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Investigation of a neural mechanism involved in suppression of reproductive neuroendocrine function during stress

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

in

Biology

by

Helen Bell

Committee in charge:

Professor Kellie Breen Church, Chair
Professor Monique Smith, Co-Chair
Professor Vineet Augustine
Professor Heidi Cook-Anderson

2024

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University of California San Diego

2024

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All the data presented in the Results section of this master's thesis may be included in future publications from the Breen Church laboratory.

ABSTRACT OF THE THESIS

Investigation of a neural mechanism involved in suppression of reproductive neuroendocrine function during stress

by

Helen Bell

Master of Science in Biology

University of California San Diego, 2024

Professor Kellie Breen Church, Chair
Professor Monique Smith, Co-Chair

Stress is a condition experienced by all living organisms. Stress can initiate psychological and physiological responses that may inhibit other systems in the body, including the reproductive system. Infertility is a concern for many individuals, and previous research has found a correlation between infertility and stress. In the mouse model, stress can inhibit normal

reproductive function; however, the neural mechanisms mediating this phenomenon are not yet fully understood. The paraventricular nucleus (PVN) is a key region activated during the response to stress and this nucleus contains corticotropin releasing hormone (CRH) neurons. Evidence supports a CRH receptor type 2 pathway to play a key role in stress-induced suppression of reproductive function. Therefore, we hypothesized that urocortin 2 (UCN2), a neuropeptide in the CRH family with high affinity for CRH receptor type 2, may be a good candidate for a mediator of stress-induced suppression of the LH surge. We observed that central administration of UCN2 was sufficient to block an estradiol-induced LH surge. Next, we looked upstream at a population of norepinephrine (NE) producing neurons in the A2 region of the nucleus of the solitary tract in the brainstem. The $A2^{NE}$ population is known to be activated during a variety of stress types, and ablation of this population restores reproductive function in a hypoglycemic stress model. We hypothesized that this population was involved in stress induced reduction of reproductive function, and we observed that chronic activation of the $A2^{NE}$ population was sufficient to inhibit estrous cyclicity. In summary, these data support a working model whereby $A2^{NE}$ neurons can activate UCN2 cells in the PVN and initiate reproductive suppression during stress.

INTRODUCTION

Stress, which can be loosely defined as a disruption or threat to homeostasis (Smith & Vale, 2006), is a universal condition; every human and every living organism experiences some type of stress at some point in their lives. There are many different types of stress including, metabolic, immune, psycho-social, etc. Depending on the type and the severity, stress can have a variety of physiological impacts including reduction of reproductive function. An estimated 48 million couples worldwide struggle with infertility (Ramya et al., 2023), and there is an abundance of research that shows a correlation between stress and infertility (Campagne, 2006; Palomba et al., 2018). Although stress has been shown to reduce reproductive function in many animal models, the neural mechanisms mediating the stress induced reduction of reproductive function are not yet well understood.

The hypothalamic-pituitary-gonadal (HPG) axis controls reproductive function in many species (Hawkins & Matzuk, 2008; Kauffman, 2022; Piet, 2023). In the hypothalamus, there are kisspeptin (Kiss) neurons which release Kiss to stimulate gonadotropin releasing hormone (GnRH) neurons which in turn release GnRH to stimulate the pituitary gland. When stimulated by GnRH, the pituitary gland releases luteinizing hormone (LH) and follicle stimulating hormone (FSH). In females, LH and FSH act on the ovary to stimulate follicular development and steroidogenesis. One of the key steroids produced by the ovary is estradiol (E_2) and it is important because it moves back up to the brain to regulate the activity of Kiss neurons.

