretion[225]. Though we found no sex steroid differences, we did not investigate progesterone levels in the pregnant rats. RFRP may be disrupting gonadal progesterone maintenance, critical for a successful pregnancy.

Much more work needs to be done to better understand the neural networks influenced by RFRP3 after stress, as well as the length of time RFRP is present in the system. Little is known about the biochemistry of RFRP, information that could be critical in understanding what happens when high levels of RFRP are released after an event like stress. Also, although RFRP has been identified in humans[25], not much is known about how it functions in humans. As we try

to identify the causes of infertility in humans, understanding how RFRP works could prove to be incredibly important and helpful in clinical work.

B. The role of RFRP3 and astrocytes

While working through how stress influences levels of RFRP, I also discovered an interesting and potentially novel function for RFRP3 in the brain. Though previously RFRP3 had only been identified in neurons in the dorsal medial hypothalamus (DMH) of the rat, I saw high intensity labeling of the RFRP3 peptide in a completely new area, and in a different cell type than neurons. RFRP3 was being expressed highly in the hippocampus, an area of the brain known for its role in learning and memory, and in astrocytes, rather than neurons. Astrocytes are the most common cell-type in the brain and research recently has shown that they are responsible for a myriad of critical regulations in the brain, including blood-brain barrier integrity, synapse development and functionality, and potassium and neurotransmitter buffering in the brain. Interestingly, RFRP3 appeared to only by in a certain population of astrocytes in the hippocampus, solely within the white matter tracts, known as fibrous astrocytes. Though much has been learned recently about the functionality of astrocytes, little is known how their populations are functionally different. The fact that not only is RFRP3 expressed in astrocytes, but that it is also specific to a small population of astrocytes is fascinating. The final part of my thesis was also investigating what a reproductive inhibitory neuronal peptide was doing in white matter astrocytes.

A first step taken in understanding RFRP3 and astrocytes was identifying where it was throughout the brain. Though the highest intensity staining was clearly within the hippocampus, we had to be sure that this wasn't overshadowing other populations. Staining for RFRP3, GFAP (a marker for astrocytes) and NeuN (a marker for neurons) was conducted on 40 micron slices of adult and juvenile rats, both male and female. Staining showed clearly that this was specific to astrocytes, as there was localization of RFRP3 to GFAP only, with no colocalization with NeuN anywhere outside of the DMH. There was no colocalization of RFRP with GFAP in either the cortex or the hypothalamus, two areas with high astrocyte populations. Intensity staining showed no differences between males or females, or adults and juveniles. PCR analysis of *in vitro* astrocyte cultures of cortex, hippocampus and hypothalamus showed an increase in hippocampal expression relative to the other two regions. This supported the idea that this was in fact specific to hippocampal astrocytes.

Astrocyte heterogeneity is a newer field within astrocyte research, and little is understood about the different populations within brain regions. However, it is becoming increasingly interesting, especially as research indicates that astrocytes play an important role in development of many neurodegenerative diseases[215,216]. Better understanding subpopulations of astrocytes is a critical new line of research in the field. That RFRP3 is expressed in only fibrous astrocytes could prove to be incredibly interesting in this field.

To gain a better understanding of RFRP3 in astrocytes, we did RNAseq transcriptome analysis of hippocampal astrocyte cultures, comparing astrocytes with either RFRP3 knocked down using our RFRP3 shRNA virus, treatment of cells with high levels of RFRP peptide, or no manipulation. We found a significant effect of RFRP3 on genes important for astrocyte communication and potassium buffering.

To investigate the functionality of RFRP's inhibition of connexin expression, a scrape-loading assay was conducted on cells treated with RFRP3 or vehicle. We found that treatment

with RFRP for 24 hours decreased spread of the dye as compared to vehicle controls. This implies that the decrease in connexin expression functionally manifests into a decrease in connectivity of the astrocytes *in vitro*, supporting our hypothesis that RFRP may be influencing astrocyte communication. Connexins are transmembrane proteins that make up gap junction channels. Astrocytes communicate almost exclusively via calcium signaling through gap junction connectivity. Understanding connexin regulation in astrocytes is tricky, as gap junctions can composed of channels made up entirely of one connexin, or the channels can be heterodimers of two different types connexins. Astrocytes predominately express connexin 30 and 43, and small levels of 26, and channels can be made up of any configuration of the three[208,211]. Different connexins can also be upregulated in compensation for the downregulation of another[226,227]. This makes it difficult to draw strong conclusions from the effect of just one of the connexins being downregulated. However, the decrease in spread of the dye with 24 hours of RFRP3 treatment indicates that decreasing connexin 43 is critical to gap junction connectivity. Though significantly more work needs to be done, this early research into RFRP and astrocytes shows that not only is the expression of RFRP real, there may be a functional role for it there. Though little is understood about astrocyte heterogeneity, the presence of RFRP in fibrous white matter astrocytes is an interesting addition to the field. Some researchers have hypothesized that there is likely a heterogeneity in gap junction connectivity as well between these populations, as their roles are likely very different, whether they are near a synapse or not[207,208,210,228]. Perhaps RFRP is critical in those differences.

C. Summary

Hormones are typically named for their function at the time they are identified. When gonadotropin-inhibitory hormone was initially identified in birds, it was named for what it did-inhibit gonadotropins. Work thus far on GnIH/RFRP3 has focused on that role, predominately examining how it controls the HPG axis, and its interaction with other hormones that also regulate the reproductive axis. While many have discussed the wide projections of RFRP3 neurons throughout the brain, few have investigated what those projections may be significant for, though research into them is growing. However, that RFRP3 may have a completely novel function in a different cell type altogether is a new field that bears investigating. RFRP3 could also play a role in how astrocytes communicate with one another, influencing how information moves throughout the hippocampus. This interesting finding could have great impact in better understanding the different sub-populations of astrocytes, and offer a new marker for identification.

References

1. Handa RJ, Weiser MJ. Gonadal steroid hormones and the hypothalamo-pituitary-adrenal axis. *Front Neuroendocrinol*. 2014;35(2):197–220. doi:10.1016/j.yfrne.2013.11.001.

2. Jennes L, Conn PM. Gonadotropin-releasing hormone and its receptors in rat brain. *Front Neuroendocrinol*. 1994;15(1):51–77. doi:10.1006/frne.1994.1003.

3. King JC, Tobet SA, Snavely FL, Arimura AA. LHRH immunopositive cells and their projections to the median eminence and organum vasculosum of the lamina terminalis. *J Comp Neurol*. 1982;209(3):287–300. doi:10.1002/cne.902090307.

4. J E Levine ACB-D. Neuroendocrine regulation of the luteinizing hormone-releasing hormone pulse generator in the rat. *Recent Prog Horm Res*. 1991;47:97 – 151.

5. Moenter SM, Anthony DeFazio R, Pitts GR, Nunemaker CS. Mechanisms underlying episodic gonadotropin-releasing hormone secretion. *Front Neuroendocrinol*. 2003;24(2):79–93. doi:10.1016/S0091-3022(03)00013-X.

6. Haisenleder DJ, Dalkin AC, Ortolano GA, Marshall JC, Shupnik MA. A pulsatile gonadotropin-releasing hormone stimulus is required to increase transcription of the gonadotropin subunit genes: evidence for differential regulation of transcription by pulse frequency in vivo. *Endocrinology*. 1991;128(1):509–17. doi:10.1210/endo-128-1-509.

7. SARKAR DK, CHIAPPA SA, FINK G, SHERWOOD NM. Gonadotropin-releasing hormone surge in pro-oestrous rats. *Nature*. 1976;264(5585):461–463. doi:10.1038/264461a0.

8. Park O-K, Ramirez VD. Spontaneous Changes in LHRH Release during the Rat Estrous Cycle, as Measured with Repetitive Push-Pull Perfusions of the Pituitary Gland in the Same Female Rats. *Neuroendocrinology*. 1989;50(1):66–72. doi:10.1159/10.1159/000125203.

9. Marshall JC, Griffin ML. The role of changing pulse frequency in the regulation of ovulation. *Hum Reprod*. 1993;8 Suppl 2:57–61. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8276970. Accessed May 27, 2014.

10. Marshall JC, Dalkin AC, Haisenleder DJ, Griffin ML, Kelch RP. GnRH pulses--the regulators of human reproduction. *Trans Am Clin Climatol Assoc*. 1993;104:31–46. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2376610&tool=pmcentrez&rendertype=abstract. Accessed May 27, 2014.

11. Herbison AE. Estrogen positive feedback to gonadotropin-releasing hormone (GnRH) neurons in the rodent: the case for the rostral periventricular area of the third ventricle (RP3V). *Brain Res Rev*. 2008;57(2):277–87. doi:10.1016/j.brainresrev.2007.05.006.

