UNIVERSITY OF CALIFORNIA SAN DIEGO

The Role of Corticosterone in Stress-Induced LH Inhibition and RFRP-3 Neuronal Activation in Male Gonadectomized Mice

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

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The Thesis of Frank Lee is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-chair

Chair

University of California San Diego

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ABSTRACT OF THE THESIS

The Role of Corticosterone in Stress-Induced LH Inhibition and RFRP-3 Neuronal Activation in Male Gonadectomized Mice

by

Frank Lee

Master of Science in Biology

University of California San Diego, 2020

Professor Alexander Kauffman, Chair
Professor Byungkook Lim, Co-chair

While stress is known to suppress reproductive activity, the underlying mechanism has not been fully elucidated. Because stress is associated with elevated corticosterone levels in mice, we investigated corticosterone's potential role in inhibiting the reproductive neuroendocrine axis. Three experiments were performed *in vivo* using gonadectomized male mice. First, with corticosterone injections in C57Bl6 mice, we observed neuronal activation of dorsal-medial hypothalamic RFamide-related peptide 3 cells (RFRP-3, the mammalian

ortholog of Gonadotropin Inhibiting Hormone) via double-label in-situ hybridization (ISH). We found that *Rfrp* and *cfos* mRNA co-expression significantly increased 30 minutes post-injection, which is also when corticosterone levels are at their highest (measured via ELISA). Because of this relationship between corticosterone and RFRP-3 neuronal activation, we conducted a second experiment using restraint stress in transgenic mice with glucocorticoid receptors (GR) knocked out of only RFRP-3 neurons. Corticosterone is known to bind to GR. With an ultra-sensitive murine LH ELISA, we found that LH pulses were inhibited even when GR was knocked out, suggesting that GR presence in RFRP-3 cells is not needed for stress-induced LH inhibition. To test whether GR presence is needed for RFRP-3 neuronal activation during stress, we utilized 45- and 180-minute restraint periods with the same transgenic line. Our ISH results indicated that GR absence did not change the expected increase in RFRP-3 neural activation after 45 minutes of restraint. All three experiments demonstrate that corticosterone is capable of activating RFRP-3 neurons, though stress-induced inhibition of the HPG axis does not depend on this relationship.

INTRODUCTION

When a stressor is introduced to an organism, the hypothalamic-pituitary-adrenal (HPA) axis becomes activated as a stress response. In the HPA axis, the hypothalamus first secretes corticotropin-releasing hormone (CRH) that traverses through the median eminence to stimulate the anterior pituitary gland. Next, the anterior pituitary secretes a different hormone (adrenocorticotropic hormone, or ACTH) that travels through the bloodstream and targets the adrenal glands, located right above the kidneys. The adrenal gland synthesizes glucocorticoids (cortisol in humans, corticosterone in rodents) that perform negative feedback to the hypothalamic and pituitary levels. Glucocorticoids are involved in glucose metabolism and assist the stress response by increasing blood glucose levels, giving the organism energy to combat the stressor [1].

Another consequence of stress is the suppression of processes that are considered unimportant at the given time. For example, the reproductive hypothalamic-pituitary-gonadal (HPG) axis becomes inhibited [1]. In brief, the HPG axis begins with secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus that stimulates the anterior pituitary to secrete luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH and FSH target the gonads (testes in males, ovaries in females) that then release sex steroids: androgens (testosterone) or estrogen. These sex steroids perform negative feedback to reduce secretion of the given sex steroid; males primarily produce testosterone while females produce estrogen [1]. Interestingly, *in vivo* studies suggest that GnRH neurons lack alpha estrogen receptors (ERα) and androgen receptors (AR) that directly mediate this negative feedback [2, 3]. Further studies have suggested that the sex steroids secreted by the gonads act upstream of GnRH neurons. One of these identified upstream targets is Kisspeptin

neurons, which not only possess ERα and AR, but also directly stimulate GnRH neurons [4-6].

There are two main populations of Kisspeptin neurons in the hypothalamus: those in the anteroventral periventricular (AVPV) nucleus and those in the arcuate nucleus (ARC) [7]. Kisspeptin neurons in the AVPV demonstrate positive feedback from sex steroids, meaning that exposure to sex steroids increases Kisspeptin activity. This specific population of neurons is implicated in the LH surge that occurs in females [7]. On the other hand, ARC Kisspeptin neurons demonstrate negative feedback from sex steroids, meaning that their activity declines upon androgen/estrogen stimulation [7]. ARC Kisspeptin neurons help activate and control the pulsatile secretion of GnRH from GnRH neurons. Because GnRH secretion occurs in pulses, LH and FSH are consequently released in pulses as well [8].

Another population of cells, RFamide-related peptide 3 (RFRP-3) neurons, exist in the dorsal-medial nucleus (DMN) of the hypothalamus. Their function is still being explored, though studies suggest that they act as the mammalian ortholog of gonadotropin-inhibiting hormone (GnIH). GnIH was first determined in birds and is known to inhibit GnRH secretion [9]. While the GnIH peptide is not present in mammals, the RFRP-3 seen in rodents has the same C-terminal LPXRFamide sequence seen in the GnIH amino acid sequence [9]. RFRP-3 is secreted from RFRP-3 neurons and binds to GPR147, which overall has an inhibitory effect. Many neural populations contain GPR147, including GnRH neurons, Kisspeptin neurons, and other cells. Apposition of RFRP-3 fibers is also seen in both GnRH and Kisspeptin cell bodies [10]. Thus, the overall effect of RFRP-3 is the opposite of ARC Kisspeptin neurons in that they inhibit GnRH and LH pulses [1, 9-13]. Because RFRP-3

fibers target many different regions, RFRP-3 may have both a direct and indirect inhibitory effect on GnRH secretion.

As mentioned before, the fact that stress inhibits reproductive activities indicates that the HPA and HPG axes seem to be interconnected in some ways [1]. Early studies have shown decreased LH plasma and testosterone levels upon immobilization stress in male rats [14]. In light of this and other early experiments, more studies have been conducted in many mammalian species (including rodents, ewes, and humans) demonstrating the decline in reproductive hormone secretion upon stress induction [11-12, 14-20]. Recently, a technological advancement has allowed us to study murine pulsatile LH activity in gonadectomized (GDX) mice [11]. Due to this advancement, more stress-related LH pulsatile studies are being performed in mice [11-12, 20-21].

While the mechanism by which stress inhibits the reproductive axis may not be not fully known, ARC Kisspeptin neurons and RFRP-3 neurons in the DMN seem to contribute to this phenomenon. A study in GDX female wildtype C57Bl6 mice used a double-label in-situ hybridization (ISH) to observe neuronal activation of *Kiss1* and *Rfrp* cells via *cfos* mRNA co-expression after 45-minutes and 180-minutes of restraint. The *cfos* silver grains indicate recent neural activation; cells with high *cfos* co-expression were activated recently while cells with little to no *cfos* were not. The study demonstrated that while the number of ARC Kisspeptin and RFRP-3 neurons does not change from stress induction, the activation of RFRP-3 neurons increases after 45 minutes of restraint [11]. After 180 minutes of restraint, activation of ARC Kisspeptin decreases. Interestingly, at this same 180-minute mark, activation of RFRP-3 neurons also decreases. These same results were also demonstrated in GDX male wildtype mice [12]. A reason for the decreased RFRP-3 neural

activation after 180-minutes of restraint has yet to be discovered, though this finding suggests that RFRP-3 neurons are quickly activated and become inactivated after prolonged exposure to stress [12]. Restraint stress in GDX female and male wildtype mice also demonstrates suppression of LH pulses by decreasing their frequency, mean levels, and peak values [11-12]. It is possible that RFRP-3 drives these stress-induced inhibitory effects on the reproductive axis, since RFRP-3 neurons are known to be activated during stress [11-12].

