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Gonadotropin-Inhibiting Hormone Rises and Luteinizing Hormone Falls in Male Rats Exposed to Stress

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Many animals, including rats and humans, show reproductive abnormalities after experiencing stress. Multiple interactions between the hypothalamic-pituitary-adrenal (HPA) axis and the hypothalamic-pituitary-gonadal (HPG) axis are responsible for the negative effects of stress on reproduction. It has recently been reported that the gonadotropin-inhibitory hormone (GnIH) in quail is a negative regulator of the HPG axis, and that its orthologous neuropeptides, RFamide-related peptides (RFRPs), have been found in rodents and primates. However, whether or whether GnIH/RFRPs affect HPG axis activity while under pressure is not well understood. Here we demonstrate that adult male rats' RFRP expression in the dorsomedial hypothalamus (DMH) increases in response to acute and chronic immobilization stress, and that this rise is accompanied by a reduction in HPG activity. Moreover, our results show that the normal stress-induced upregulation of RFRP expression is blocked by adrenalectomy. Around 53% of RFRP cells have glucocorticoid (GC) receptors, according to immunohistochemistry, indicating that adrenal GCs may directly act on RFRP cells to modulate the stress effect. It is hypothesized that GnRH-secreting neurons play a mediating role between stress and the central regulation of reproduction. Our findings demonstrate that elevated RFRP contributes to hypothalamic regulation of reproductive function by increasing the activity of GCs in the adrenal glands in response to stress. These novel insights into the HPA-HPG connection imply that the role of GnIH/RFRP has to be addressed in future investigations of the interplay between stress and the reproductive system, shifting the paradigm for research into stress-related reproductive dysfunction and infertility.

Keywords: HPA-HPG, gonadotropin-inhibitory hormone, GnIH/RFRP, adrenal GCs.

INTRODUCTION

Numerous physiological and behavioral features of vertebrates are affected both temporarily and permanently by stress. Stresses in one's life, both physical and mental, may have a negative impact on reproduction, a crucial part of every organism's life cycle. The hypothalamic-pituitary-gonadal (HPG) axis in vertebrates is affected negatively by stress on several fronts. By stimulating the hypothalamic-pituitary-adrenal (HPA) axis, gonadotropin-releasing hormone (GnRH) production is blocked and, in turn, HPG activity is suppressed. As a functional consequence of stress on reproduction, decreased pituitary luteinizing hormone (LH) secretion

and reduced sexual activity are seen. Stress chemicals produced by the adrenal glands, called glucocorticoids, seem to be responsible for the negative impact of stress on HPG function (GCs). Systemic treatment of GC reduces circulating gonadotropin levels, reduces the weight of the male mammal's seminal vesicles, and causes fewer implantation sites and fewer viable embryos in female partners. On the other hand, corticotropin-releasing hormone (CRH) secreted by the hypothalamus has been linked to stress-induced birth defects.

Recently identified in the hypothalamus, gonadotropin inhibitory hormone (GnIH) blocks the production and release of gonadotropins. This neuropeptide's inhibitory effect prompts us to wonder whether the GnIH system is involved in stress-induced reproductive failure. If this were really the case, it would reveal a layer of central HPG regulation that is controlled by stress but has not been studied before. To connect with GnRH neurons in the DMH, the rat dorsomedial hypothalamic (DMH) neurons that generate Similar to avian GnIH (RFamide-related peptide), which has fibers that reach the median eminence and the preoptic region, the mammalian version does as well. Full-length precursor peptides are produced during mammalian RFRP production; they are then cleaved in vivo to provide RFRP-1 and RFRP-3 are the two functional peptides. The peptides generated from the GnIH precursor in the quail and white-crowned sparrow are very similar to the rat RFRP peptides in terms of their amino acid sequence. In the same way as the hypothalamus, pituitary, and testes are the only locations in birds where the GnIH receptor is found. The RFRP receptor (OT7T022) is also restricted to these regions in mammals. The in vivo reduction of HPG function by RFRP is important in both birds and mammals. Stress-like suppression of LH release and sexual activity in male rats after systemic administration of RFRP-3. Furthermore, stress changes RFRP expression in breeding house sparrows. In sum, the evidence so far points to RFRP's potential role as a powerful mediator of stress effects on mammalian reproductive.

We hypothesized that stress would alter HPG function in mammals by elevating RFRP expression, and set out to test this hypothesis in the current research. We were also interested in learning if stress hormones like GCs or CRH influence the impact we found. We discovered that both short-term and long-term stress increase RFRP expression and cause HPG malfunction. We also observed that RFRP cells had receptors that are activated in response to stress hormones. Furthermore, adrenalectomy (ADX) suppresses the upregulation of RFRP expression that occurs in response to stress. Consequently, our findings offer substantial evidence that stress-induced adrenal hormone release directly influences RFRP expression and HPG function.