12. CHAZAL G, FAUDON M, GOGAN F, LAPLANTE E. NEGATIVE AND POSITIVE EFFECTS OF OESTRADIOL UPON LUTEINIZING HORMONE SECRETION IN THE FEMALE RAT. *J Endocrinol*. 1974;61(3):511–512. doi:10.1677/joe.0.0610511.

13. Shupnik MA. Gonadotropin gene modulation by steroids and gonadotropin-releasing hormone. *Biol Reprod*. 1996;54(2):279–286. doi:10.1095/biolreprod54.2.279.

14. Knobil E. The neuroendocrine control of the menstrual cycle. *Recent Prog Horm Res*. 1980;36:53 – 88.

15. Ferin M, Tempone A, Zimmering PE, Van de Wiele RL. Effect of antibodies to 17beta-estradiol and progesterone on the estrous cycle of the rat. *Endocrinology*. 1969;85(6):1070–8. doi:10.1210/endo-85-6-1070.

16. Labhsetwar AP. Role of estrogens in ovulation: A study using the estrogen-antagonist, I.C.I. 46,474. *Endocrinology*. 1970;87(3):542–51. doi:10.1210/endo-87-3-542.

17. Chappell PE, Levine JE. Stimulation of gonadotropin-releasing hormone surges by estrogen. I. Role of hypothalamic progesterone receptors. *Endocrinology*. 2000;141(4):1477–85. doi:10.1210/endo.141.4.7428.

18. Micevych P, Sinchak K, Mills RH, Tao L, LaPolt P, Lu JKH. The luteinizing hormone surge is preceded by an estrogen-induced increase of hypothalamic progesterone in ovariectomized and adrenalectomized rats. *Neuroendocrinology*. 2003;78(1):29–35. doi:71703.

19. Kuo J, Hamid N, Bondar G, Prossnitz ER, Micevych P. Membrane estrogen receptors stimulate intracellular calcium release and progesterone synthesis in hypothalamic astrocytes. *J Neurosci*. 2010;30(39):12950–7. doi:10.1523/JNEUROSCI.1158-10.2010.

20. Micevych P, Soma KK, Sinchak K. Neuroprogesterone: key to estrogen positive feedback? *Brain Res Rev*. 2008;57(2):470–80. doi:10.1016/j.brainresrev.2007.06.009.

21. Micevych PE, Chaban V, Ogi J, Dewing P, Lu JKH, Sinchak K. Estradiol stimulates progesterone synthesis in hypothalamic astrocyte cultures. *Endocrinology*. 2007;148(2):782–9. doi:10.1210/en.2006-0774.

22. Micevych P, Sinchak K. The Neurosteroid Progesterone Underlies Estrogen Positive Feedback of the LH Surge. *Front Endocrinol (Lausanne)*. 2011;2:90. doi:10.3389/fendo.2011.00090.

23. Chaban V V, Lakhter AJ, Micevych P. A membrane estrogen receptor mediates intracellular calcium release in astrocytes. *Endocrinology*. 2004;145(8):3788–95. doi:10.1210/en.2004-0149.

24. Ubuka T, Inoue K, Fukuda Y, et al. Identification, expression, and physiological functions of Siberian hamster gonadotropin-inhibitory hormone. *Endocrinology*. 2012;153(1):373–385. Available at: papers://cf7c60b8-94a1-4c79-88e4-6c57345fd583/Paper/p1434.

25. Ubuka T, Morgan K, Pawson A, et al. Identification of human GnIH homologs, RFRP-1 and RFRP-3, and the cognate receptor, GPR147 in the human hypothalamic pituitary axis. *PLoS One*. 2009;4(12):1334–1339. Available at: papers://cf7c60b8-94a1-4c79-88e4-6c57345fd583/Paper/p1233.

26. Ubuka T, Lai H, Kitani M, et al. Gonadotropin-inhibitory hormone identification, cDNA cloning, and distribution in rhesus macaque brain. *J Comp Neurol*. 2009;517:841–855. doi:10.1002/cne.22191.

27. Ukena K, Iwakoshi E, Minakata H, Tsutsui K. A novel rat hypothalamic RFamide-related peptide identified by immunoaffinity chromatography and mass spectrometry. *FEBS Lett*. 2002;512(1-3):255–258. Available at: papers://cf7c60b8-94a1-4c79-88e4-6c57345fd583/Paper/p1299.

28. Tsutsui K, Saigoh E, Ukena K, et al. A novel avian hypothalamic peptide inhibiting gonadotropin release. *Biochem Biophys Res Commun*. 2000;275(2):661–7. doi:10.1006/bbrc.2000.3350.

29. De Roux N, Genin E, Carel J-C, Matsuda F, Chaussain J-L, Milgrom E. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. *Proc Natl Acad Sci U S A*. 2003;100(19):10972–6. doi:10.1073/pnas.1834399100.

30. Thompson EL, Patterson M, Murphy KG, et al. Central and peripheral administration of kisspeptin-10 stimulates the hypothalamic-pituitary-gonadal axis. *J Neuroendocrinol*. 2004;16(10):850–8. doi:10.1111/j.1365-2826.2004.01240.x.

31. Gottsch ML, Cunningham MJ, Smith JT, et al. A role for kisspeptins in the regulation of gonadotropin secretion in the mouse. *Endocrinology*. 2004;145(9):4073–7. doi:10.1210/en.2004-0431.

32. Navarro VM, Castellano JM, Fernández-Fernández R, et al. Effects of KiSS-1 peptide, the natural ligand of GPR54, on follicle-stimulating hormone secretion in the rat. *Endocrinology*. 2005;146(4):1689–97. doi:10.1210/en.2004-1353.

33. Dhillo WS, Chaudhri OB, Patterson M, et al. Kisspeptin-54 stimulates the hypothalamic-pituitary gonadal axis in human males. *J Clin Endocrinol Metab*. 2005;90(12):6609–15. doi:10.1210/jc.2005-1468.

34. Li X-F, Kinsey-Jones JS, Cheng Y, et al. Kisspeptin signalling in the hypothalamic arcuate nucleus regulates GnRH pulse generator frequency in the rat. Tena-Sempere M, ed. *PLoS One*. 2009;4(12):e8334. doi:10.1371/journal.pone.0008334.

35. Maeda K-I, Ohkura S, Uenoyama Y, et al. Neurobiological mechanisms underlying GnRH pulse generation by the hypothalamus. *Brain Res*. 2010;1364:103–15. doi:10.1016/j.brainres.2010.10.026.

36. Roseweir AK, Kauffman AS, Smith JT, et al. Discovery of potent kisspeptin antagonists delineate physiological mechanisms of gonadotropin regulation. *J Neurosci*. 2009;29(12):3920–9. doi:10.1523/JNEUROSCI.5740-08.2009.

37. Messager S, Chatzidaki EE, Ma D, et al. Kisspeptin directly stimulates gonadotropin-releasing hormone release via G protein-coupled receptor 54. *Proc Natl Acad Sci U S A*. 2005;102(5):1761–1766. Available at: papers://cf7c60b8-94a1-4c79-88e4-6c57345fd583/Paper/p1232.

38. Millar RP, Roseweir AK, Tello JA, et al. Kisspeptin antagonists: unraveling the role of kisspeptin in reproductive physiology. *Brain Res*. 2010;1364:81–9. doi:10.1016/j.brainres.2010.09.044.

39. Pineda R, Garcia-Galiano D, Sanchez-Garrido MA, et al. Characterization of the inhibitory roles of RFRP3, the mammalian ortholog of GnIH, in the control of gonadotropin secretion in the rat: in vivo and in vitro studies. *Am J Physiol Endocrinol Metab*. 2010;299(1):E39–46. Available at: papers://cf7c60b8-94a1-4c79-88e4-6c57345fd583/Paper/p1269.

40. Khan AR, Kauffman AS. The role of kisspeptin and RFamide-related peptide-3 neurones in the circadian-timed preovulatory luteinising hormone surge. *J Neuroendocrinol*. 2012;24(1):131–143. Available at: papers://cf7c60b8-94a1-4c79-88e4-6c57345fd583/Paper/p1435.

41. Clarke I, Smith J, Henry B, et al. Gonadotropin-Inhibitory Hormone Is a Hypothalamic Peptide That Provides a Molecular Switch between Reproduction and Feeding. *Neuroendocrinology*. 2012;95(4):305–16. Available at: papers://cf7c60b8-94a1-4c79-88e4-6c57345fd583/Paper/p1596.

42. Wu M, Dumalska I, Morozova E, Van Den Pol A, Alreja M. Gonadotropin inhibitory hormone inhibits basal forebrain vGluT2-gonadotropin-releasing hormone neurons via a direct postsynaptic mechanism. *J Physiol*. 2009;587(7):1401. Available at: papers://cf7c60b8-94a1-4c79-88e4-6c57345fd583/Paper/p1234.