However, the mechanism by which RFRP-3 neurons become activated during stress is currently unknown. Since RFRP-3 neuronal activation and corticosterone levels both increase from stress induction, it may be possible that corticosterone binds to glucocorticoid receptors (GR) to enable RFRP-3 neuronal activation. Furthermore, unpublished preliminary data collected from our lab suggests that after injecting corticosterone in GDX C57Bl6 male mice, LH pulses are inhibited for about 30-45 minutes and recover approximately an hour later. Coincidentally, corticosterone levels are shown to be greatly elevated during this period of LH inhibition and recover back to normal levels after 90 minutes. In light of all the above, the main question we ask is: does corticosterone directly activate RFRP-3 neurons through GR to enable stress-induced reproductive symptoms like inhibited LH pulses?

EXPERIMENT 1: RFRP-3 NEURONAL ACTIVATION FROM CORTICOSTERONE INJECTIONS IN GDX WILDTYPE MALE MICE

Brief Overview

The first experiment will be based on a previous experiment performed in our lab that utilized subcutaneous corticosterone injections in GDX C57Bl6 male mice. After the injection, LH inhibition occurred for about 30-45 minutes, with pulsing returning back to normal about an hour later. So, our aim was to examine the reproductive neural populations that may have contributed to this brief LH inhibition. We focus primarily on the RFRP-3 neural population in the DMN since RFRP-3 is known to inhibit GnRH [14]. We hypothesized that at the 30-minute mark (when LH was known to be suppressed), RFRP-3 neuronal activation would increase. Because pulsing appears to recover an hour after injection, we predict that the RFRP-3 neuronal activation would be decreased 60 minutes post-injection compared to 30 minutes.

Using GDX wildtype male mice, we either: injected corticosterone, or sacrificed them immediately (the latter being the "no injection" group). For those that were injected, we either sacrificed them 30-minutes later or 60-minutes later. Later, a double-label in-situ hybridization was performed, and we looked at *Rfrp* cells and their levels of *cfos* activation. We also measured corticosterone levels at sacrifice via an ELISA.

Materials and Methods

Adult wildtype C57Bl6 male mice were housed in a vivarium undergoing a 12-hour light/darkness cycle, with lights off at 1800. Food and water were available *ad libitum*. At 8-10 weeks of age, the mice were gonadectomized for two reasons: to increase LH levels, and

to prevent gonadal sex steroids from potentially interfering with the HPG axis and the stress response [11-12]. During surgery, mice were anesthetized under 4% isoflurane and a heat pad was placed underneath the mice. Both testes were removed from a small incision made on the ventral skin line above the penis. Afterwards, the muscle underneath the skin was sutured and staples were placed on the skin to cover the incision. An analgesic dose of buprenorphine was given subcutaneously shortly after and again a day later. Staples were removed 14 days after surgery. Surgeries and experiments were performed under approval of the Institutional Animal Care and Use Committee.

After a week of recovery from the surgery, the mice were handled daily for 3 weeks so that they were prepared for the experimental injection. During this handling period, a pipet tip was used to mimic the injection each day for multiple rounds. Ideally, the handling should have prevented the mice from becoming stressed by the injection. The rise in corticosterone measured from the ELISA should occur from just the corticosterone contained in the injection and not the HPA axis becoming activated due to stress. The latter would be an unintended consequence from our experimental setup.

Preliminary unpublished data from our lab suggests that the 1 mg/kg dosage of corticosterone best models the corticosterone rise when restraint stress occurs. So, to create this dosage, corticosterone was dissolved in sesame oil (1 mg CORT/1 mL sesame oil) on the day before the experiment. For this thesis, corticosterone and CORT can be used interchangeably. 100% ethanol was also added to allow for better dissolving. The volume of ethanol used was 4% of the total mixture. The mixture was spun overnight in a fume hood to allow for the ethanol to evaporate. The next morning, the 1 mg CORT/1 mL sesame oil mixture was diluted 1:4 with sesame oil, and so a 100-microliter (μL) injection would contain

25 µL of the 1mg/1mL CORT solution, which would be equivalent to 0.025 mg of CORT. 0.025 mg of CORT would be used for a mouse that weighs 0.025 kilograms, or 25 grams. With this standardization, a mouse that weighed 24 grams, for example, would be injected with 96 µL of our 1:4 diluted CORT solution. Mice in the "no injection" group did not experience any injection and were sacrificed immediately with brains and blood collected.

There were three groups: no injection mice (n=10) that were immediately sacrificed; mice that were sacrificed 30 minutes after corticosterone injection (n=9); and mice that were sacrificed 60 minutes after injection (n=7). Vehicle-treated groups still need to be collected for the data to be completely solidified. At time of sacrifice, retro-orbital blood and brains were collected. Sacrifices occurred before noon to avoid the circadian rise of corticosterone. For all mice, the retro-orbital blood was spun down two hours after sacrifice and the plasma was extracted and kept at -20° Celsius. This plasma was used to determine corticosterone levels via DetectX Corticosterone Enzyme Immunoassay Kit (Arbor Assays). Brains were stored at -80°C.

When the brains were ready to be cut, they were placed in a cryostat at -14°C. After an hour of thawing in the cryostat, the brain was mounted onto a chuck. 20 micrometer coronal slices were collected on slides, which were stored at -80°C until ready for the in-situ hybridization (ISH) procedure. A double-label ISH procedure was used to detect DIG-labeled *Rfrp* neurons and P33 *cfos* silver grains. Once the procedure was completed, we waited for approximately 7-10 days. Afterwards, the slides were developed, fixed, and cover-slipped. Once cover-slipped, the slides were left to dry overnight and were eventually scraped, labeled, and coded so that the investigator was blinded to the treatment. Using a microscope and a quantification software program (Dr. Don Clifton, University of Washington), red

fluorescent DIG-containing *Rfrp* cells were first counted under a microscope. Then, with these labeled neurons, *cfos* silver grains levels were measured within these cells with respect to background. If there were enough *cfos* silver grains inside these identified cells, then the cell was considered to be activated. The co-expression of *cfos* and the *Rfrp* cells can also be referred to as double-labeling, co-labeling, co-localization, or co-activation. For this experiment, a cell was considered to be double-labeled if the signal-to-background ratio was greater than 4.

Results

Figure 1 shows a bar graph of the corticosterone levels of all mice at the time of sacrifice. The treatment groups are shown in the x-axis. A one-way ANOVA (group) was followed by *post hoc* Bonferroni's multiple comparison test with a $P<0.05$ considered to be statistically significant. The 30 min CORT group (n=9) had the highest level of corticosterone and was statistically different from all other groups (shown by ***, or $P<0.001$). The 60 min CORT group (n=7) had corticosterone levels that were higher than the no injection group (n=10), indicated by **, or $P<0.01$. This figure is consistent with our unpublished preliminary data that showed a drastic increase in corticosterone 30 minutes post-injection and slightly elevated levels an hour after the injection.

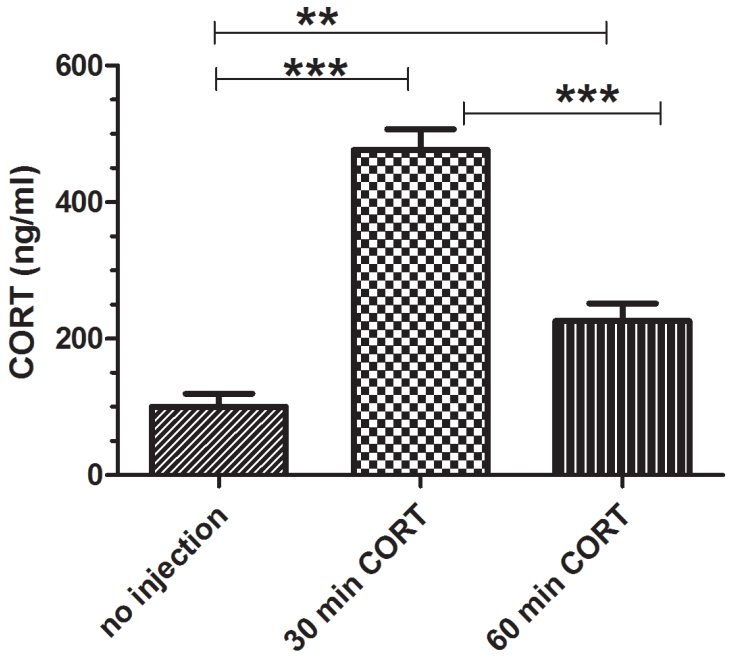

Figure 1: Corticosterone Levels After Injection for the GDX C57Bl6 Wildtype Male Mice. Treatment groups are shown in the x-axis and the measured corticosterone level (from the DetectX Corticosterone Enzyme Immunoassay Kit) is shown in the y-axis. Animals that lacked DIG expression were excluded from this graph: no injection: n=10; 30 min CORT: n=9; 60-min CORT: n=7. ***P<0.001 and **P<0.01 vs. group.