1-2- Results Expression of RFRP in the hypothalamus is upregulated in response to acute stress

To determine whether or not RFRP is involved in the regulation of the HPG axis by stress, we examined its expression in adult male rats in response to acute stress (Fig. 1A). Immediately after a three-hour immobilization stress, hypothalamus RFRP mRNA and peptide levels increased (Fig. 1 B–E). To analyze the expression of this gene, we performed employing a probe that identified the full-length rat RFRP precursor mRNA, in-situ hybridization was performed.

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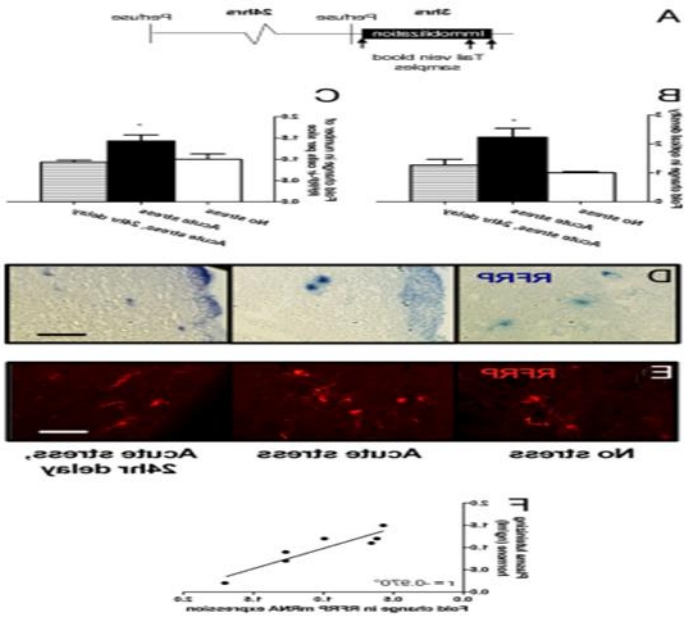


Fig.1. Impact of short-term stress on RFRP expression. Time line of experiments, type A. (B) Immediately after stress, stressed rats' mRNA expression levels for RFRP were significantly greater than those of control rats mean standard error of the mean, $P < 0.002$. The number of RFRP peptide-positive cells was considerably larger in stressed rats (mean SEM; $P = 0.006$) immediately after the stressor was removed. Images (D) depict some of the cells in the DMH that test positive for RFRP mRNAE. Examples of some of cells with an RFRP-ir signature that were found in the DMH. Conclusion (F) the fold change in RFRP mRNA was negatively correlated with plasma-luteinizing hormone had a correlation coefficient of -0.920 and a significance level of 0.003. *Statistical significance greater than or equal to 0.05 compared to the no-stress and acute-stress, 24-hour-delay controls. Stakes at 40 micrometers (°$P < 0.05$) indicate a level of importance.

We found that 2.3 (0.3 SEM)-fold more RFRP mRNA was present in the DMH immediately after immobilization compared to no-stress controls. No significant difference was seen between stressed animals and controls in terms of RFRP mRNA levels 24 hours after immobilization was removed (Fig. 1 B and D). For the RFRP precursor peptide, a similar trend was seen (Fig. 1 C and E). When stressed rats were immobilized, the number of RFRP-ir cells rose by 1.4% immediately after immobilization (0.1 SEM). After 24 hours, there was no difference in the RFRP of stressed and unstressed rats in terms of RFRP when the rats were killed.

In order to determine how abundant RFRP mRNA transcripts are in the hypothalamus, we used real-time reverse transcriptase polymerase chain reaction (RT-PCR). In order to ascertain whether or whether stress-induced changes in RFRP expression are crucial for HPG function, we linked the mRNA expression level with circulating LH levels. There was a statistically significant inverse relationship between plasma RFRP mRNA expression levels and