43. Kriegsfeld LJ, Gibson EM, Williams WP, et al. The roles of RFamide-related peptide-3 in mammalian reproductive function and behaviour. *J Neuroendocrinol*. 2010;22(7):692–700. doi:10.1111/j.1365-2826.2010.02031.x.

44. Clarkson J, d'Anglemont de Tassigny X, Moreno AS, Colledge WH, Herbison AE. Kisspeptin-GPR54 signaling is essential for preovulatory gonadotropin-releasing hormone neuron activation and the luteinizing hormone surge. *J Neurosci*. 2008;28(35):8691–7. doi:10.1523/JNEUROSCI.1775-08.2008.

45. Sinchak K, Wagner EJ. Estradiol signaling in the regulation of reproduction and energy balance. *Front Neuroendocrinol*. 2012;33(4):342–63. doi:10.1016/j.yfrne.2012.08.004.

46. Christensen A, Bentley GE, Cabrera R, et al. Hormonal regulation of female reproduction. *Horm Metab Res*. 2012;44(8):587–91. doi:10.1055/s-0032-1306301.

47. Giuliani FA, Yunes R, Mohn CE, Laconi M, Rettori V, Cabrera R. Allopregnanolone induces LHRH and glutamate release through NMDA receptor modulation. *Endocrine*. 2011;40(1):21–6. doi:10.1007/s12020-011-9451-8.

48. Sim JA, Skynner MJ, Herbison AE. Direct regulation of postnatal GnRH neurons by the progesterone derivative allopregnanolone in the mouse. *Endocrinology*. 2001;142(10):4448–53. doi:10.1210/endo.142.10.8451.

49. el-Etr M, Akwa Y, Fiddes RJ, Robel P, Baulieu EE. A progesterone metabolite stimulates the release of gonadotropin-releasing hormone from GT1-1 hypothalamic neurons via the gamma-aminobutyric acid type A receptor. *Proc Natl Acad Sci U S A*. 1995;92(9):3769–73. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=42043&tool=pmcentrez&rendertype=abstract. Accessed May 27, 2014.

50. Oakley AE, Breen KM, Clarke IJ, Karsch FJ, Wagenmaker ER, Tilbrook AJ. Cortisol reduces gonadotropin-releasing hormone pulse frequency in follicular phase ewes: influence of ovarian steroids. *Endocrinology*. 2009;150(1):341–9. doi:10.1210/en.2008-0587.

51. Cates PS, Li XF, O'Byrne KT. The influence of 17beta-oestradiol on corticotrophin-releasing hormone induced suppression of luteinising hormone pulses and the role of CRH in hypoglycaemic stress-induced suppression of pulsatile LH secretion in the female rat. *Stress*. 2004;7(2):113–8. doi:10.1080/1025389042000218988.

52. Bowe JE, Li XF, Kinsey-Jones JS, Brain SD, Lightman SL, O'Byrne KT. The role of corticotrophin-releasing hormone receptors in the calcitonin gene-related peptide-induced suppression of pulsatile luteinising hormone secretion in the female rat. *Stress*. 2008;11(4):312–9. doi:10.1080/10253890701801448.

53. Li XF, Bowe JE, Kinsey-Jones JS, Brain SD, Lightman SL, O'Byrne KT. Differential role of corticotrophin-releasing factor receptor types 1 and 2 in stress-induced suppression of pulsatile luteinising hormone secretion in the female rat. *J Neuroendocrinol*. 2006;18(8):602–610. Available at: papers://cf7c60b8-94a1-4c79-88e4-6c57345fd583/Paper/p701.

54. Li XF, Bowe JE, Lightman SL, O'Byrne KT. Role of corticotropin-releasing factor receptor-2 in stress-induced suppression of pulsatile luteinizing hormone secretion in the rat. *Endocrinology*. 2005;146(1):318–322. Available at: papers://cf7c60b8-94a1-4c79-88e4-6c57345fd583/Paper/p710.

55. Kinsey-Jones J, Li X, Knox A, et al. Corticotrophin-Releasing Factor Alters the Timing of Puberty in the Female Rat. *J Neuroendocrinol*. 2010;22(2):102–109. Available at: file:///Users/annageraghty/Documents/Papers/2010/Kinsey-Jones/Journal of Neuroendocrinology 2010 Kinsey-Jones.pdf.

56. Rivier C, Rivest S. Effect of stress on the activity of the hypothalamic-pituitary-gonadal axis: peripheral and central mechanisms. *Biol Reprod*. 1991;45(4):523–32. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1661182. Accessed September 9, 2013.

57. RIVEST S, RIVIER C. Central Mechanisms and Sites of Action Involved in the Inhibitory Effects of CRF and Cytokines on LHRH Neuronal Activity. *Ann N Y Acad Sci*. 1993;697(1 Corticotropin):117–141. doi:10.1111/j.1749-6632.1993.tb49928.x.

58. Calogero AE, Burrello N, Bosboom AM, Garofalo MR, Weber RF, D'Agata R. Glucocorticoids inhibit gonadotropin-releasing hormone by acting directly at the hypothalamic level. *J Endocrinol Invest*. 1999;22(9):666–70. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10595829. Accessed May 28, 2014.

59. Ahima RS, Harlan RE. Glucocorticoid receptors in LHRH neurons. *Neuroendocrinology*. 1992;56(6):845–50. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1369593. Accessed May 27, 2014.

60. Dondi D, Piccolella M, Messi E, et al. Expression and differential effects of the activation of glucocorticoid receptors in mouse gonadotropin-releasing hormone neurons. *Neuroendocrinology*. 2005;82(3-4):151–63. doi:10.1159/000091693.

61. DeFranco DB, Attardi B, Chandran UR. Glucocorticoid receptor-mediated repression of GnRH gene expression in a hypothalamic GnRH-secreting neuronal cell line. *Ann N Y Acad Sci*. 1994;746:473–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7825918. Accessed May 28, 2014.

62. Tellam DJ, Perone MJ, Dunn IC, et al. Direct regulation of GnRH transcription by CRF-like peptides in an immortalized neuronal cell line. *Neuroreport*. 1998;9(14):3135–3140. doi:10.1097/00001756-199810050-00003.

63. Tellam DJ, Mohammad YN, Lovejoy DA. Molecular integration of hypothalamo-pituitary-adrenal axis-related neurohormones on the GnRH neuron. 2011. Available at: http://www.nrcresearchpress.com/doi/abs/10.1139/o00-060#.U4Z5rlhdX-Y. Accessed May 29, 2014.

64. Mellon PL, Windle JJ, Goldsmith PC, Padula CA, Roberts JL, Weiner RI. Immortalization of hypothalamic GnRH by genetically targeted tumorigenesis. *Neuron*. 1990;5(1):1–10. doi:10.1016/0896-6273(90)90028-E.

65. Wetsel WC, Mellon PL, Weiner RI, Negro-Vilar A. Metabolism of pro-luteinizing hormone-releasing hormone in immortalized hypothalamic neurons. *Endocrinology*. 1991;129(3):1584–95. doi:10.1210/endo-129-3-1584.

66. Chandran UR, Attardi B, Friedman R, Dong KW, Roberts JL, DeFranco DB. Glucocorticoid receptor-mediated repression of gonadotropin-releasing hormone promoter activity in GT1 hypothalamic cell lines. *Endocrinology*. 1994;134(3):1467–74. doi:10.1210/endo.134.3.8119188.

67. Attardi B, Tsujii T, Friedman R, et al. Glucocorticoid repression of gonadotropin-releasing hormone gene expression and secretion in morphologically distinct subpopulations of GT1-7 cells. *Mol Cell Endocrinol*. 1997;131(2):241–55. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9296383. Accessed May 28, 2014.

68. Gore A, Attardi B, DeFranco D. Glucocorticoid repression of the reproductive axis: effects on GnRH and gonadotropin subunit mRNA levels. *Mol Cell Endocrinol*. 2006;256(1-2):40–48. Available at: papers://cf7c60b8-94a1-4c79-88e4-6c57345fd583/Paper/p1253.

69. Maeda K, Cagampang FR, Coen CW, Tsukamura H. Involvement of the catecholaminergic input to the paraventricular nucleus and of corticotropin-releasing hormone in the fasting-induced suppression of

luteinizing hormone release in female rats. *Endocrinology*. 1994;134(4):1718–22. doi:10.1210/endo.134.4.8137735.

70. Tsukamura H, Nagatani S, Cagampang FR, Kawakami S, Maeda K. Corticotropin-releasing hormone mediates suppression of pulsatile luteinizing hormone secretion induced by activation of alpha-adrenergic receptors in the paraventricular nucleus in female rats. *Endocrinology*. 1994;134(3):1460–6. doi:10.1210/endo.134.3.8119187.