Normal female reproductive function involves a cyclical increase and decrease of E_2. In low E_2 conditions, Kiss neurons in the arcuate nucleus of the hypothalamus (ARC) reduce their activity (Kauffman, 2022) which creates negative feedback on the system resulting in a pulsatile secretion pattern of GnRH and subsequently LH. LH pulses are a normal characteristic of the

female reproductive cycle, and as the cycle progresses the development of larger follicles leads to an increase in E_2. In high E_2 conditions, E_2 signaling to Kiss neurons in the rostral periventricular area of the third ventricle (RP3V) creates positive feedback which generates a robust secretion of GnRH and subsequently LH (Piet, 2023; Herbison, 2008). This physiological event is known as the LH surge. In the absence of fertilization, this ovulatory cycle will repeat and continuously transition from "pulse mode" to "surge mode" and back again.

The mouse model is a helpful tool for studying reproductive function because in females the cycles of ovulatory function, referred to as the estrous cycle, can easily be non-invasively monitored. The female mouse estrous cycle can be divided into four stages: diestrus, proestrus, estrus, and metestrus. Estrus refers to the stage of behavior receptivity which occurs following ovulation. Each stage of the cycle can be determined based on morphology and qualification of cells from the vaginal opening (McLean et al., 2012). Samples can be collected daily to track the progression of the estrus cycle over time. Often, a female mouse with normal reproductive function will progress through one cycle in 4-7 days.

The LH surge is a key area of study for fertility because it is a physiological cue that triggers ovulation. In the mouse model, the LH surge is under circadian control and the surge typically occurs at the time of lights out (Piet, 2023). The surge can be experimentally induced by ovariectomizing (OVX) the female mouse and administering a high dose of E_2. After receiving the high E_2 treatment, a mouse will show surge-like levels of LH at the time of lights out every day until exogenous E_2 is reduced. Stress can inhibit both the naturally occurring and experimentally induced LH surge. Stress is thought to inhibit the LH surge primarily in two ways. The first is through a reduction of ovarian function. Stress inhibits activity at every level of the HPG axis (Dobson & Smith, 2000), meaning the ovary is producing less E_2 and the lower

level of E_2 is insufficient to trigger an LH surge. The second is through a neural mechanism. In the high E_2 LH surge induced model, where the ovary is removed and not a factor, stress can still have a suppressive effect on the LH surge. This would indicate an upstream neural pathway is involved in the suppression of E_2 positive feedback. This research is focused on uncovering the neural mechanism responsible for the suppression of the LH surge and disruption of ovulatory cyclicity during stress.

Stress is correlated with infertility in humans and has been shown to inhibit reproductive function in the mouse model (Karunyam et al., 2023). Multiple types of metabolic stress have been found to reduce reproductive function in the mouse model. Chronic undernutrition has been found to reduce ovarian cyclicity, the LH surge and LH pulses in female mice (Kreisman et al., 2021). Insulin-induced hypoglycemia, an acute type of metabolic stress, has been found to inhibit LH pulses (McCosh et.at, 2019). Understanding how these types of metabolic stress impact the LH surge and ovarian cyclicity leads to questions about the neural populations that communicate metabolic cues to the neural centers controlling reproductive neuroendocrine function.

Multiple areas of the brain have been shown to be altered during metabolic stress. One area of interest is located in the brainstem within the nucleus of the solitary tract (NTS), known as the A2 region which contains norepinephrine (NE) producing cells. The NTS receives rich communication from the periphery, either via spinal nerves, the vagus nerve, or via circulating factors that communicate via the area postrema, a circumventricular organ receiving cues in the blood (Appleyard et al., 2007). Previous research found that A2 neurons in rats are activated during food deprivation (Rinaman, 2011) and hypoglycemic stress also activates $A2^{NE}$ neurons. Additionally, lesion of this area restores reproductive function during chronic glucoprivation, a model of metabolic stress (I'Anson et al., 2003). This suggests that this region is involved in the

signaling pathway that conveys stress cues, at least those related to low glucose levels, to the HPG axis during stress.