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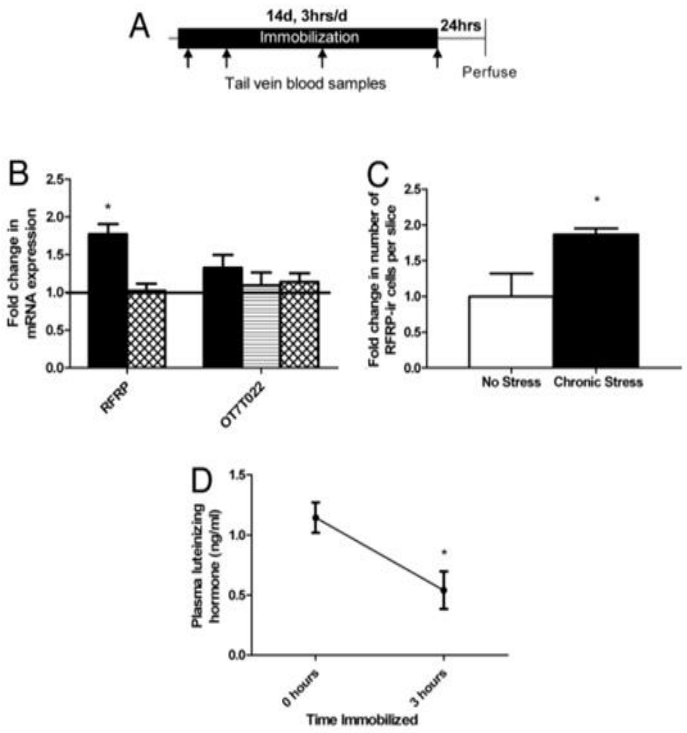


Fig. 2. How RFRP expression is affected by chronic stress. Typically, an experimental procedure of type A would take the following amount of time. Normalized to no-stress controls, gene expression changes in the hypothalamus (solid bars), pituitary (lined bar), and testes (crosshatched bars) after long-term immobilization (B). RFRP mRNA expression in the hypothalamus was elevated while participants were immobilized (mean SEM, P 0.007). The hypothalamus, pituitary, and testes showed no significant differences variations in RFRP expression ($P > 0.10$). (C) After long-term fixing, the number of RFRP-ir cells in the DMH of the hypothalamus increased (mean ± SEM, P 0.041). There was a drop in LH after being immobile for the duration of the stress period. After stress, plasma LH levels dropped significantly by day 14 ($P = 0.023$). Stress-free vs stress-filled ($^*P < 0.05$) and the effects of sitting still for a chunk of the day. Scale bar represents (40 µM) impact of daily immobilization ($\dagger P < 0.10$).

LH levels (Fig. 1F), corroborating earlier studies indicating that RFRP reduces circulating LH levels via inhibiting GnRH neuron activity or decreasing pituitary cells' sensitivity to hypophysial GnRH. As determined Acute stress did not affect RFRP (1.2 ± 0.1-fold change, $P = 0.35$) or OT7T022 (1.0 ± 0.1-fold change, $P = 0.82$) testicular mRNA transcript expression by real-time reverse transcription-polymerase chain reaction. Both of these genes are involved in the response to testosterone (RT-PCR). According to these data, it seems that acute immobilization stress causes a brief rise in hypothalamic RFRP expression, which is accompanied by a reduction in the function of downstream HPG.

1-3-There is a correlation between prolonged stress and Expression of RFRP in the Hypothalamus

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Because chronic stress is also known to decrease reproductive function, we next looked into whether or not RFRP expression is similarly affected by chronic stress, and whether or not the effects would be more lasting or stronger than those of acute stress. This was done due to the established fact that prolonged exposure to stress inhibits reproductive capacity. Adult male rats were immobilized for three hours each day for a period of 14 consecutive days, and then they were put to death 24 hours later (Fig.2A). The expression of RFRP mRNA as well as peptides in the hypothalamus was shown to be up-regulated for up to 24 hours following the termination of prolonged immobilization. According to the findings of our real-time reverse transcription PCR (Fig. 2B), RFRP mRNA levels were increased by 1.8 (± 0.1 SEM) fold after prolonged immobilization stress as compared to controls. Stress over an extended period of time also led to an increase in RFRP peptide. Stressed rats had 1.9 (± 0.1 SEM) times as many RFRP-ir cells in their DMH as did control rats, as shown by immunohistochemistry (Fig. 2C). The level of RFRP mRNA in the testicles was not affected by stress. Similarly,