Figure 2 shows an example of DIG-labeled *Rfrp* cells co-labeled with *cfos* from this experiment. The top image shows four *Rfrp* cells, the middle image shows only *cfos* expression, and the bottom image shows both the cells and the *cfos* merged together. Again, the criteria for a co-labeled cell is that the signal-to-background ratio has to be greater than 4. In the bottom image, two of the four cells are identified with white arrows to show that these were considered to be double-labeled due to their high *cfos* co-expression.

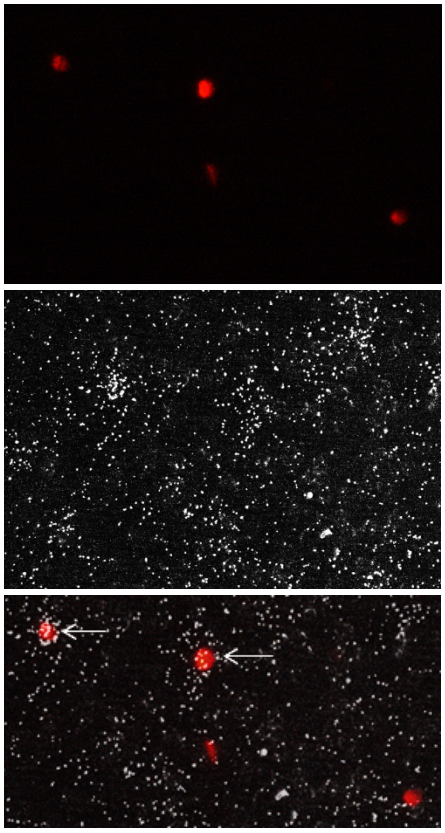

Figure 2: Examples of ISH imaging for *Rfrp* cells with *cfos* for Experiment 1. The top row shows only the DIG-labeled cells, the middle row shows *cfos* silver grains, and the bottom row shows the DIG-labeled cells and *cfos* both merged together (the white arrows indicate double-labeled cells). A cell is considered co-activated (demonstrates neuronal activation) if the signal-to-background ratio is greater than 4.

Figure 3 shows the percentage of *Rfrp* cells co-expressing *cfos* silver grains. A one-way ANOVA (group) was followed by *post hoc* Bonferroni's multiple comparison test with a $P<0.05$ considered to be statistically significant. The RFRP-3 neuronal activation significantly increased in the 30 min CORT group compared to all the other groups. There was no statistical difference between the 60 min CORT group and the no injection group. Some animals were not included in this figure because they lacked DIG expression.

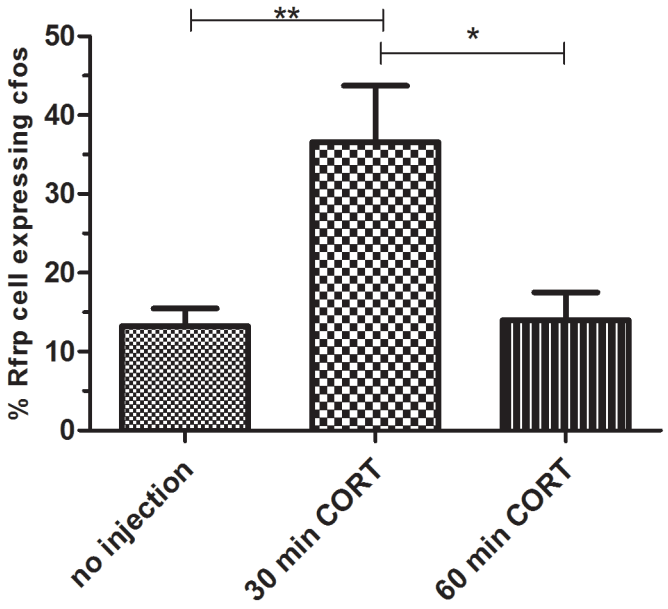

Figure 3: Percentage of cells activated for *Rfrp*. The co-expression of the cell with *cfos* indicated recent neural activation. The three groups are: no injection (n=10), 30 min CORT (n=7), and 60 min CORT (n=6). Animals that lacked DIG expression were excluded from this figure. **P<0.01 and *P<0.05 vs. group.

Discussion

Figure 1 demonstrates that corticosterone levels were highest in the 30 min CORT group and declined 60 minutes after the injection. This clear difference in corticosterone levels in the blood is consistent with the unpublished preliminary data that showed this same trend. The 60 min CORT group is still statistically different from the no injection group, so it cannot be concluded that corticosterone levels return back to normal baseline levels in an hour. The preliminary data also showed LH pulsatile inhibition for 30-45 minutes post-injection and eventually recovered an hour later. Thus, it may be possible that this rise in corticosterone levels may be related to the LH suppression observed after the injection.

Figure 3 demonstrates statistical differences in RFRP-3 neuronal activation. The percent co-localization of *Rfrp* and *cfos* in the 30 min CORT was statistically different from

all the other groups. The increased activation of RFRP-3 neurons suggests that GnRH pulsing may have been suppressed, which would have consequently led to inhibited LH pulsing. Therefore, there may be a possible relationship between corticosterone and RFRP-3 neuronal activation. It may then be possible that corticosterone directly activates RFRP-3 neurons by binding to GR, which would lead to LH inhibition. Or, perhaps corticosterone indirectly activates RFRP-3 neurons through a different pathway that can then lead to increased RFRP-3 activity. Further experiments would need to be performed to determine the exact mechanism of how corticosterone may interact with RFRP-3 neurons.

This data is missing vehicle treatment groups. Thus, two additional groups will be needed for this experiment to be fully solidified: a 30- and 60-min vehicle injection. These vehicle groups would likely be injections of approximately 100 uL sesame oil, though the exact volume of oil used is not crucial for the vehicle-treated mice. Vehicle injection groups would isolate the effect seen in neuronal activation to corticosterone alone, since it may be possible that the effects observed from this experiment were caused by the stressful injection. Hopefully, the vehicle groups show corticosterone levels similar to the no injection group. To prevent increased corticosterone from the stress-activated HPA axis, we need to ensure that our daily handling calms the mice so that they do not become stressed by the injection on the experimental day.

Another future study may involve observing activation of Kisspeptin cells in the ARC, as these are known to stimulate GnRH and LH pulsing. We would predict that their activation would be attenuated upon corticosterone injection in light of the consequent inhibition of LH pulses. While a study in GDX female C57Bl6 mice showed that kisspeptin activation in the ARC decreased after 45, 90, and 180 minutes of restraint, a study in GDX

male C57Bl6 mice only showed decreased activation after 180 minutes of restraint [11-12]. It may be possible that for males, Kisspeptin activation in the ARC decreases only after prolonged stress. Regardless, we could still repeat this experiment and see how ARC Kisspeptin activity changes when the mice are sacrificed 30 minutes or 60 minutes post-injection.