The paraventricular nucleus in the hypothalamus (PVN) is another key region activated during stress (Herman & Tasker, 2016; López et al., 1999) including metabolic stress (Ritter & Dinh, 1994). When activated by stress, the PVN releases corticotropin releasing hormone (CRH) and initiates an increase in adrenal corticosteroids, which initiates stress response pathways in the whole body (Dobson & Smith, 2000). Previous research found that a CRH receptor type 2 antagonist, administered into the lateral ventricle, was sufficient to restore LH secretion during metabolic stress (Li et al., 2005). This indicates that a central CRH receptor type 2 pathway is a key player in stress induced suppression of the HPG axis. There is a distinct population of neurons called urocortin 2 (UCN2) neurons, which have a high affinity for CRH receptor type 2 (Reyes et al., 2001), making them a good candidate as a mediator of stress induced reduction of reproductive function. Evidence that UCN2 neurons in the PVN are activated during stress, suggests an intriguing location for stress to induce both CRH and UCN2 pathways to play a role in stress responses, with UCN2 being important for HPG suppression and CRH being important for HPA axis activation.

This master's thesis is focused on the role of a brainstem to hypothalamic pathway that may mediate the suppression of the preovulatory surge and ovarian cyclicity during stress. Based on the literature and preliminary studies in the Breen Church Laboratory, we test two interrelated hypotheses. First, we hypothesize that UCN2 signaling is involved in the suppression of the HPG axis during stress, so we tested if central administration of UCN2 was sufficient to block an induced LH surge. Next, we focused on the brainstem NTS NE population which are activated

during stress and have the potential to communicate to PVN UCN2 neurons. Our second hypothesis is that chronic activation of the $A2^{NE}$ population will inhibit ovarian cyclicity.

METHODS

Animals

Adult female C57bl6 wildtype, Kiss GFP, and DBH Cre mice were used. Animals were housed either 1 or 2 per cage, and on a12 hour light-12 hour dark cycle, with lights off at 6 PM. Mice were fed Harlan irradiated chow number 2920X and given ad libitum access. All procedures were performed at UCSD and performed in accordance with NIH and IACUC guidelines.

Experiment 1A: Is central administration of UCN2 sufficient to block an induced LH surge?

This experiment tested the sufficiency of central administration of UCN2 via intracerebroventricular (ICV) injection to impede an induced LH surge (Figure 1A). WT and Kiss GFP animals were ovariectomized and received a high E_2 implant (OVX+E_2), following a protocol from the Breen Church Laboratory from previous research (Kreisman et al., 2021). All animals were anesthetized with isoflurane and both ovaries were surgically removed. A high E_2 implant consisting of 1.0 μg 17-β estradiol in a silastic capsule was then inserted subcutaneously. Surgeries were completed before 12 PM on day 0 of the experiment.

On day 1 of the experiment, a needle was used to perform a craniotomy on all animals to prepare an ICV injection site into the lateral ventricle. On day 2 of the experiment at 10 AM, either saline or 7.23 nmol of UCN2 was centrally administered via ICV injection into the lateral ventricle. On day 2 of the experiment at 5 PM, at the time of the expected surge, 6.4 μl of tail blood was collected from each animal and then animals were sacrificed, and brains were collected. An enzyme linked immunosorbent assay (Kreisman et al., 2022) was performed to determine the concentration of LH in the blood sample from each animal.

Assessment of correct targeting of the lateral ventricle (i.e. "a hit") was determined by inspecting the injection site. Brains were frozen on dry ice directly after collection then sectioned coronally and during the sectioning process the injection site was observed for accuracy.

Experiment 1B: Does central administration of UCN2 inhibit activation of RP3V Kiss cells?

Following administration of UCN2 or saline as in experiment 1a, Kiss GFP animals were bled to assess LH surge secretion and then were perfused with paraformaldehyde (PFA) and their brains collected and post-fixed in PFA. Fixed neural tissue was cut coronally in 40μm sections in three series. Then the rostral areas, including the RP3V region, of one of the series was stained via immunohistochemistry. The protocol used rabbit anti c-Fos and Alexa 647 to label c-Fos expression and anti-rabbit and rabbit-anti GFP Alexa 488 to label the Kiss GFP neurons. After staining, sections were mounted on slides then viewed and imaged under a fluorescent microscope. Co-expression of Kiss and c-Fos in the RP3V region was quantified in each section from saline and UCN2 treated animals to determine activation of the $RP3V^{Kiss}$ population.