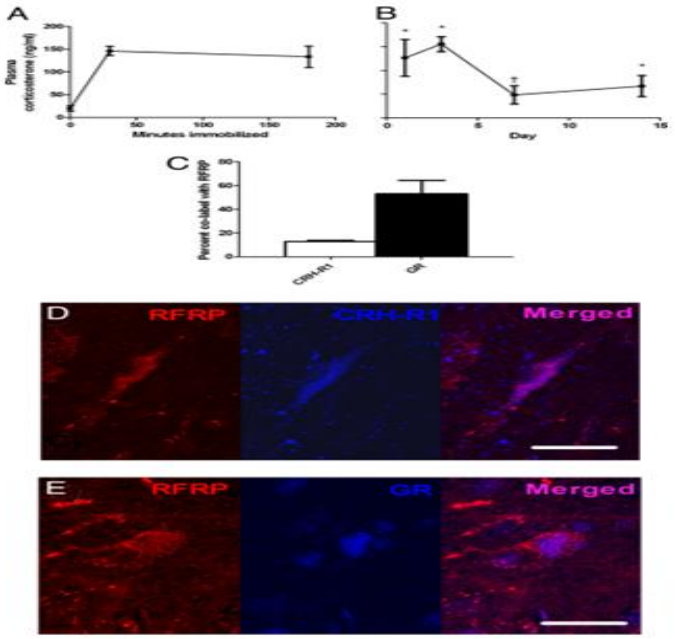


Fig. 3. RFRP cells' sensitivity to the HPA axis. A significant rise in plasma corticosterone was seen after just three hours of immobilization (mean standard error of the mean, P 0.001). (B) The change in corticosterone levels after a stressful situation. Increases in plasma corticosterone were seen after daily periods of immobilization (effects of immobility over the course of a day, $P < 0.002$; time-by-day interaction, $P < 0.001$). On days 1, 3, and 14, corticosterone levels were significantly higher as a result of immobilization (average deviation from the first day's values; $P = 0.023$, $P < 0.002$, and $P = 0.029$). There was no statistically significant spike on day 7 (P=0.053). (C) The proportion of RFRP cells that also express GR whilst expressing CRH-R1. An adult rat DMH displaying a cell with an RFRP-ir structure (first panel, red) that also expresses CRH-R1 (second panel, blue). A cell that not only expresses RFRP-ir but also GR (shown in red in the first panel) (blue, second panel). Impact of immobility compared to Day 1 baseline (*$P < 0.05$). The impact of immobility on subsequent days compared to day 1 (†$P < 0.10$). Indicates the size of the scale (20 M).

None of the three brain regions (hypothalamus, pituitary, and testes) showed any variation in RFRP receptor (OT7T022) expression (Fig. 2B). Plasma LH concentrations were shown to be lower as a result of our chronic stressor, which demonstrated stress-induced impairment of HPG function in rats (Fig. 2D).

1-4- The HPA axis becomes active in response to both short-term and long-term stress

Adequate plasma was obtained from the tail veins of acutely and chronically stressed animals to confirm HPA axis activity during the immobilization stress test. Rapid and persistent increases in plasma corticosterone were seen in acutely agitated mice (Fig. 3A). Elevated plasma corticosterone levels in reaction to restraint were seen in chronically stressed rats during the whole 14-day restraint period, suggesting that these animals did not develop a complete habituation to the stressor (Fig. 3B).

Researchers have found corticosterone peaks of similar magnitudes following exposure to either chronic or acute stress, indicating that HPG function is decreased in both situations.

1-5- There are stress hormone receptors on RFRP cells in the hypothalamus

The hypothalamic RFRP was shown to be affected by stress, and the HPA axis was consistently triggered by the stress paradigm. We examined receptor expression in hypothalamic RFRP cells to see whether they directly respond to corticotropin-releasing hormone (CRH) and corticosterone (cortisol), the two primary hormones controlled by the hypothalamic-pituitary-adrenal (HPA) (the major rat GC). Using confocal imaging and double immunohistochemistry, we determined that RFRP-positive cells express both the CRH receptor-1 (CRH-R1) and the glucocorticoid receptor (GR) (Fig. 3 C–E). A little over 11% (2.25%, SEM) of RFRP-positive cells were also tagged with CRH-R1, whereas over 49% (11.4%, SEM) of RFRP-positive cells were also labeled with GR (Fig. 3 D–E).

1-6-Stress-Induced RFRP Up regulation is suppressed by Adrenalectomy

This research aimed to see whether stress-induced increases in RFRP were also influenced by the presence of higher GCs levels in the blood. About half of the RFRP cells we examined in the hypothalamus were GR-positive. After that, we looked to see whether the stress-induced rise in RFRP remained when the adrenal glands, which are in charge of GC production, were eliminated. Two weeks after adrenalectomy, male rats were tested (ADX). Control groups consisted of animals subjected to fake surgeries.

The rats experienced the daily immobility stress for a total of two weeks. After one day of no exposure to the stressor, the rats were put to sleep and their RFRP mRNA expression was evaluated. Confirmation that ADX successfully reduced corticosterone release in response to stress on day 1 was obtained by measuring the corticosterone response to inhibition (Fig. 4A). Since hypothalamic RFRP mRNA expression was significantly upregulated in sham-operated rats after continuous immobilization stress (Fig. 4B), we conclude that stress-induced elevation of RFRP expression is dependent on a large circulating quantity of GCs.peripheral muscarinic and nicotinic receptors to interfere with synoptic transmission.