EXPERIMENT 2: LH PULSATILE INHIBITION VIA RESTRAINT STRESS IN TRANSGENIC GDX MALE MICE

Brief Overview

The results from Experiment 1 demonstrated that RFRP-3 neuronal activation increased 30 minutes after injecting corticosterone. To further study the potential relationship between corticosterone and RFRP-3, we performed a second experiment to test the necessity of corticosterone binding to glucocorticoid receptors (GR) in RFRP-3 neurons to inhibit LH pulsing. To do this, we used transgenic male mice with GR flanked by loxP sites. Mice that also tested positive for Cre Recombinase (Cre+ mice) that is specifically activated in RFRP-3 cells would have the GR gene removed from these neurons only. Mice lacking Cre Recombinase (Cre- mice) would not have GR removed because they do not possess the enzyme to knock out the receptor, thereby mimicking wildtype mice.

If stress-induced LH suppression requires direct activation of RFRP-3 neurons from corticosterone binding to GR, then the Cre+ mice would still maintain regular LH pulsing during restraint. However, if the obstruction of LH pulses occurs through a different mechanism, then the Cre+ mice would likely demonstrate inhibited pulses during stress.

Materials and Methods

All mice were housed in the same vivarium setting: 12-hour light/darkness cycle, lights off at 1800, and food and water available *ad libitum*. Transgenic mice were used as breeders; these mice had GR flanked by loxP sites. All mice used therefore had "GR floxed" (floxed refers to the fact that the DNA coding sequence for GR is surrounded by two loxP

sites). One breeder would be positive for Cre Recombinase that specifically becomes activated in only the RFRP-3 cells, so the mice born would be either Cre+ or Cre-.

Mice were weaned at three weeks of age and were either paired or tripled in cages. Only males were used. Tail samples were collected to test for GR floxing and the presence (or absence) of Cre Recombinase specifically activated in RFRP-3 neurons. Polymerase Chain Reactions (PCRs) and gel electrophoresis were used to determine their genotype. The primers used for the PCR were: (1) RFRP-3 Cre Forward: GAC CAG GTT CGT TCA CTC AT; (2) RFRP-3 Cre Reverse: GTT CAG CAC TAC GCA TAC TT; (3) GR Flox Forward: GGC ATC CAC ATT ACT GGC CTT CT; and (4) GR Flox Reverse: CCT TCT CAT TCC ATG TCA GCA TGT. Brain samples (DMN and cerebellum) were collected from a Cre+ mouse to test the successful removal of GR. The GR recombination primers used were: GR Flox Forward, and GR Recomb Reverse: GTG TAG CAG CCA GCT TAC AGG A.

At 7-9 weeks of age, the mice were gonadectomized in the same manner explained in Experiment 1. After a week of recovery from the surgery, the mice were handled daily for 3 weeks so that they were prepared for the experimental day of tail-blood collection. This handling involved the massaging of their tails to allow for better blood collection on the day of the experiment.

On the experimental day, starting at approximately 0850 hours, mice were handled for habituation purposes. At 0908, a small portion of their tail was cut to allow for blood collection. At 0914 and 0920, 3 μL of blood was collected from the tail, though these samples were discarded. Starting at 0926 and onward, 3 μL of blood was collected and mixed with 57 μL of assay buffer, stored on ice. Blood was collected every 6 minutes. Blood collection continued for another 198 minutes.

The 198-minute duration had two components: the first 72 minutes were used to establish a baseline (noted as "pre"), and afterwards, the remaining 126 minutes reflected the treatment effect (noted as "post"). After the 72-minute mark (at 1032 hours), the mice were either: restrained and isolated in a separate cage or left as is (the latter being the "no restraint" group). After the 198-minute mark, mice were sacrificed: tail samples, retro-orbital blood, and brains were collected. Sacrifices occurred at approximately 1240. Tail samples were stored at -20°C until they were lysed and neutralized. After neutralization, they were stored at 4°C. The genotypes of the mice were confirmed again with these tail samples via PCR and gel electrophoresis. Retro-orbital blood was spun down two hours later with the plasma extracted and also kept at -20°C. Brains were stored at -80°C in case they were needed to be used. However, both plasma and brains ended up not being used for this particular experiment.

Tail-blood samples collected throughout the experiment were stored at -20°C until shipped to the University of Virginia Ligand Assay Core. An ultra-sensitive murine LH ELISA was used to measure LH levels. Assay conditions are specified in Jennifer Yang's paper published in 2018:

> "The intra- and inter-assay % CVs were 2.3 and <7% respectively. Functional sensitivity was 0.320 ng/mL. Values are reported as ng/mL of whole blood. Serial blood LH measures were analyzed for endogenous LH pulses. An LH value was determined to be a pulse if the value had a >20% increase from one of the two previous points and LH subsequently decreased by >10% in the subsequent point" [12].

LH pulses were defined with the following parameters: (1) pulse frequency (the number of pulses per hour); (2) interpulse interval (the amount of time between two pulse peaks); (3) pulse amplitude (peak value of pulse subtracted by the preceding nadir); (4) pulse

peak (the absolute value of the peak); (5) basal LH levels (the average LH value of the nadirs), and (6) mean LH levels. These six parameters were used to analyze LH pulses before and after the treatment. If there was only one pulse observed during the given time period, the interpulse interval was defined as the time from that pulse's peak to the end of the given time group. If there were no pulses observed, then the interpulse interval was the entire duration of the given time group. These parameters were all analyzed with a two-way repeated measures ANOVA (time x group) followed by *post hoc* Bonferroni's multiple comparison test; $P<0.05$ was considered to be statistically significant.

Results

Figure 4 demonstrates evidence of GR being successfully floxed. Using a Cre+ mouse, the DMN and cerebellum brain samples show a band when GR flox primers were utilized, indicating that all cells throughout the mouse had GR floxed. However, only the DMN shows a band when GR recombination primers were used, indicating that only the cells in the DMN (ideally just the RFRP-3 neurons) had GR knocked out. Water was used as a negative control and no bands were seen here as expected.

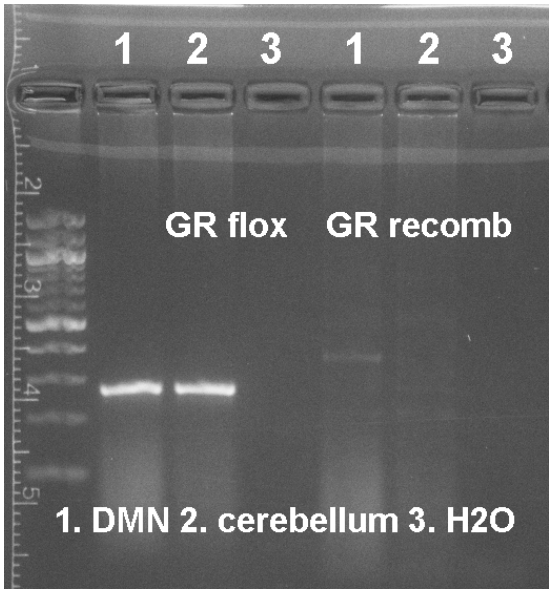

Figure 4: Evidence of GR Floxing and Recombination Working After Running PCRs. Brain samples from a Cre+ mouse were collected from the DMN and cerebellum. Water was used as a negative control. Columns labeled "1" contained the DMN sample; columns labeled "2" contained the cerebellum sample; columns labeled "3" contained water. The first three columns (going left to right) after the ladder utilized GR Flox primers and demonstrate that GR was floxed throughout the mouse. The last three columns utilized GR Flox Recombination primers and only detect successful removal of GR. Only a band is shown in the DMN column, indicating that only RFRP-3 neurons in the DMN had GR removed.

Figure 5 shows examples of how the LH data looked after receiving the results from the ultrasensitive LH murine ELISA. The asterisks represent the peak of a given pulse. With the no restraint conditions, both Cre+ and Cre- mice show regular pulsing throughout the entire experiment, which makes sense because there was no stressor to induce LH inhibition. In our restraint groups, LH pulses were suppressed after restraint and isolation onset (indicated by the dashed line at the 72-minute mark. Although one Cre+ mouse maintained regular LH pulsing upon restraint onset, all other Cre+ and Cre- restraint mice showed LH pulsatile inhibition.