71. DeFazio RA, Heger S, Ojeda SR, Moenter SM. Activation of A-type gamma-aminobutyric acid receptors excites gonadotropin-releasing hormone neurons. *Mol Endocrinol*. 2002;16(12):2872–91. doi:10.1210/me.2002-0163.

72. Klöhn P-C, Farmer M, Linehan JM, et al. PrP antibodies do not trigger mouse hippocampal neuron apoptosis. *Science*. 2012;335(6064):52. doi:10.1126/science.1215579.

73. Kinsey-Jones J, Li X, Knox A, et al. Down-Regulation of Hypothalamic Kisspeptin and its Receptor, Kiss1r, mRNA Expression is Associated with Stress-Induced Suppression of Luteinising Hormone Secretion in the Female Rat. *J Neuroendocrinol*. 2009;21(1):20–29. Available at: file:///Users/annageraghty/Documents/Papers/2009/Kinsey-Jones/Journal of Neuroendocrinology 2009 Kinsey-Jones-1.pdf.

74. Iwasa T, Matsuzaki T, Murakami M, et al. Decreased expression of kisspeptin mediates acute immune/inflammatory stress-induced suppression of gonadotropin secretion in female rat. *J Endocrinol Invest*. 2008;31(7):656–9. Available at: http://europepmc.org/abstract/MED/18787387. Accessed May 28, 2014.

75. Grachev P, Li XF, O'Byrne K. Stress regulation of kisspeptin in the modulation of reproductive function. *Adv Exp Med Biol*. 2013;784:431–54. doi:10.1007/978-1-4614-6199-9_20.

76. Takumi K, Iijima N, Higo S, Ozawa H. Immunohistochemical analysis of the colocalization of corticotropin-releasing hormone receptor and glucocorticoid receptor in kisspeptin neurons in the hypothalamus of female rats. *Neurosci Lett*. 2012;531(1):40–5. doi:10.1016/j.neulet.2012.10.010.

77. Grachev P, Li XF, Hu MH, et al. Neurokinin B signaling in the female rat: a novel link between stress and reproduction. *Endocrinology*. 2014:en20132038. doi:10.1210/en.2013-2038.

78. Goodman RL, Hileman SM, Nestor CC, et al. Kisspeptin, neurokinin B, and dynorphin act in the arcuate nucleus to control activity of the GnRH pulse generator in ewes. *Endocrinology*. 2013;154(11):4259–69. doi:10.1210/en.2013-1331.

79. Okamura H, Tsukamura H, Ohkura S, Uenoyama Y, Wakabayashi Y, Maeda K. Kisspeptin and GnRH pulse generation. *Adv Exp Med Biol*. 2013;784:297–323. doi:10.1007/978-1-4614-6199-9_14.

80. Wakabayashi Y, Yamamura T, Sakamoto K, Mori Y, Okamura H. Electrophysiological and morphological evidence for synchronized GnRH pulse generator activity among Kisspeptin/neurokinin B/dynorphin A (KNDy) neurons in goats. *J Reprod Dev*. 2013;59(1):40–8. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3943231&tool=pmcentrez&rendertype=abstract. Accessed May 28, 2014.

81. Bowe JE, Li XF, Kinsey-Jones JS, et al. Calcitonin gene-related peptide-induced suppression of luteinizing hormone pulses in the rat: the role of endogenous opioid peptides. *J Physiol*. 2005;566(Pt 3):921–8. doi:10.1113/jphysiol.2005.085662.

82. Nabeshima T, Katoh A, Wada M, Kameyama T. Stress-induced changes in brain Met-enkephalin, Leu-enkephalin and dynorphin concentrations. *Life Sci*. 1992;51(3):211–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1352028. Accessed May 28, 2014.

83. Petraglia F, Vale W, Rivier C. Opioids act centrally to modulate stress-induced decrease in luteinizing hormone in the rat. *Endocrinology*. 1986;119(6):2445–50. doi:10.1210/endo-119-6-2445.

84. Ukena K, Iwakoshi E, Minakata H, Tsutsui K. A novel rat hypothalamic RFamide-related peptide identified by immunoaffinity chromatography and mass spectrometry. *FEBS Lett*. 2002;512(1-3):255–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11852091. Accessed September 9, 2013.

85. Sari IP, Rao A, Smith JT, Tilbrook AJ, Clarke IJ. Effect of RF-amide-related peptide-3 on luteinizing hormone and follicle-stimulating hormone synthesis and secretion in ovine pituitary gonadotropes. *Endocrinology*. 2009;150(12):5549–5556. Available at: papers://cf7c60b8-94a1-4c79-88e4-6c57345fd583/Paper/p1243.

86. Kirby ED, Geraghty AC, Ubuka T, Bentley GE, Kaufer D. Stress increases putative gonadotropin inhibitory hormone and decreases luteinizing hormone in male rats. *Proc Natl Acad Sci U S A*. 2009;106(27):11324–11329. Available at: papers://cf7c60b8-94a1-4c79-88e4-6c57345fd583/Paper/p1273.

87. Lee Son Y, Ubuka T, Narihiro M, et al. Molecular Basis for the Activation of Gonadotropin-Inhibitory Hormone Gene Transcription by Corticosterone. *Endocrinology*. 2014:en20132076. doi:10.1210/en.2013-2076.

88. Gojska NM, Belsham DD. Glucocorticoid receptor-mediated regulation of Rfrp (GnIH) and Gpr147 (GnIH-R) synthesis in immortalized hypothalamic neurons. *Mol Cell Endocrinol*. 2014;384(1-2):23–31. doi:10.1016/j.mce.2013.12.015.

89. Kaiser UB, Jakubowiak A, Steinberger A, Chin WW. Differential effects of gonadotropin-releasing hormone (GnRH) pulse frequency on gonadotropin subunit and GnRH receptor messenger ribonucleic acid levels in vitro. *Endocrinology*. 1997;138(3):1224–31. doi:10.1210/endo.138.3.4968.

90. Vale W, Rivier C, Brown M. Regulatory peptides of the hypothalamus. *Annu Rev Physiol*. 1977;39:473–527. doi:10.1146/annurev.ph.39.030177.002353.

91. Pierce JG, Parsons TF. Glycoprotein hormones: structure and function. *Annu Rev Biochem*. 1981;50:465–95. doi:10.1146/annurev.bi.50.070181.002341.

92. Kononen J, Honkaniemi J, Gustafsson JA, Pelto-Huikko M. Glucocorticoid receptor colocalization with pituitary hormones in the rat pituitary gland. *Mol Cell Endocrinol*. 1993;93(1):97–103. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8319836. Accessed May 28, 2014.

93. Breen KM, Thackray VG, Hsu T, Mak-McCully RA, Coss D, Mellon PL. Stress levels of glucocorticoids inhibit LHβ-subunit gene expression in gonadotrope cells. *Mol Endocrinol*. 2012;26(10):1716–31. doi:10.1210/me.2011-1327.

94. Armario A, Lopez-Calderon A, Jolin T, Balasch J. Response of anterior pituitary hormones to chronic stress. The specificity of adaptation. *Neurosci Biobehav Rev*. 1986;10(3):245–250. doi:10.1016/0149-7634(86)90011-4.

95. Lopez-Calderon A, Gonzalez-Quijano MI, Tresguerres JAF, Ariznavarreta C. Role of LHRH in the gonadotrophin response to restraint stress in intact male rats. *J Endocrinol*. 1990;124(2):241–246. doi:10.1677/joe.0.1240241.

96. Blake CA. Effects of “stress” on pulsatile luteinizing hormone release in ovariectomized rats. *Proc Soc Exp Biol Med*. 1975;148(3):813–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/165534. Accessed May 29, 2014.

97. Kamel F, Kubajak CL. Modulation of gonadotropin secretion by corticosterone: interaction with gonadal steroids and mechanism of action. *Endocrinology*. 1987;121(2):561–8. doi:10.1210/endo-121-2-561.

98. Rivier C, Vale W. Influence of corticotropin-releasing factor on reproductive functions in the rat. *Endocrinology*. 1984;114(3):914–21. doi:10.1210/endo-114-3-914.

99. Baldwin DM, Sawyer CH. Effects of dexamethasone on LH release and ovulation in the cyclic rat. *Endocrinology*. 1974;94(5):1397–403. doi:10.1210/endo-94-5-1397.

100. Vreeburg JT, de Greef WJ, Ooms MP, van Wouw P, Weber RF. Effects of adrenocorticotropin and corticosterone on the negative feedback action of testosterone in the adult male rat. *Endocrinology*. 1984;115(3):977–83. doi:10.1210/endo-115-3-977.