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1-7-Argumentation

Suppression of reproductive function in adult male rats by hypothalamic secretion for the Rational Expression of Regulatory Proteins (considered a GnIH). Our results further show that adrenal hormones are required for the stress-induced upregulation of RFRP expression. Hypothalamic RFRP mRNA and protein levels went increased in response to acute and chronic immobilization stress. The effects of acute stress on RFRP expression were short-lived, with most of them disappearing within 24 hours of the stressor's removal, but the effects of chronic stress lingered for much longer. This second finding is consistent with research demonstrating that stress effects tend to increase as stress continues to be imposed.

Stress and RFRP both contribute to the inhibition of sexual activity in adult male rats. Similarly, stress and the administration of RFRP both reduce plasma LH levels in mature male rats. Here, we demonstrate that elevated RFRP expression due to stress is associated with decreased plasma LH levels. By demonstrating the presence of stress hormone receptors on RFRP neurons, Stress has medial effects on the RFRP system, and we detail the neuroanatomical basis for these effects. All together, our results highlight the significance of RFRP in the stress-induced modification of gonadotropin release and consequent HPG dysfunction.

Whether in mammals or birds, the effects of stress on reproductive function are well seen. Stress has been linked to a variety of reproductive issues, including male adult infertility, Medical students had worse sperm motility, male rats had fewer impregnations, and baby chickens had a lower chance of survival.

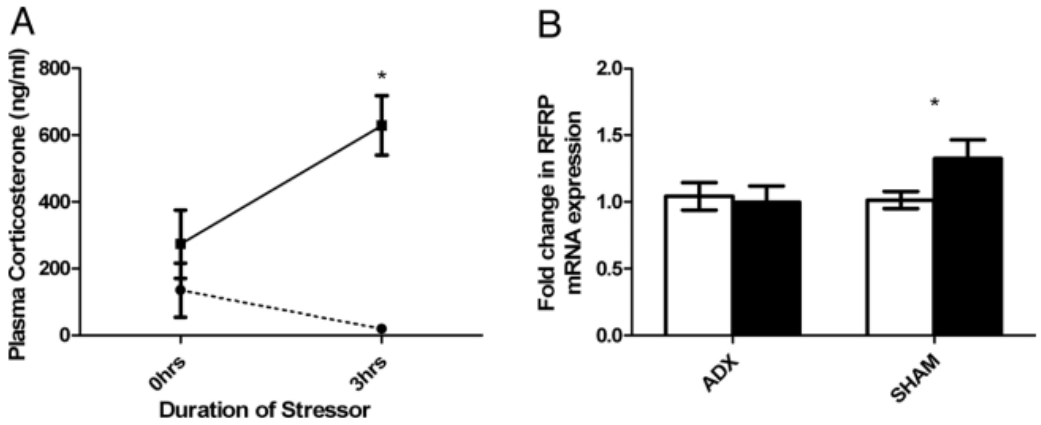


Fig. 4. The stress-induced upregulation of RFRP expression in the hypothalamus may be blocked by adrenalectomy. Compared to ADX rats (solid line; P = 0.891), sham-operated rats showed a substantial rise in plasma corticosterone in response to stress (P = 0.001). (A). (B) After two weeks of daily immobilization, RFRP mRNA expression was significantly the hypothalamus in rats that had sham operations was significantly (P = 0.050) greater than in animals that had ADX (P = 0.794). Limitless tension control using an open regulator. Comparison of stress to no stress or zero to three hours of stress, if appropriate, *P < 0.05.

European starlings that are allowed to live in the wild. Furthermore, the stress system may be activated by starvation, resulting in negative effects on reproduction Therefore; stress may have far-reaching consequences on the HPG, even beyond the typical sources of tension. Aging and the decline in fertility that comes with it have both been linked to the HPA axis.