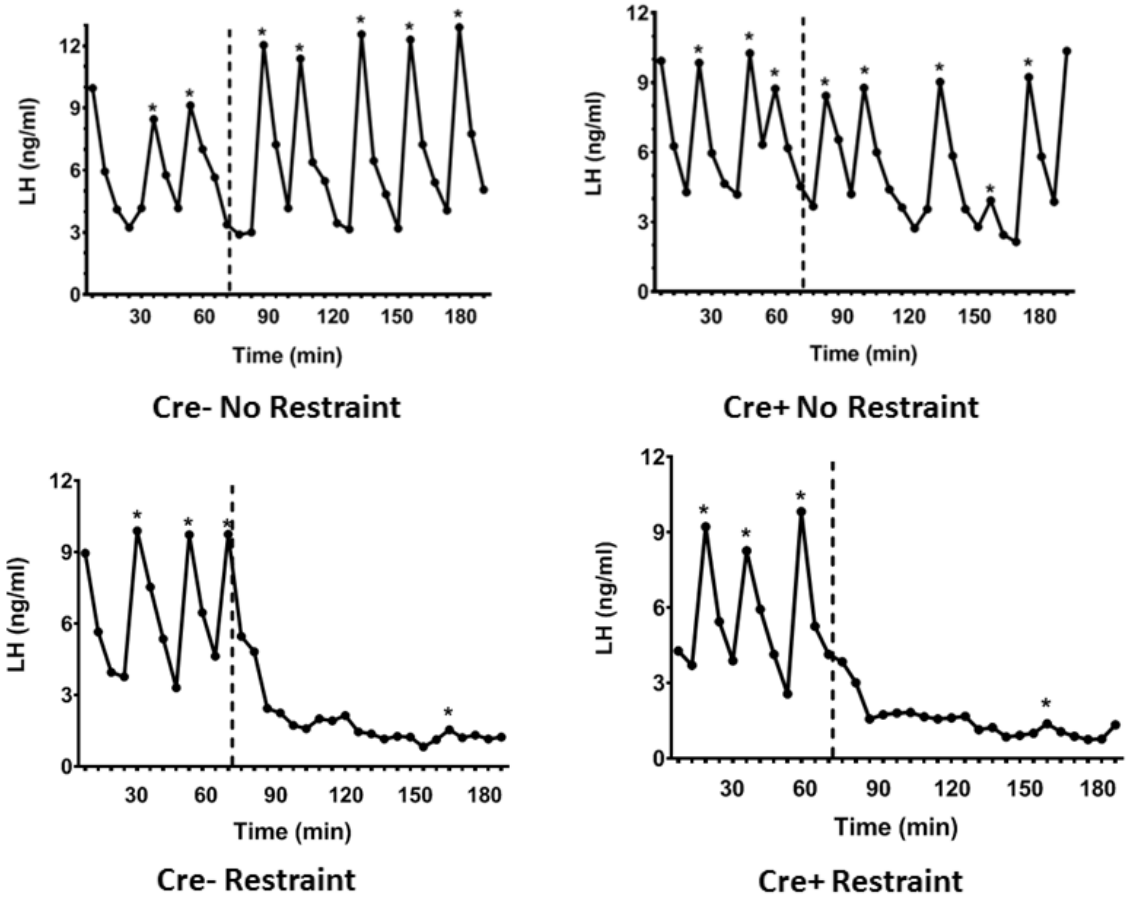

Figure 5: Examples of LH pulses for our four groups: Cre- No Restraint (n=4), Cre+ No Restraint (n=6), Cre- Restraint (n=5), and Cre+ Restraint (n=6). The dashed line at the 72-minute mark indicates treatment (either restraint with isolation or no restraint). An asterisk indicates the peak of a given pulse.

Figure 6 summarizes the LH data results in the six different parameters mentioned in the Materials & Methods section. A two-way repeated measures ANOVA (time x group) followed by *post hoc* Bonferroni's multiple comparison test was used. The "pre" data represents the baseline conditions (the first 72 minutes) and the "post" represents either restraint/isolation or, for the case of the no restraint mice, regular conditions (the latter 126 minutes). With the exception of the single Cre+ restraint outlier, all restraint mice demonstrated the following after restraint onset: decreased mean LH, decreased frequency

(pulses per hour), increased interpulse intervals, decreased pulse amplitude, decreased pulse peaks, and decreased basal LH levels. The Cre+ Restraint mouse that maintained regular LH pulsing was still included in the data. For the control groups, the “post” conditions are not statistically different from the “pre” conditions. Again, this makes sense because there was no stressor to induce LH suppression. Furthermore, we see no genotype difference between the Cre+ and Cre- mice for both restraint and no stress groups.

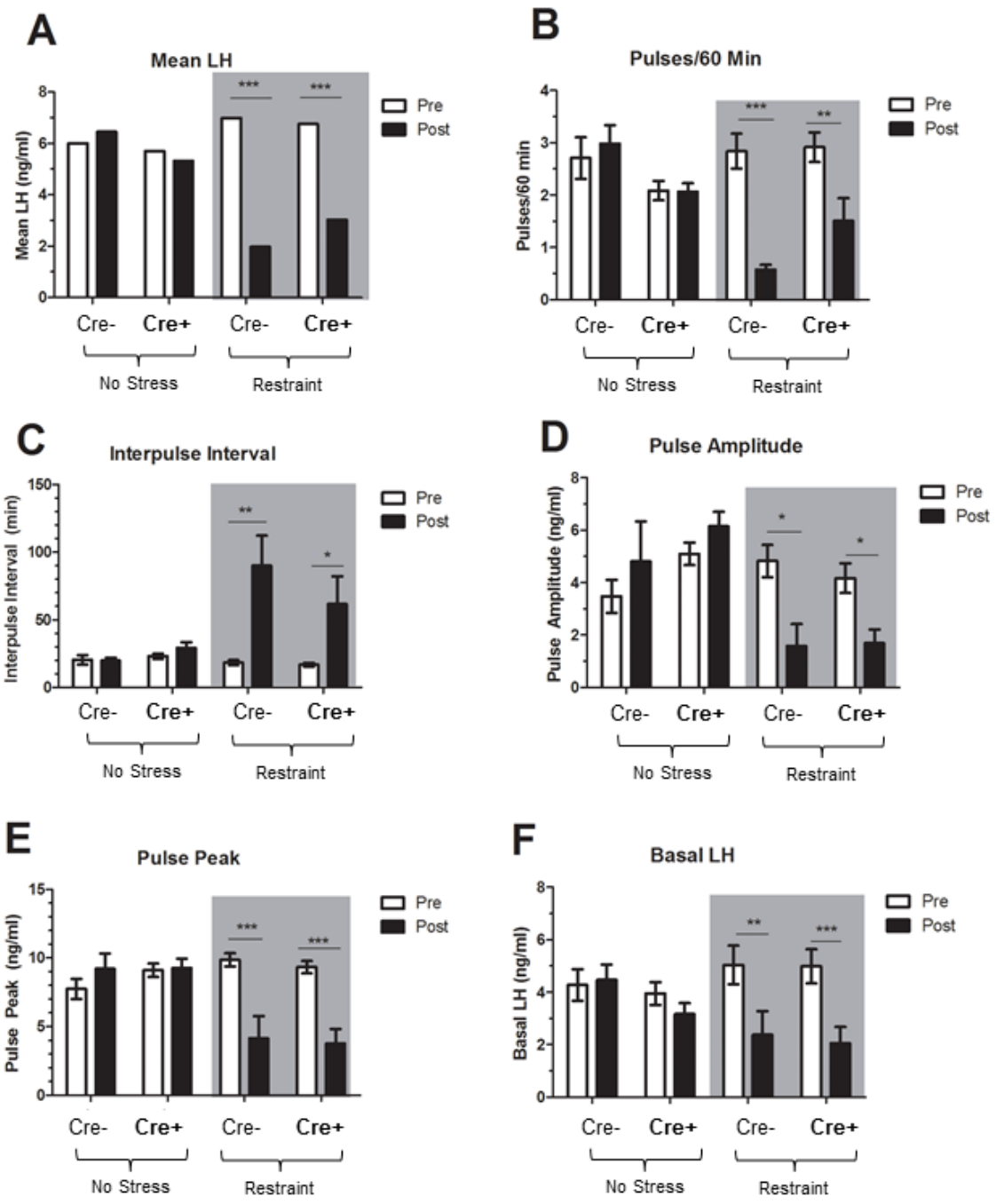

Figure 6: LH pulsing parameters of the four treatment groups: Cre- No Restraint (n=4), Cre+ No Restraint (n=6), Cre- Restraint (n=5), and Cre+ Restraint (n=6). The two restraint groups are differentiated by a grey box. A two-way repeated-measures ANOVA (time x group) was followed by *post hoc* Bonferroni's multiple comparisons test. *P<0.05, **P<0.01, ***P<0.001 vs. baseline ("pre") value. No genotype differences were observed.