101. Taché Y, Ducharme JR, Charpenet G, Haour F, Saez J, Collu R. Effect of chronic intermittent immobilization stress on hypophyso-gonadal function of rats. *Acta Endocrinol (Copenh)*. 1980;93(2):168–74. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7376788. Accessed May 28, 2014.

102. Briski KP, Sylvester PW. Differential impact of naltrexone on luteinizing hormone release during single versus repetitive exposure to restraint stress. *Psychoneuroendocrinology*. 17(2-3):125–33. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1332097. Accessed May 28, 2014.

103. Li XF, Edward J, Mitchell JC, et al. Differential effects of repeated restraint stress on pulsatile lutenizing hormone secretion in female Fischer, Lewis and Wistar rats. *J Neuroendocrinol*. 2004;16(7):620–7. doi:10.1111/j.1365-2826.2004.01209.x.

104. Briski KP, Sylvester PW. Effects of repetitive daily acute stress on pituitary LH and prolactin release during exposure to the same stressor or a second novel stress. *Psychoneuroendocrinology*. 1987;12(6):429–37. Available at: http://www.ncbi.nlm.nih.gov/pubmed/3441582. Accessed May 28, 2014.

105. Sakakura M, Takebe K, Nakagawa S. Inhibition of luteinizing hormone secretion induced by synthetic LRH by long-term treatment with glucocorticoids in human subjects. *J Clin Endocrinol Metab*. 1975;40(5):774–9. doi:10.1210/jcem-40-5-774.

106. Du Ruisseau P, Taché Y, Brazeau P, Collu R. Effects of chronic immobilization stress on pituitary hormone secretion, on hypothalamic factor levels, and on pituitary responsiveness to LHRH and TRH in female rats. *Neuroendocrinology*. 1979;29(2):90–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/116141. Accessed September 9, 2013.

107. Rivier C, Vale W. Effect of the long-term administration of corticotropin-releasing factor on the pituitary-adrenal and pituitary-gonadal axis in the male rat. *J Clin Invest*. 1985;75(2):689–94. doi:10.1172/JCI111748.

108. Collu R, Taché Y, Ducharme J. Hormonal modifications induced by chronic stress in rats. *J Steroid Biochem*. 1979;11(1):989–1000. doi:10.1016/0022-4731(79)90042-6.

109. Suter DE, Schwartz NB, Ringstrom SJ. Dual role of glucocorticoids in regulation of pituitary content and secretion of gonadotropins. *Am J Physiol*. 1988;254(5 Pt 1):E595–600. Available at: http://ajpendo.physiology.org/content/254/5/E595.abstract. Accessed May 20, 2014.

110. Baldwin DM. The effect of glucocorticoids on estrogen-dependent luteinizing hormone release in the ovariectomized rat and on gonadotropin secretin in the intact female rat. *Endocrinology*. 1979;105(1):120–8. doi:10.1210/endo-105-1-120.

111. Maya-Núñez G, Conn PM. Transcriptional regulation of the GnRH receptor gene by glucocorticoids. *Mol Cell Endocrinol*. 2003;200(1-2):89–98. doi:10.1016/S0303-7207(02)00419-7.

112. Kotitschke A, Sadie-Van Gijsen H, Avenant C, Fernandes S, Hapgood JP. Genomic and nongenomic cross talk between the gonadotropin-releasing hormone receptor and glucocorticoid receptor signaling pathways. *Mol Endocrinol*. 2009;23(11):1726–45. doi:10.1210/me.2008-0462.

113. Ringstrom SJ, Schwartz NB. Differential effect of glucocorticoids on synthesis and secretion of Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH). *J Steroid Biochem*. 1987;27(1-3):625–630. doi:10.1016/0022-4731(87)90362-1.

114. Ringstrom SJ, McAndrews JM, Rahal JO, Schwartz NB. Cortisol in vivo increases FSH beta mRNA selectively in pituitaries of male rats. *Endocrinology*. 1991;129(5):2793–5. doi:10.1210/endo-129-5-2793.

115. Thackray VG, McGillivray SM, Mellon PL. Androgens, progestins, and glucocorticoids induce follicle-stimulating hormone beta-subunit gene expression at the level of the gonadotrope. *Mol Endocrinol*. 2006;20(9):2062–79. doi:10.1210/me.2005-0316.

116. Smals AG, Kloppenborg PW, Benraad TJ. Plasma testosterone profiles in Cushing's syndrome. *J Clin Endocrinol Metab*. 1977;45(2):240–5. doi:10.1210/jcem-45-2-240.

117. Whirledge S, Cidlowski JA. Glucocorticoids, stress, and fertility. *Minerva Endocrinol*. 2010;35(2):109–25. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3547681&tool=pmcentrez&rendertype=abstract. Accessed May 20, 2014.

118. Schultz R, Isola J, Parvinen M, et al. Localization of the glucocorticoid receptor in testis and accessory sexual organs of male rat. *Mol Cell Endocrinol*. 1993;95(1-2):115–120. doi:10.1016/0303-7207(93)90036-J.

119. Silva EJR, Queiróz DBC, Honda L, Avellar MCW. Glucocorticoid receptor in the rat epididymis: expression, cellular distribution and regulation by steroid hormones. *Mol Cell Endocrinol*. 2010;325(1-2):64–77. doi:10.1016/j.mce.2010.05.013.

120. Bernier M, Gibb W, Collu R, Ducharme JR. Effect of glucocorticoids on testosterone production by porcine Leydig cells in primary culture. *Can J Physiol Pharmacol*. 1984;62(9):1166–1169. doi:10.1139/y84-195.

121. ORR T. Effects of restraint stress on plasma LH and testosterone concentrations, Leydig cell LH/HCG receptors, and in vitro testicular steroidogenesis in adult rats. *Horm Behav*. 1990;24(3):324–341. doi:10.1016/0018-506X(90)90013-N.

122. ORR T. Role of glucocorticoids in the stress-induced suppression of testicular steroidogenesis in adult male rats. *Horm Behav*. 1992;26(3):350–363. doi:10.1016/0018-506X(92)90005-G.

123. Cumming DC, Quigley ME, Yen SS. Acute suppression of circulating testosterone levels by cortisol in men. *J Clin Endocrinol Metab*. 1983;57(3):671–3. doi:10.1210/jcem-57-3-671.

124. Marić D, Kostić T, Kovačević R. Effects of acute and chronic immobilization stress on rat Leydig cell steroidogenesis. *J Steroid Biochem Mol Biol*. 1996;58(3):351–355. doi:10.1016/0960-0760(96)00044-1.

125. Saez JM, Morera AM, Haour F, Evain D. Effects of in vivo administration of dexamethasone, corticotropin and human chorionic gonadotropin on steroidogenesis and protein and DNA synthesis of testicular interstitial cells in prepuberal rats. *Endocrinology*. 1977;101(4):1256–63. doi:10.1210/endo-101-4-1256.

126. Bambino TH, Hsueh AJ. Direct inhibitory effect of glucocorticoids upon testicular luteinizing hormone receptor and steroidogenesis in vivo and in vitro. *Endocrinology*. 1981;108(6):2142–8. doi:10.1210/endo-108-6-2142.

127. Hales DB, Payne AH. Glucocorticoid-mediated repression of P450scc mRNA and de novo synthesis in cultured Leydig cells. *Endocrinology*. 1989;124(5):2099–104. doi:10.1210/endo-124-5-2099.

128. Payne AH, Sha LL. Multiple mechanisms for regulation of 3 beta-hydroxysteroid dehydrogenase/delta 5----delta 4-isomerase, 17 alpha-hydroxylase/C17-20 lyase cytochrome P450, and cholesterol side-chain cleavage cytochrome P450 messenger ribonucleic acid levels in primary cul. *Endocrinology*. 1991;129(3):1429–35. doi:10.1210/endo-129-3-1429.

129. Martin LJ, Tremblay JJ. Glucocorticoids antagonize cAMP-induced Star transcription in Leydig cells through the orphan nuclear receptor NR4A1. *J Mol Endocrinol*. 2008;41(3):165–75. doi:10.1677/JME-07-0145.

130. Sasagawa I, Yazawa H, Suzuki Y, Nakada T. STRESS AND TESTICULAR GERM CELL APOPTOSIS. 2009. Available at: http://informahealthcare.com/doi/abs/10.1080/014850101753145924. Accessed May 20, 2014.

131. Yazawa H. Apoptosis of testicular germ cells induced by exogenous glucocorticoid in rats. *Hum Reprod*. 2000;15(9):1917–1920. doi:10.1093/humrep/15.9.1917.

132. Gao H-B, Tong M-H, Hu Y-Q, Guo Q-S, Ge R, Hardy MP. Glucocorticoid Induces Apoptosis in Rat Leydig Cells. 2013. Available at: http://press.endocrine.org/doi/abs/10.1210/endo.143.1.8604?url_ver=Z39.88-2003&rfr_id=ori:rid:crossref.org&rfr_dat=cr_pub=pubmed. Accessed May 20, 2014.