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There is a complicated interaction between the hypothalamic-pituitary-adrenal (HPA) axis and the hypothalamic-gonadal (HPG) axis that seems to be conserved throughout a broad range of mammals. Adrenocorticotropic hormone (ACTH) is secreted by the anterior pituitary in response to CRH from the brain through the hypophysial portal system during times of stress (ACTH). GCs are secreted into the circulation in response to a hormone called adrenocorticotropin (ACTH), which activates the adrenal cortex. In males, the hypothalamus gonadotropin-releasing hormone (GnRH) promotes the pituitary luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which in turn drive the formation of testosterone-secreting Leydig cells and sperm-producing Sertoli cells (FSH). Forming the HPG axi as shown in (Figure 5A, together with [Supplemental Information (SI) Fig. S1], GCs and CRH both have effects on the HPG axis at numerous levels in a variety of species. Both GC and CRH receptor antagonists may prevent stress from lowering When injected into the circulation, plasma LH has the same effect on rats, rhesus monkeys, and humans. Gene expression in GnRH neurons changes in response to GCs in culture, however this may not necessarily be because of a stressor that suppresses subsequent reproductive activity. We found that inhibition of plasma LH secretion did not result in any alterations in GnRH expression. Reduced GnRH synthesis and/or reduced pituitary sensitivity to GnRH may explain the stress-induced reductions in circulating gona-dotrophin, as shown by our results. Our results offer solid evidence for a molecular basis for this response and add to the literature supporting both of these pathways of stress-mediated decrease of HPG activity.

GnIH/RFRP fibers reach the median eminence and congregate around GnRH cells in both avian and rodent models. GnRH cells have GnIH receptors and express GnRH. Combined, our findings imply that GnIH/RFRP may control the HPG axis by modulating pituitary sensitivity to GnRH and/or hypothalamic GnRH production. GnIH/RFRP controls HPG function by influencing the excitability/release of hypothalamic GnRH neurons and pituitary gonadotropin output, and evidence suggests that this occurs in mammals as well as birds. In vitro studies by Ducret et al. showed that RFRP causes GnRH neurons in mice to hyperpolarize, the research carried out by Rizwan and colleagues shown that the injection of RFRP-3 through the method inhibited the LH response to GnRH in rats (suggesting RFRP functions via pituitary processes). It is possible that the GnIH/RFRP pathway is the one that mediates the relationship between stress and reproduction; nevertheless, it is more likely that both routes are involved. Our data on changed RFRP after acute and chronic stress in rats suggests that GnIH/RFRP is a shared mechanism by which stress attenuates GnRH release and/or gonadotropin secretion in response to GnRH. Our findings of altered RFRP in rats after both forms of stress support this interpretation (Fig. 5 A and B).

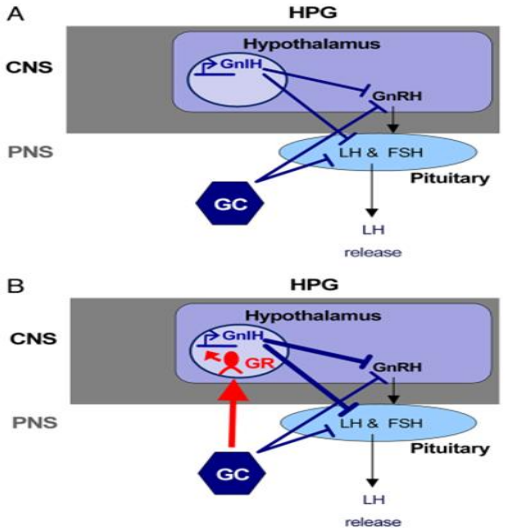


Fig. 5. Exemplifying the stress-induced GnIH-HPG axis theoretically.

The hypothesis that (A) GCs and GnIH block the HPG axis in distinct but complementary ways has been disproved. The bluish color represents these connections. We postulate that GCs activated by stress reduce LH release via increasing GnIH's inhibitory effects on GnRH synthesis and/or pituitary sensitivity. Our proposed red channel of GC-GnIH interaction adds to the well-established roles of GCs and GnIH in regulating reproduction on their own. Stimulation is shown by arrows, whereas inhibition is shown by Ts. The brain and spinal cord are the two main parts of the nervous system, whereas the rest of the body is considered the peripheral nervous system.

GnIH/RFRP is thought to be present in the testes of rats and birds in addition to the hypothalamus. We discovered no evidence of stress-induced alterations in RFRP expression in the testes of adult male rats, suggesting that the impact of stress on RFRP expression is an entirely central, hypothalamic phenomena. Similarly, we did not observe any shift in the expression of RFRP receptors along the HPG axis. Thus, it seems that stress primarily affects RFRP production rather than the sensitivity of other cell types to RFRP through changes in the expression of RFRP receptors.