Discussion

Because there was one Cre+ mouse that experienced normal LH pulsing during restraint, we initially thought that GR presence in RFRP-3 neurons was needed for LH inhibition to occur. However, this one mouse appeared to be an outlier as the remaining Cre+ Restraint mice experienced inhibited LH pulses. The reason for its regular pulsing during restraint is unknown and difficult to explain.

All the parameters shown in Figure 6 demonstrated suppressed LH pulses in Cre+ mice: decreased mean LH, decreased frequency, increased interpulse interval, decreased amplitudes of pulses, decreased peak values, and decreased basal levels. The only parameter that increased was the interpulse interval, which makes sense because the inhibition of pulsing made the pulses less frequent, thereby making the time between the pulse peaks even longer. The Cre+ Restraint group demonstrated that even when GR is removed from RFRP-3 neurons, restraint stress can still suppress LH pulsing. It may have been possible that our knockout model was not 100% successful, in which case some GR may have still been present in RFRP-3 neurons. But nonetheless, the overall conclusion from Figure 6 is that the inhibition of LH pulses is not directly mediated by the binding of corticosterone to GR in RFRP-3 neurons.

While corticosterone may not directly obstruct LH pulsing by binding to GR in RFRP-3 neurons, corticosterone may indirectly influence RFRP-3 neuronal activation. It could be possible that corticosterone acts through another mediator (perhaps an interneuron) that then presumably activates RFRP-3 neurons to suppress GnRH activity, leading to suppressed LH pulses. However, not only is this mediator unknown, but the purpose of an indirect pathway is also a mystery.

It is also possible that corticosterone acts directly on GnRH neurons to suppress LH pulses. GnRH neurons are known to have GR, though this should not be surprising because many cells throughout the body possess GR [22]. Even with GR removed from RFRP-3 neurons, corticosterone can still bind to GR in GnRH neurons to suppress their activity. To test this, we can repeat the same study but have GR flanked in GnRH neurons as well.

Another scenario that could explain the data is that corticotropin-releasing hormone (CRH) may act on RFRP-3 neurons (and/or GnRH neurons). In the HPA axis, CRH is synthesized in the paraventricular nucleus of the hypothalamus. Because of its close proximity to RFRP-3 neurons in the DMN, it may be possible that CRH activates RFRP-3. To test this, we could repeat the same experiment except knock out CRH receptors in RFRP-3 neurons instead of GR. A study demonstrated in ovariectomized female mice that with estradiol treatment, higher doses of CRH can inhibit GnRH firing activity [23]. However, it has yet to be clarified whether this is a direct or indirect effect. Further studies show that in certain cases, CRH Receptor Type 2 antagonists can prevent LH pulse suppression [24]. Because there seems to be some evidence showing that CRH may potentially play a role in LH inhibition, it may be worthwhile to investigate this hormone. Overall, additional studies will need to be performed to better understand the mechanism required to inhibit LH pulsing.

EXPERIMENT 3: RFRP-3 NEURONAL ACTIVATION FOR VARIOUS RESTRAINT DURATIONS IN TRANSGENIC GDX MALE MICE

Brief Overview

So far, we have determined that corticosterone may potentially increase RFRP-3 neuronal activation (Experiment 1), though corticosterone does not need to directly bind to GR in these neurons to suppress LH pulses (Experiment 2). For our third experiment, we wondered whether GR is needed in RFRP-3 neurons for these cells to become activated during restraint stress. A previous study using GDX wildtype male mice showed that RFRP-3 neuronal activation increased after 45-minutes of restraint stress but decreased below no-stress conditions after 180-minutes [11-12]. To better understand this phenomenon, we repeated this study but instead used the transgenic line mentioned in Chapter 2. While we used three different treatments (no restraint, 45 minutes of restraint, and 180 minutes of restraint), we also wanted to compare genotype differences (Cre- and Cre+ mice). Therefore, there were six groups total: Cre- and Cre+ mice that experienced no restraint; Cre- and Cre+ 45-minute restraint mice; and Cre- and Cre+ 180-minute restraint mice.

The purpose of this experiment is twofold: (1) to confirm the trend previously seen at these two time points (45-minutes and 180-minutes); and (2) to see whether the presence or absence of GR will affect RFRP-3 neuronal activation. Restraint was used instead of corticosterone injection because corticosterone levels decay over time from the injection. Restraint allows corticosterone levels to remain constantly elevated during our acute periods of stress.

Materials and Methods

The same transgenic line used in experiment 2 was used for experiment 3. They were housed in the same vivarium setting: 12-hour light/darkness cycle, lights off at 1800, and food and water available *ad libitum*. Mice were weaned at 3 weeks of age and only the males were kept. Tail samples were taken at weaning to test for their genotype. The same PCR and gel electrophoresis procedures were used from Experiment 1. We tested for the successful floxing of GR and for the presence or absence of Cre Recombinase that specifically gets activated in RFRP-3 cells.

At 8-9 weeks of age, the mice were gonadectomized in the same manner explained in Experiment 1. No daily handling was used for this particular experiment. Two weeks after surgery, the experiment was performed. To monitor their health, body weights were taken three times: on the day of surgery, a week after surgery, and on the experimental day. There were three different treatments: 45-minute restraint, 180-minute restraint, and no-restraint (the control). But as mentioned earlier, because there were both Cre- and Cre+ mice, there were a total of six different groups. All restraint mice were isolated. After the given treatment, the mice were immediately sacrificed with brains and blood collected. Sacrifices were performed before noon to avoid circadian increase of corticosterone levels. Mice that were not restrained were sacrificed immediately. Brains, retro-orbital blood, and tail samples were collected at sacrifice. Brains were stored at -80°C. Two hours after sacrifice, retro-orbital blood was spun down and the plasma was extracted, kept at -20°C, and eventually used for a corticosterone ELISA. Again, a DetectX Corticosterone Enzyme Immunoassay Kit (Arbor Assays) was used. Tail samples were lysed and re-genotyped to confirm again (1) the

presence or absence of Cre Recombinase specifically activated in RFRP-3 neurons, and (2) whether GR was floxed.

The brain cutting protocol was the same as that explained in Experiment 1. A double-label ISH was run for DIG-labeled *Rfrp* neurons and *cfos* silver grains. The same ISH protocol was used from Experiment 1. The procedures for developing, fixing, labeling, coding, and counting of slides were also the same from Experiment 1, though for this assay we developed the slides 3 weeks after ISH procedure was completed. For this experiment, the criteria for a co-labeled cell was that the grains-per-cell is greater than 2 and the signal-to-background ratio is greater than 3.

Results

Figure 7 shows the corticosterone levels of all mice at the time of sacrifice. Two separate graphs are shown: the left for Cre- mice and the right for Cre+ mice. The treatment groups are organized and labeled on the x-axis. Corticosterone levels are elevated for all restraint groups and are much lower in the No Restraint groups. A one-way ANOVA (group) was followed by *post hoc* Bonferroni's multiple comparison test with a $P<0.05$ considered to be statistically significant.