133. Almeida SA, Petenusci SO, Anselmo-Franci JA, Rosa-e-Silva AAM, Lamano-Carvalho TL. Decreased spermatogenic and androgenic testicular functions in adult rats submitted to immobilization-induced stress from prepuberty. *Brazilian J Med Biol Res*. 1998;31(11):1443–1448. doi:10.1590/S0100-879X1998001100013.

134. Zorn B, Auger J, Velikonja V, Kolbezen M, Meden-Vrtovec H. Psychological factors in male partners of infertile couples: relationship with semen quality and early miscarriage. *Int J Androl*. 2008;31(6):557–64. doi:10.1111/j.1365-2605.2007.00806.x.

135. Tetsuka M. Expression of 11 -Hydroxysteroid Dehydrogenase, Glucocorticoid Receptor, and Mineralocorticoid Receptor Genes in Rat Ovary. *Biol Reprod*. 1999;60(2):330–335. doi:10.1095/biolreprod60.2.330.

136. Benediktsson R, Yau JLW, Brett LP, Cooke BE, Edwards CRW, Seckl JR. 11β-Hydroxysteroid dehydrogenase in the rat ovary: High expression in the oocyte. *J Endocrinol*. 1992;135(1):53–58. Available at: http://www.scopus.com/inward/record.url?eid=2-s2.0-0026757114&partnerID=tZOtx3y1.

137. McDonald SE, Henderson TA, Gomez-Sanchez CE, Critchley HOD, Mason JI. 11Beta-hydroxysteroid dehydrogenases in human endometrium. *Mol Cell Endocrinol*. 2006;248(1-2):72–8. doi:10.1016/j.mce.2005.12.010.

138. Michael AE, Evagelatou M, Norgate DP, et al. Isoforms of 11β-hydroxysteroid dehydrogenase in human granulosa-lutein cells. *Mol Cell Endocrinol*. 1997;132(1-2):43–52. doi:10.1016/S0303-7207(97)00118-4.

139. Schreiber JR, Nakamura K, Erickson GF. Rat ovary glucocorticoid receptor: Identification and characterization. *Steroids*. 1982;39(5):569–584. doi:10.1016/0039-128X(82)90057-5.

140. MICHAEL A, COOKE B. A working hypothesis for the regulation of steroidogenesis and germ cell development in the gonads by glucocorticoids and 11β-hydroxysteroid dehydrogenase (11βHSD). *Mol Cell Endocrinol*. 1994;100(1-2):55–63. doi:10.1016/0303-7207(94)90279-8.

141. Albiston AL, Smith RE, Krozowski ZS. Changes in the levels of 11β-hydroxysteroid dehydrogenase mRNA over the oestrous cycle in the rat. *J Steroid Biochem Mol Biol*. 1995;52(1):45–48. doi:10.1016/0960-0760(94)00154-E.

142. HILLIER SG. *Molecular Biology of the Female Reproductive System*. Elsevier; 1994:1–37. doi:10.1016/B978-0-08-091819-8.50005-9.

143. Hillier S., Tetsuka M. An anti-inflammatory role for glucocorticoids in the ovaries? *J Reprod Immunol*. 1998;39(1-2):21–27. doi:10.1016/S0165-0378(98)00011-4.

144. Hales DB, Payne AH. Glucocorticoid-mediated repression of P450scc mRNA and de novo synthesis in cultured Leydig cells. *Endocrinology*. 1989;124(5):2099–104. doi:10.1210/endo-124-5-2099.

145. Michael AE, Pester LA, Curtis P, Shaw RW, Edwards CR, Cooke BA. Direct inhibition of ovarian steroidogenesis by cortisol and the modulatory role of 11 beta-hydroxysteroid dehydrogenase. *Clin Endocrinol (Oxf)*. 1993;38(6):641–4. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8334750. Accessed May 29, 2014.

146. Hsueh A. Glucocorticoid inhibition of fsh-induced estrogen production in cultured rat granulosa cells. *Steroids*. 1978;32(5):639–648. doi:10.1016/0039-128X(78)90074-0.

147. Schoonmaker JN, Erickson GF. Glucocorticoid modulation of follicle-stimulating hormone-mediated granulosa cell differentiation. *Endocrinology*. 1983;113(4):1356–63. doi:10.1210/endo-113-4-1356.

148. Yang J-G. Effects of Glucocorticoids on Maturation of Pig Oocytes and Their Subsequent Fertilizing Capacity In Vitro. *Biol Reprod*. 1999;60(4):929–936. doi:10.1095/biolreprod60.4.929.

149. Jimena P, Castilla JA, Peran F, et al. Adrenal hormones in human follicular fluid. *Eur J Endocrinol*. 1992;127(5):403–406. doi:10.1530/acta.0.1270403.

150. González R, Ruiz-León Y, Gomendio M, Roldan ERS. The effect of glucocorticoids on ERK-1/2 phosphorylation during maturation of lamb oocytes and their subsequent fertilization and cleavage ability in vitro. *Reprod Toxicol*. 2010;29(2):198–205. doi:10.1016/j.reprotox.2009.10.009.

151. Andersen CY. Effect of glucocorticoids on spontaneous and follicle-stimulating hormone induced oocyte maturation in mouse oocytes during culture. *J Steroid Biochem Mol Biol*. 2003;85(2-5):423–427. doi:10.1016/S0960-0760(03)00190-0.

152. Liang B, Wei D-L, Cheng Y-N, et al. Restraint stress impairs oocyte developmental potential in mice: role of CRH-induced apoptosis of ovarian cells. *Biol Reprod*. 2013;89(3):64. doi:10.1095/biolreprod.113.110619.

153. Liu Y-X, Cheng Y-N, Miao Y-L, et al. Psychological stress on female mice diminishes the developmental potential of oocytes: a study using the predatory stress model. *PLoS One*. 2012;7(10):e48083. doi:10.1371/journal.pone.0048083.

154. Rabin DS. Glucocorticoids inhibit estradiol-mediated uterine growth: possible role of the uterine estradiol receptor. *Biol Reprod*. 1990;42(1):74–80. doi:10.1095/biolreprod42.1.74.

155. BEVER AT, HISAW FL, VELARDO JT. Inhibitory action of desoxycorticosterone acetate, cortisone acetate, and testosterone on uterine growth induced by estradiol-17beta. *Endocrinology*. 1956;59(2):165–9. doi:10.1210/endo-59-2-165.

156. JOHNSON DC, DEY SK. Role of Histamine in Implantation: Dexamethasone Inhibits Estradiol-Induced Implantation in the Rat. *Biol Reprod*. 1980;22(5):1136–1141. Available at: http://www.biolreprod.org/content/22/5/1136.abstract. Accessed May 26, 2014.

157. Zhao L-H, Cui X-Z, Yuan H-J, et al. Restraint Stress Inhibits Mouse Implantation: Temporal Window and the Involvement of HB-EGF, Estrogen and Progesterone. *PLoS One*. 2013;8(11):e80472. doi:10.1371/journal.pone.0080472.

158. Chien EJ, Liao C-F, Chang C-P, et al. The non-genomic effects on Na+/H+-exchange 1 by progesterone and 20alpha-hydroxyprogesterone in human T cells. *J Cell Physiol*. 2007;211(2):544–50. doi:10.1002/jcp.20962.

159. Szekeres-Bartho J, Barakonyi A, Miko E, Polgar B, Palkovics T. The role of gamma/delta T cells in the feto-maternal relationship. *Semin Immunol*. 2001;13(4):229–33. doi:10.1006/smim.2000.0318.

160. Arck P, Hansen PJ, Mulac Jericevic B, Piccinni MP, Szekeres-Bartho J. Progesterone during pregnancy: endocrine-immune cross talk in mammalian species and the role of stress. *Am J Reprod Immunol (New York, NY 1989)*. 2007;58(3):268–279. Available at: papers://cf7c60b8-94a1-4c79-88e4-6c57345fd583/Paper/p1437.

161. Szekeres-Bartho J, Wegmann TG. A progesterone-dependent immunomodulatory protein alters the Th1/Th2 balance. *J Reprod Immunol*. 1996;31(1-2):81–95. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8887124. Accessed May 29, 2014.

162. Polgar B, Kispal G, Lachmann M, et al. Molecular cloning and immunologic characterization of a novel cDNA coding for progesterone-induced blocking factor. *J Immunol*. 2003;171(11):5956–63. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14634107. Accessed May 29, 2014.

163. Wiebold JL, Stanfield PH, Becker WC, Hillers JK. The effect of restraint stress in early pregnancy in mice. *Reproduction*. 1986;78(1):185–192. doi:10.1530/jrf.0.0780185.