We discovered that many of the RFRP-expressing cells in the hypothalamus also expressed GR and CRH-R1. Both GCs and CRH have been related to stress-induced changes in the HPG. Reproductive function may be impaired by stress, however CRH receptor blockers (including CRH-R1-specific blockers) may mitigate these effects. We anticipated that GCs would mediate the effects of stress on RFRP expression after finding that GR was expressed by a higher percentage of RFRP cells than CRH-R1, and we then stressed adrenal- lectomized rats. Our findings imply that changes in RFRP are sensitive to increases in GCs since ADX suppressed the stress-induced elevation of RFRP expression in the hypothalamus (Fig. 5B). Previous research has shown that GCs modulate the effects of stress on HPG function; our findings give a molecular foundation for this relationship. Approximately 4990 base pairs (bp) upstream of the rat RFRP gene, with the closest one being ≈ 1198 kb upstream, are predicted glucocorticoid response elements (GREs). In conclusion, our data showing GC-dependent changes in RFRP, coexpression of RFRP and GR, and the presence of putative GREs in the RFRP promoter strongly suggest that stress-induced increases in circulating GCs activate GR in hypothalamic RFRP cells, resulting in increased transcription of the RFRP gene (Fig. 5B).

Consequences for the HPG axis are indirect as well. Stress has been shown to influence reproduction through many, possibly overlapping routes, including as modifications to dopaminergic, serotonergic, and cholinergic transmission. Different stimuli trigger different responses in the HPG-HPA axis, it is likely that both mechanisms evolved concurrently. As a result, our findings suggesting a link between RFRP and HPA suggest a potential mechanism through which some people are more vulnerable to the negative effects of stress than others do. Discrepant results in the stress and reproduction literature may be explained by the fact that various stressors activate distinct HPA-HPG interaction pathways.

Collectively, our findings provide light on how activation of the HPA axis, and consequent consequences on reproductive function, may be influenced by GC-induced increases in RFRP transmission to GnRH neurons and/or the anterior pituitary. Due to the conservation of the HPA-HPG axis and the similarity of GnIH/RFRP activity in birds and mammals, it is likely that the participation of RFRP in the HPA-HPG interaction seen here in rats is also present in most other vertebrates. The hypothalamic-pituitary-adrenal (HPA) axis has been linked to chronic stress and malnutrition, both of which may have a major impact on the success rate of assisted reproductive technologies in the human population. Agricultural breeding operations and captive breeding facilities also face the challenge of chronic stress. Consequently, the present results illuminate a hitherto unknown but vitally significant step in the mechanism behind one of the most prevalent suppressors of HPG activity.

1-8-Making Plans for Use of Resources and Methods

1-8-1-Humans used as test subjects

Twelve hours of daylight and twelve hours of darkness every day, was utilized to house adult male Sprague-Dawley rats in pairs, and the rats had access to food and water at all times. The lights were turned on at 07:00 each day. Five animals (n=) were immobilized and murdered instantly, while four animals (n=) were let to suffer for 24 hours to simulate acute stress. The control group (n = 3) was left alone in their cages until perfusion. Tissue LH and RFRP expression were correlated in a different set of rats (n = 3 con, n = 4 str). Acute stress-induced changes in testes transcript expression were evaluated using real-time RT-PCR in male and female con (n = 4) and str (n = 4) rats. Rats (n = 5 for PCR, n = 3 for IHC) were either immobilized everyday (chronic stress) or left alone in their cages (acute stress). Tissue from three control animals was analyzed using confocal microscopy. Fourteen animals (six stressed, eight non-stressed) were given ADX, while sixteen animals (eight stressed, eight non-stressed) were given sham surgery in the ADX experiment. The Animal Care and Use Committee of the University of California, Berkeley, reviewed all animal care and procedures and gave its approval.

1-8-2-The strain of immobilization

Individual cages of rats were put in a fume hood and the rodents were immobilized for three hours in Decapicone bags (Braintree scientific). The chronic trial included 14 consecutive days of immobility.

1-8-3-Hormone Levels in Plasma Specimens

Plasma was collected from all blood samples and kept at 20 °C until analysis. The centrifugation process took 15 minutes at 2000 g. A Corticosterone EIA kit was used to determine cortico-sterone levels (Cayman Chemical). The National Hormone and Peptide Program RIA determined the LH concentrations in the plasma. As part of a larger study on chronic stress, researchers mixed blood from two cagemates' tails. Research on acute stress relied on the examination of isolated samples. Lowest measurable values from samples were also included.

1-8-4-Adrenalectomy

Rats of adult male gender had their adrenal glands removed on both sides or had fake surgery performed. After spending a week apart to heal, the rats were put back in with their old cage mates. As indicated earlier, immobili-zation stress manifested itself a week later. The ADX rats were fed a diet of water supplemented with 1.0% for the course of the study, participants were given NaCl and 25 g/ml of corticosterone. One ADX stress rat was not included in the analysis because its corticosterone reaction to day one of confinement was inadequate.