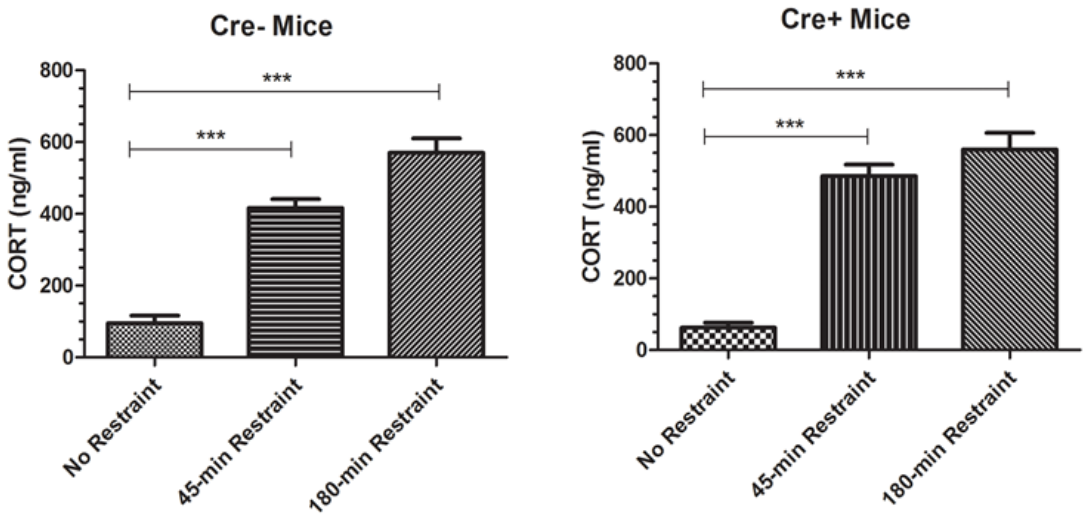

Figure 7: Corticosterone Levels After Restraint for Cre- and Cre+ GDX Male Mice. The left graph shows CORT levels for Cre- mice that still have GR in RFRP-3 neurons: No Restraint (n=7), 45-Min Restraint (n=9), 180-Min Restraint (n=9). The right graph shows CORT levels for Cre+ mice that have GR knocked out of RFRP-3 neurons: No Restraint (n=8), 45-Min Restraint (n=9), 180-Min Restrain (n=9). ***P<0.001.

Figure 8 shows an example of a DIG-labeled *Rfrp* cell co-labeled with *cfos* from this experiment. The left image shows 3 *Rfrp* cells, the middle image shows only *cfos* expression, and the right image shows both the cells and *cfos* merged together. Again, the criteria for a co-labeled cell is that the grains-per-cell is greater than 2 and the signal-to-background ratio is greater than 3. Cells that are considered double-labeled have a white arrow shown in the right image.

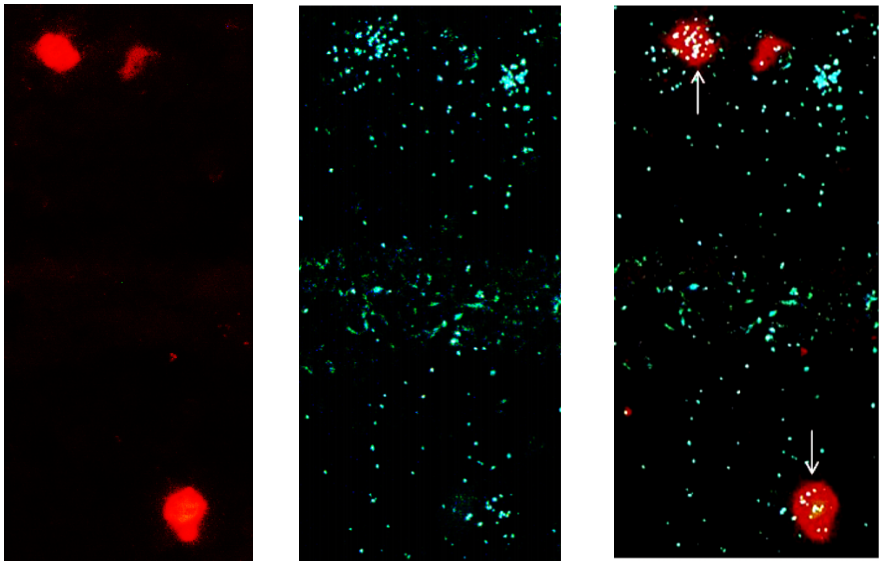

Figure 8: Example of ISH Imaging for *Rfrp* cells and *cfos* for Experiment 3. The left image shows the DIG-labeled *Rfrp* cells, the middle image shows *cfos* silver grains, and the right image shows the DIG-labeled cells and *cfos* merged together. The white arrows in the right image indicate a cell with high *cfos* expression, suggesting its recent activation. A cell is considered co-labeled (aka demonstrates neuronal activation) if the grains-per-cell is greater than 2 and the signal-to-background ratio is greater than 3.

Figure 9 shows the percentage of RFRP-3 neurons activated among the six different groups. A two-way ANOVA (group x genotype) was followed by *post hoc* Bonferroni's multiple comparison test with a $P<0.05$ considered to be statistically significant. We did not see any genotype differences between Cre- and Cre+ mice for any of the treatments. The 45-Min Restraint group had the highest percentage of RFRP-3 neurons activated for both Cre- and Cre+ mice. The percentage of RFRP-3 cells activated in the 180-Min Restraint group was statistically different from the 45-Min Restraint group but not the No Restraint group.

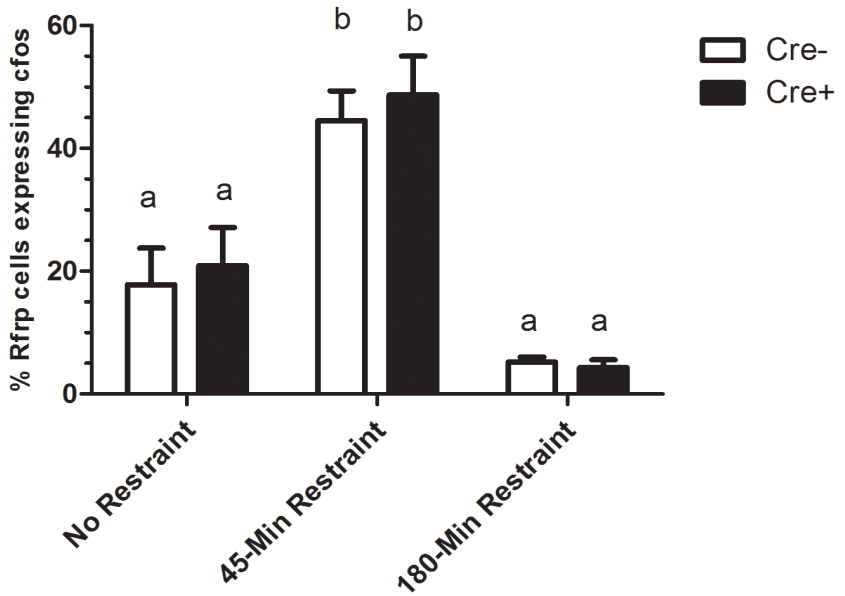

Figure 9: Percentage of *Rfrp* Cells Co-Expressing *cFos*. A two-way ANOVA (group x genotype) was followed by *post hoc* Bonferroni's multiple comparisons test. The white bars represent Cre- mice and the black bars represent Cre+ mice. The 45-Min Restraint group has the highest percentage of Rfrp cells activated for both genotypes. The No Restraint and 180-Min Restraint groups did not show statistical differences between each other regardless of genotype. For the Cre- mice: No Restraint (n=5), 45-Min Restraint (n=7), 180-Min Restraint (n=8). For the Cre+ mice: No Restraint (n=7), 45-Min Restraint (n=7), 180-Min Restraint (n=7). Not all the mice were used from Figure 7 because some lacked DIG expression. Different letters indicate statistical difference, $P<0.05$.