164. Joachim R, Zenclussen AC, Polgar B, et al. The progesterone derivative dydrogesterone abrogates murine stress-triggered abortion by inducing a Th2 biased local immune response. *Steroids*. 2003;68(10-13):931–940. doi:10.1016/j.steroids.2003.08.010.

165. Blois SM, Joachim R, Kandil J, et al. Depletion of CD8+ Cells Abolishes the Pregnancy Protective Effect of Progesterone Substitution with Dydrogesterone in Mice by Altering the Th1/Th2 Cytokine Profile. *J Immunol*. 2004;172(10):5893–5899. doi:10.4049/jimmunol.172.10.5893.

166. Kalinka J, Szekeres-Bartho J. The impact of dydrogesterone supplementation on hormonal profile and progesterone-induced blocking factor concentrations in women with threatened abortion. *Am J Reprod Immunol*. 2005;53(4):166–71. doi:10.1111/j.1600-0897.2005.00261.x.

167. Raghupathy R, Al Mutawa E, Makhseed M, Azizieh F, Szekeres-Bartho J. Modulation of cytokine production by dydrogesterone in lymphocytes from women with recurrent miscarriage. *BJOG*. 2005;112(8):1096–101. doi:10.1111/j.1471-0528.2005.00633.x.

168. Ferin M. Clinical review 105: Stress and the reproductive cycle. *J Clin Endocrinol Metab*. 1999;84(6):1768–74. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10372662. Accessed September 9, 2013.

169. Tilbrook AJ, Turner AI, Clarke IJ. Effects of stress on reproduction in non-rodent mammals: the role of glucocorticoids and sex differences. *Rev Reprod*. 2000;5(2):105–13. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10864855. Accessed September 9, 2013.

170. Louis GMB, Lum KJ, Sundaram R, et al. Stress reduces conception probabilities across the fertile window: evidence in support of relaxation. *Fertil Steril*. 2011;95(7):2184–9. doi:10.1016/j.fertnstert.2010.06.078.

171. Wasser SK, Barash DP. Reproductive suppression among female mammals: implications for biomedicine and sexual selection theory. *Q Rev Biol*. 1983;58(4):513–38. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6686686. Accessed September 9, 2013.

172. Young AJ, Carlson AA, Monfort SL, Russell AF, Bennett NC, Clutton-Brock T. Stress and the suppression of subordinate reproduction in cooperatively breeding meerkats. *Proc Natl Acad Sci U S A*. 2006;103(32):12005–10. doi:10.1073/pnas.0510038103.

173. Practice Committee of the American Society for Reproductive Medicine in collaboration with the Society for Reproductive Endocrinology and Infertility. Optimizing natural fertility. *Fertil Steril*. 2008;90(5 Suppl):S1–6. doi:10.1016/j.fertnstert.2008.08.122.

174. Ukena K, Tsutsui K. Distribution of novel RFamide-related peptide-like immunoreactivity in the mouse central nervous system. *Neurosci Lett*. 2001;300(3):153–156. Available at: papers://cf7c60b8-94a1-4c79-88e4-6c57345fd583/Paper/p1304.

175. Kriegsfeld L, Mei D, Bentley G, et al. Identification and characterization of a gonadotropin-inhibitory system in the brains of mammals. *Proc Natl Acad Sci U S A*. 2006;103(7):2410. Available at: papers://cf7c60b8-94a1-4c79-88e4-6c57345fd583/Paper/p1216.

176. Ducret E, Anderson GM, Herbison AE. RFamide-related peptide-3, a mammalian gonadotropin-inhibitory hormone ortholog, regulates gonadotropin-releasing hormone neuron firing in the mouse. *Endocrinology*. 2009;150:2799–2804. doi:10.1210/en.2008-1623.

177. Johnson M, Tsutsui K, Fraley G. Rat RFamide-related peptide-3 stimulates GH secretion, inhibits LH secretion, and has variable effects on sex behavior in the adult male rat. *Horm Behav*. 2007;51(1):171–180. Available at: papers://cf7c60b8-94a1-4c79-88e4-6c57345fd583/Paper/p1258.

178. Murakami M, Matsuzaki T, Iwasa T, et al. Hypophysiotropic role of RFamide-related peptide-3 in the inhibition of LH secretion in female rats. *J Endocrinol*. 2008;199(1):105. Available at: papers://cf7c60b8-94a1-4c79-88e4-6c57345fd583/Paper/p1255.

179. Son YL, Ubuka T, Millar RP, Kanasaki H. Gonadotropin-Inhibitory Hormone Inhibits GnRH- Induced Gonadotropin Subunit Gene Transcriptions by Inhibiting AC/cAMP/PKA-Dependent ERK Pathway in L[beta]T2 Cells. *Neuroendocrinology*. 2012;153:2332–2343. doi:10.1210/en.2011-1904.

180. Pineda R, Garcia-Galiano D, Sanchez-Garrido M, et al. Characterization of the potent gonadotropin-releasing activity of RF9, a selective antagonist of RF-amide-related peptides and neuropeptide FF

receptors: physiological and pharmacological implications. *Endocrinology*. 2010;151(4):1902. Available at: papers://cf7c60b8-94a1-4c79-88e4-6c57345fd583/Paper/p1214.

181. Rizwan MZ, Porteous R, Herbison AE, Anderson GM. Cells expressing RFamide-related peptide-1/3, the mammalian gonadotropin-inhibitory hormone orthologs, are not hypophysiotropic neuroendocrine neurons in the rat. *Endocrinology*. 2009;150(3):1413–20. doi:10.1210/en.2008-1287.

182. Rizwan MZ, Poling MC, Corr M, et al. RFamide-related peptide-3 receptor gene expression in GnRH and kisspeptin neurons and GnRH-dependent mechanism of action. *Endocrinology*. 2012;153(8):3770–9. doi:10.1210/en.2012-1133.

183. Ciccone NA, Dunn IC, Boswell T, et al. Gonadotrophin inhibitory hormone depresses gonadotrophin alpha and follicle-stimulating hormone beta subunit expression in the pituitary of the domestic chicken. *J Neuroendocrinol*. 2004;16(12):999–1006. Available at: papers://cf7c60b8-94a1-4c79-88e4-6c57345fd583/Paper/p1278.

184. Ubuka T, Inoue K, Fukuda Y, et al. Identification, Expression, and Physiological Functions of Siberian Hamster Gonadotropin-Inhibitory Hormone. *Endocrinology*. 2011. Available at: papers://cf7c60b8-94a1-4c79-88e4-6c57345fd583/Paper/p1335.

185. Anderson G, Relf H, Rizwan M, Evans J. Central and peripheral effects of RFamide-related peptide-3 on luteinizing hormone and prolactin secretion in rats. *Endocrinology*. 2009;150(4):1834. Available at: papers://cf7c60b8-94a1-4c79-88e4-6c57345fd583/Paper/p1212.

186. Blaustein JD. Neuroendocrine regulation of feminine sexual behavior: lessons from rodent models and thoughts about humans. *Annu Rev Psychol*. 2008;59:93–118. doi:10.1146/annurev.psych.59.103006.093556.

187. Sirinathsinghji DJS, Rees LH, Rivier J, Vale W. Corticotropin-releasing factor is a potent inhibitor of sexual receptivity in the female rat. *Nature*. 1983;305(5931):232–235. doi:10.1038/305232a0.

188. Sato Y, Suzuki N, Horita H, et al. Effects of long-term psychological stress on sexual behavior and brain catecholamine levels. *J Androl*. 17(2):83–90. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8723430. Accessed September 17, 2013.

189. Piekarski DJ, Zhao S, Jennings KJ, et al. Gonadotropin-inhibitory hormone reduces sexual motivation but not lordosis behavior in female Syrian hamsters (Mesocricetus auratus). *Horm Behav*. 2013;64(3):501–10. doi:10.1016/j.yhbeh.2013.06.006.

190. Hardy DF, Debold JF. Effects of mounts without intromission upon the behavior of female rats during the onset of estrogen-induced heat. *Physiol Behav*. 1971;7(4):643–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/5167162. Accessed September 9, 2013.

191. Conaway CH, Conaway BYCH. Embryo Resorption and Placental Scar Formation in the Rat. *J Mammal*. 1955;36(4):516–532.

192. Goosens KA, Maren S. Contextual and auditory fear conditioning are mediated by the lateral, basal, and central amygdaloid nuclei in rats. *Learn Mem*. 8(3):148–55. doi:10.1101/lm.37601.

193. Zhao S, Fernald RD. Comprehensive algorithm for quantitative real-time polymerase chain reaction. *J Comput Biol*. 2005;12(8):1047–64. doi:10.1089/cmb.2005.12.1047.