1-8-5-Using Immunohistochemical Staining and In-Situ Hybridization to Investigate Stress

Paraformaldehyde (4% concentration) was transcardially infused into the rats. After being postfixed in 4% paraformaldehyde, the brains were equilibrated in 29% sucrose in 0.2 M phosphate buffered saline (PBS) at 79 degrees Celsius. This was done before they were examined. More details on the DIG-labeled probe we used for in situ hybridization against the rat RFRP precursor full length are available in SI Methods. To perform immunohistochemical labeling of RFRP peptide, sections were washed in PBS, blocked for 1 hour with a solution containing 3% normal goat serum and 0.5% tritonX-100 in PBS, and then incubated overnight at 5 °C with primary antibody against GnIH (13) (PAC121/123, 1:5200 in PBS plus 0.5% TritonX-100 [PBS-T]). After overnight incubation with goat anti-rabbit Alexafluor 570 (1:490, Molecular Probes Inc.), PBS-T was used to clean the slides, and then left at room temperature for 3 hours. Coverslipped with DABCO antifading material after a wash in PBS-T, the slides were stored at 4 degrees Celsius in the dark.

1-8-6-The Use of Immunohistochemistry in Confocal Microscopy

After a quick rinse in 0.1 M PBS, the slices were incubated in Tris buffer (pH 8.0), 59 °C, for 19 minutes. A blocking solution of 2% normal donkey serum and 0.3% Triton X 100 in phosphate-buffered saline was applied to the tissue after it had been cleaned (PBS). For this experiment, we diluted each antibody with the blocking solution. The primary antibody was applied at 5 °C overnight (rabbit anti-RFRP with mouse anti-GR [1:198, Affinity BioReagents] or goat anti-CRHR1 [1:198, Santa Cruz]). After being incubated in primary antibody for a period of one night, the sections were rinsed in PBS before being subjected to treatment with secondary antibody for a period of three hours while the temperature was kept constant (Cy3 anti-mouse with FITC anti-rabbit or Cy5 anti-goat with Cy3 anti-rabbit, 1:498, Jackson ImmunoResearch). Following a quick wash in PBS, sections were mounted on gelatin-coated slides and coverslipped/stored as mentioned above

1-8-7-Analysis using Confocal Microscopy

To test if CRH-R1 or GR was co-labeled, we isolated 25 RFRP-ir cells from each animal's DMH and analyzed them in a z-series of 0.9-m slices. The Zeiss 498 META/NLO confocal microscope with a 39 oil objective was used to obtain the confocal pictures, and LSM Image Browser was used to enhance the contrast and brightness of the acquired images. Specifically, 500 nm, 600 nm, and 680 nm were employed.

1-9- Quantitative polymerase chain reaction using real-time reverse transcription

Rats had their heads chopped, hypothalamic, pituitaries, and testes removed, and then flash-frozen in liquid nitrogen after being put under isoflurane anesthesia for a short period. TRIzol-extracted RNA was subjected to real-time reverse transcriptase PCR using primers specific for rat RFRP and OT7T022. PCR miner was used to calculate the Ct values, which were then normalized using the RPLP reference gene. Primer1 was used to create the primer sequences, and BLAST was used to verify their specificity. For all of the stress studies, we followed the instructions provided by the manufacturer and utilized the SYBR Green One-step PCR kit (BioRad). After treating the RNA obtained for the ADX study with DNase DNA-free, Ambion, a two-step PCR was performed using the iScript cDNA synthesis kit (BioRad) and iQ SYBR Green Supermix. The samples were analyzed using a BioRad IQ5 real-time PCR system. Following the PCR, melt curve analysis was used to verify the specificity of each primer pair. Primer sequences may be seen in the SI Methods.

1-10- Quantitative Research

We used two-tailed T-tests to see whether there were statistically significant differences in RFRP mRNA and peptide expression across groups. No statistically significant difference in mRNA ($P = 0.398$) or peptide expression ($P = 0.590$) was found between the acute stress, 0-hour delay rats and the no-stress controls, thus these animals were included with the acute stress, 24-hour delay rats for the purpose of comparison. Repeated-measures analysis of variance in an acute stress experiment showed that immobilization time affected plasma corticosterone levels (ANOVA). We used a 33 (time of immobilization by day) repeated measures (within-day) ANOVA in the chronic stress experiment to examine the impact of immobilization on plasma corticosterone and paired t tests to detect daily deviations from the day 1 baseline. A two-sample t test was used to determine the impact of stress on plasma LH levels on day 14. One-sample t tests were conducted to determine whether stress influenced RFRP expression in sham and ADX animals. Planned comparisons were also used to examine the differences between sham and ADX animals when it came to stress-induced variations in plasma corticosterone (two-sample paired t tests). P 0.05 was chosen as the threshold of statistical significance.

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