Because there did not seem to be a genotype difference, we combined the Cre- and Cre+ for Figure 10 to see if we could see a statistical difference in neuronal activation between the No Restraint and 180-Min Restraint groups, as seen in previous studies [11-12]. Doing so would raise the statistical power by increasing the number of samples per group. Figure 10 shows that there is a statistical difference between these two groups. A one-way ANOVA (group) was followed by *post hoc* Bonferroni's multiple comparison test.

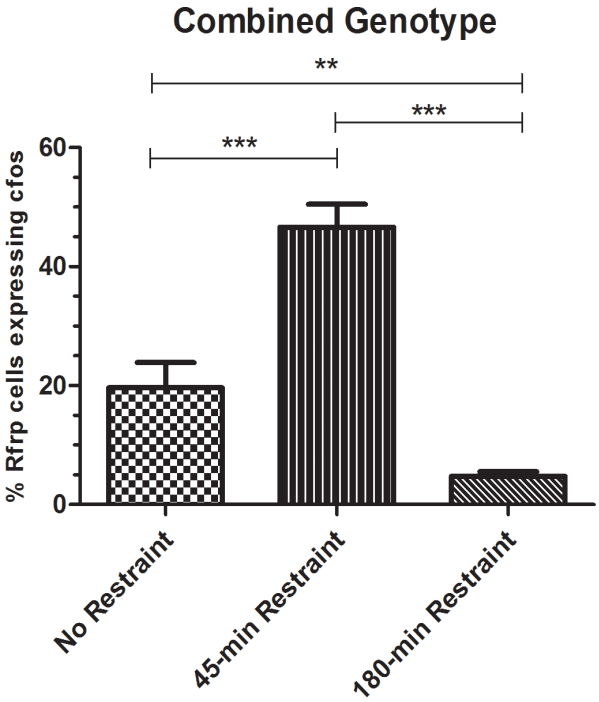

Figure 10: Observing RFRP-3 Neuronal Activation After Combining Cre- and Cre+ mice to Increase Statistical Power. A one-way ANOVA (group) was followed by *post hoc* Bonferroni's multiple comparisons test. When we combine the genotypes and then compare the three treatment groups, we see a statistical difference between the No Restraint and 180-min Restraint groups. No Restraint (n=12), 45-min Restraint (n=14), 180-min Restraint (n=15). **P<0.01, ***P<0.001.

Discussion

According to Figure 7, whether the duration was 45 minutes or 180 minutes of restraint, corticosterone levels are greatly elevated for all stressed mice. While there may be some statistical differences within the restraint groups, the differences between the restraint mice and the control mice are much more drastic.

Additionally, Figure 8 demonstrates that our in-situ hybridization procedure worked. After counting our slides, Figure 9 shows that there was no genotype difference in any of the treatment groups. Again, the Cre+ mice had GR knocked out of RFRP-3 neurons while Cre-

mice still had GR in these cells. The No Restraint Cre- and Cre+ groups were both similar in activation; the 45-Minute Restraint groups were similar to each other; and so were the 180-Minute Restraint groups to each other. It did not matter whether the mice were Cre- or Cre+ because after 45 minutes of restraint, we see a significant increase in RFRP-3 neuronal activation in both genotypes. This data is consistent with Experiment 2, which demonstrated that GR was not needed in RFRP-3 neurons to suppress LH pulsing. This finding is also consistent with previous studies that demonstrated increased RFRP-3 neural activation after 45 minutes of restraint [11-12]. This further solidifies the notion that elevated corticosterone levels during stress may quickly activate RFRP-3 neuronal activation. Therefore, one conclusion we can draw from this experiment is that during restraint stress, GR presence is not needed in RFRP-3 neurons for their increased activation after 45 minutes of restraint.

We also see that the percentage of *Rfrp* cells activated at the 180-minute restraint time point is statistically different from the 45-minute restraint group. While we did not see the 180-minute restraint group be statistically different from the No Restraint mice, we find that combining the genotypes within the same treatment group (in Figure 10) brought up statistical power and demonstrated a significant difference. The reason why we combined the genotypes together is because Figure 9 demonstrated no statistical differences between Cre+ and Cre- in any of the three treatment groups. Figure 10 is consistent with the previous studies [11-12].

However, the purpose for this decreased activation after 180 minutes of restraint is currently unknown. Both Experiment 1 and our 45-Min Restraint groups from Figures 9 and 10 show that increased corticosterone levels can activate RFRP-3 neurons. However, Figure 7 shows greatly increased corticosterone levels at the 180-minute mark. This is intriguing

because while corticosterone levels are still elevated after prolonged restraint, the neural activation of RFRP-3 is diminished compared to the 45-min activation levels. Perhaps RFRP-3 functions as a quick responder to stress and does not need to be activated for such a long period of time. It could also be the case where RFRP-3 serves to trigger rapid transcriptional activity and once it has completed its job, the neurons no longer need to be activated. It may also be possible that RFRP-3 neurons need a period of rest before becoming activated again, and prolonged stress onset may prevent this recovery period thus leading to diminished activation levels. In light of these possibilities, the purpose for this 180-minute phenomenon has yet to be determined and further studies will need to be performed to understand why it occurs.

CONCLUSION

While much is still unknown about how stress suppresses the reproductive axis, our three experiments provide insight on how corticosterone plays a role in this phenomenon. First, we followed up a previous study done in our lab that had demonstrated brief LH suppression after direct subcutaneous corticosterone injections. By measuring corticosterone levels at two different time points (30 minutes and 60 minutes post-injection), we found that these levels were much higher in the former. Furthermore, we also found increased RFRP-3 neuronal activation at the 30-minute mark. Although this experiment needs vehicle groups for the data to be fully solidified, the results seem to suggest a potential connection between corticosterone and RFRP-3 neurons.

We then wondered if, during stress, corticosterone directly binds to RFRP-3 neurons to result in LH suppression. Thus, our second experiment measured LH pulses in transgenic mice that had GR removed from RFRP-3 neurons. Although one Cre+ mouse showed regular pulsing, the remaining Cre+ mice that were restrained showed inhibited LH pulses, indicating that stress-induced LH suppression does not require corticosterone directly binding to GR in RFRP-3 neurons. LH suppression may either occur through an indirect pathway or by a different neurohormone like CRH. Or, what may be likely is that LH suppression can occur through many mechanisms in addition to corticosterone binding to GR in RFRP-3 neurons.

To see if GR is even needed for RFRP-3 neuronal activation during stress, we conducted a third experiment using the same transgenic line. This experiment also tested the temporal component of RFRP-3 neuronal activation that was observed in recent studies [11-12]. We performed 45-minute and 180-minute restraint sessions and observed neuronal activation of RFRP-3 neurons. We found that the absence of GR in RFRP-3 cells did not

affect the restraint stress-induced activation of these neurons. Furthermore, we confirmed the trends seen in previous studies where RFRP-3 neural activation significantly increases after 45 minutes of restraint and decreases after 180-minutes of restraint. After confirming that there was no genotype difference from Figure 9, we combined the genotypes together to see if the neural activation after 180 minutes of restraint would be lower than the no restraint levels. Figure 10 indeed demonstrated significant decrease in neural activation in the 180-minute restraint group compared to the No Restraint group.

Overall, the three experiments suggest that corticosterone may activate RFRP-3 neurons during stress, though corticosterone does not need to bind to GR in these neurons to inhibit the reproductive axis. This then suggests that this relationship between corticosterone, GR, and RFRP-3 neurons is not essential for the stress-induced inhibition of the reproductive axis. There may be other factors like CRH or other interneurons that can also suppress the reproductive axis during stress. We are, however, still left with many questions. Will vehicle groups solidify the findings from Experiment 1? Does our second experiment suggest an indirect corticosterone pathway to activate RFRP-3 neurons? Or do other pathways occur to compensate for the knockout of GR in these neurons? Why does the 180-minute restraint group show decreased activation in RFRP-3 neurons? We hope to answer these questions in the near future.

This thesis is coauthored with Lee, Frank; Yang, Jennifer; and Kauffman, Alexander. The thesis author was the primary author of this material.

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