194. Barres BA. The Mystery and Magic of Glia: A Perspective on Their Roles in Health and Disease. *Neuron*. 2008;60(3):430–440. Available at: http://dx.doi.org/10.1016/j.neuron.2008.10.013.

195. Zhang Y, Barres BA. Astrocyte heterogeneity: an underappreciated topic in neurobiology. *Curr Opin Neurobiol*. 2010;20(5):588–94. doi:10.1016/j.conb.2010.06.005.

196. Wang DD, Bordey A. The astrocyte odyssey. *Prog Neurobiol*. 2008;86(4):342–67. doi:10.1016/j.pneurobio.2008.09.015.

197. Ramón y Cajal S. *Histologie du système nerveux de l'homme & des vertébrés.* Paris : Maloine,; 1909:1 – 1012. doi:10.5962/bhl.title.48637.

198. Andriezen WL. The Neuroglia Elements in the Human Brain. *Br Med J*. 1893;2(1700):227–30. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2422013&tool=pmcentrez&rendertype=abstract. Accessed April 27, 2015.

199. Miller RH, Raff MC. Fibrous and protoplasmic astrocytes are biochemically and developmentally distinct. *J Neurosci*. 1984;4(2):585–92. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6366155. Accessed May 5, 2015.

200. Kettenmann H, Verkhratsky A. Neuroglia: the 150 years after. *Trends Neurosci*. 2008;31(12):653–9. doi:10.1016/j.tins.2008.09.003.

201. Matthias K, Kirchhoff F, Seifert G, et al. Segregated expression of AMPA-type glutamate receptors and glutamate transporters defines distinct astrocyte populations in the mouse hippocampus. *J Neurosci*. 2003;23(5):1750–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12629179. Accessed March 28, 2015.

202. Seifert G, Steinhäuser C. Glial Cells in the Mouse Hippocampus Express AMPA Receptors with an Intermediate Ca 2+ Permeability. *Eur J Neurosci*. 1995;7(9):1872–1881. doi:10.1111/j.1460-9568.1995.tb00708.x.

203. Steinhäuser C, Gallo V. News on glutamate receptors in glial cells. *Trends Neurosci*. 1996;19(8):339–345. doi:10.1016/0166-2236(96)10043-6.

204. Zhou M, Kimelberg HK. Freshly Isolated Hippocampal CA1 Astrocytes Comprise Two Populations Differing in Glutamate Transporter and AMPA Receptor Expression. *J Neurosci*. 2001;21(20):7901–7908. Available at: http://www.jneurosci.org/content/21/20/7901.abstract?ijkey=e2ab76f255bc16f4f315a96e3caa91400b24e38b&keytype2=tf_ipsecsha. Accessed May 5, 2015.

205. Theis M, Söhl G, Eiberger J, Willecke K. Emerging complexities in identity and function of glial connexins. *Trends Neurosci*. 2005;28(4):188–195. Available at: http://www.sciencedirect.com/science/article/pii/S0166223605000500.

206. Theis M, Giaume C. Connexin-based intercellular communication and astrocyte heterogeneity. *Brain Res*. 2012;1487:88–98. doi:10.1016/j.brainres.2012.06.045.

207. Anders S, Minge D, Griemsmann S, Herde MK, Steinhäuser C, Henneberger C. Spatial properties of astrocyte gap junction coupling in the rat hippocampus. *Philos Trans R Soc Lond B Biol Sci*. 2014;369(1654):20130600. doi:10.1098/rstb.2013.0600.

208. Wallraff A, Odermatt B, Willecke K, Steinhäuser C. Distinct types of astroglial cells in the hippocampus differ in gap junction coupling. *Glia*. 2004;48(1):36–43. doi:10.1002/glia.20040.

209. Micevych PE, Abelson L. Distribution of mRNAs coding for liver and heart gap junction proteins in the rat central nervous system. *J Comp Neurol*. 1991;305(1):96–118. doi:10.1002/cne.903050110.

210. Giaume C, McCarthy KD. Control of gap-junctional communication in astrocytic networks. *Trends Neurosci*. 1996;19(8):319–325. doi:10.1016/0166-2236(96)10046-1.

211. Nagy J., Patel D, Ochalski PA., Stelmack G. Connexin30 in rodent, cat and human brain: selective expression in gray matter astrocytes, co-localization with connexin43 at gap junctions and late developmental appearance. *Neuroscience*. 1999;88(2):447–468. doi:10.1016/S0306-4522(98)00191-2.

212. Nagy JI, Ionescu A-V, Lynn BD, Rash JE. Coupling of astrocyte connexins Cx26, Cx30, Cx43 to oligodendrocyte Cx29, Cx32, Cx47: Implications from normal and connexin32 knockout mice. *Glia*. 2003;44(3):205–18. doi:10.1002/glia.10278.

213. McCarthy KD, de Vellis J. Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. *J Cell Biol*. 1980;85(3):890–902. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2111442&tool=pmcentrez&rendertype=abstract. Accessed April 14, 2015.

214. Geraghty AC, Muroy SE, Zhao S, Bentley GE, Kriegsfeld LJ, Kaufer D. Knockdown of hypothalamic RFRP3 prevents chronic stress-induced infertility and embryo resorption. *Elife*. 2015;4. doi:10.7554/eLife.04316.

215. Molofsky A V, Krencik R, Krenick R, et al. Astrocytes and disease: a neurodevelopmental perspective. *Genes Dev*. 2012;26(9):891–907. doi:10.1101/gad.188326.112.

216. Maragakis NJ, Rothstein JD. Mechanisms of Disease: astrocytes in neurodegenerative disease. *Nat Clin Pract Neurol*. 2006;2(12):679–89. doi:10.1038/ncpneuro0355.

217. Walz W. Controversy surrounding the existence of discrete functional classes of astrocytes in adult gray matter. *Glia*. 2000;31(2):95–103. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10878596. Accessed May 5, 2015.

218. Son YL, Ubuka T, Narihiro M, et al. Molecular basis for the activation of gonadotropin-inhibitory hormone gene transcription by corticosterone. *Endocrinology*. 2014;155:1817–1826. doi:10.1210/en.2013-2076.

219. Beery AK, Zucker I. Sex bias in neuroscience and biomedical research. *Neurosci Biobehav Rev*. 2011;35(3):565–72. doi:10.1016/j.neubiorev.2010.07.002.

220. Rapakko S, Menke M. Factors Associated With Baseline Stress Levels at Time of Referral to an Infertility Clinic [160]. *Obstet Gynecol*. 2015;125 Suppl :55S. doi:10.1097/01.AOG.0000463700.25833.d0.

221. Lynch CD, Sundaram R, Maisog JM, Sweeney AM, Buck Louis GM. Preconception stress increases the risk of infertility: results from a couple-based prospective cohort study--the LIFE study. *Hum Reprod*. 2014;29(5):1067–75. doi:10.1093/humrep/deu032.

222. Kokay IC, Grattan DR. Expression of mRNA for prolactin receptor (long form) in dopamine and pro-opiomelanocortin neurones in the arcuate nucleus of non-pregnant and lactating rats. *J Neuroendocrinol*. 2005;17(12):827–35. doi:10.1111/j.1365-2826.2005.01374.x.

223. Bugnon C, Bloch B, Lenys D, Gouget A, Fellmann D. Comparative study of the neuronal populations containing β-endorphin, corticotropin and dopamine in the arcuate nucleus of the rat hypothalamus. *Neurosci Lett*. 1979;14(1):43–48. doi:10.1016/0304-3940(79)95341-2.

224. Voogt JL, Lee Y, Yang S, Arbogast L. Regulation of prolactin secretion during pregnancy and lactation. *Prog Brain Res*. 2001;133:173–85. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11589129. Accessed May 5, 2015.

225. Oishi H, Klausen C, Bentley GE, et al. The human gonadotropin-inhibitory hormone ortholog RFamide-related peptide-3 suppresses gonadotropin-induced progesterone production in human granulosa cells. *Endocrinology*. 2012;153(7):3435–45. doi:10.1210/en.2012-1066.

226. Musil LS, Goodenough DA. Gap junctional intercellular communication and the regulation of connexin expression and function. *Curr Opin Cell Biol*. 1990;2(5):875–880. doi:10.1016/0955-0674(90)90086-T.

227. Oyamada M, Oyamada Y, Takamatsu T. Regulation of connexin expression. *Biochim Biophys Acta*. 2005;1719(1-2):6–23. doi:10.1016/j.bbamem.2005.11.002.

228. Rouach N, Koulakoff A, Giaume C. Neurons set the tone of gap junctional communication in astrocytic networks. *Neurochem Int*. 2004;45(2-3):265–272. Available at: http://www.sciencedirect.com/science/article/pii/S0197018603002912.