Experiment 2: Does chronic activation of the $A2^{NE}$population disrupt estrous cyclicity?

A Cre-dependent stimulatory DREADD virus was injected into the A2 region via stereotaxic surgery of WT (Cre-) and DBH-Cre (Cre+) animals. Transduction of the virus leads to expression of designer receptors that are exclusively activated by a designer drug called clozapine-N-oxide (CNO). Dopamine beta hydroxylase (DBH) is an enzyme which catalyzes the synthesis of NE meaning that all NE producing cells express DBH. In a Cre+ animal, NE producing cells in the A2 region would have viral transduction and be activated by CNO treatment. Cell type specificity is determined by the genotype, only Cre+ cells will accept the virus, and regional specificity is determined by injection site accuracy. Cre- animals should have

no transduction and CNO treatment would not cause any neural activation while Cre+ positive animals should have transduction in DBH expressing cells in the A2 region.

After two weeks of recovery from the neural surgery, we began monitoring estrous cyclicity. Every day between 3PM and 5PM, vaginal lavage was performed; 30 µl of distilled water was flushed within the vaginal opening to collect cells and then smeared on a slide. Slides were then stained using indigo blue and observed under a microscope. Each sample was assigned a cycle stage based on qualification of cells. Body weight and food intake were also monitored daily. After a two week pre period, clozapine-N-oxide (CNO) was added to drinking water at 5 mg/kg per day and ovarian cyclicity, food intake, and body weight were continued to be monitored. After two weeks of CNO in drinking water, animals were sacrificed in diestrus, and ovaries and uteruses were weighed and collected. Then all animals were perfused and fixed brains were collected.

Fixed neural tissue was cut coronally in 40µm sections in three series, then the caudal areas, including the NTS region, of one of the series was stained via immunohistochemistry. The protocol used rabbit anti c-Fos and Alexa 647 to label c-Fos expression and rabbit anti-tyrosine hydroxylase (TH) and Dylight 448 Goat anti-rabbit to label TH expression. TH is another enzyme required to catalyze norepinephrine and therefore present in all DBH expressing cells and it was used to confirm cell type specificity of the virus. The stimulatory DREADD virus contained mCherry so cells with viral transduction will also express mCherry.

After staining, sections were mounted on slides, then viewed and imaged under a fluorescent microscope to assess A2 cell transduction and stimulatory DREADD activation only in the experimental Cre+ animals. Co-expression of TH, mCherry, and c-Fos in the A2 region confirmed successful transduction and stimulatory DREADD activation. Animals without

mCherry in the A2 were deemed ineffective transduction and disqualified; animals with mCherry transduction in regions other than the A2 were deemed to express off target transduction and also disqualified.

All statistical analysis was performed in excel. To compare quantitative data from one time point between a control group and an experimental group, a two-tailed t-test was used to determine statistical significance.

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RESULTS

Experiment 1A: Central administration of UCN2 is sufficient to block an induced LH surge

Central administration of UCN2 was sufficient to block the induced LH surge (Figure 1B). Control animals received a high E_2 treatment and then saline on the morning of the expected surge. Of the 9 controls, 8 expressed an LH surge (>0.5 ng/ml) with a group average of 1.21 +/- SEM of 0.34 ng/ml. In contrast, the UCN2 treatment group received a high E_2 treatment and then UCN2 on the morning of the expected surge; 0 of the 7 animals expressed an LH surge. UCN2 treated animals had an average LH concentration of 0.07 +/- SEM of 0.01 ng/ml. Saline-treated animals expressed significantly higher levels of LH than UCN2-treated animals (p-value = 0.009).

Experiment 1B: Central administration of UCN2 inhibits activation of RP3V Kiss cells

Kiss activation in the $RP3V^{Kiss}$ population was lower in UCN2 treated animals than in saline-treated animals (Figure 2A). Brains were collected immediately following blood collection, sectioned, stained, and imaged to quantify c-Fos expression in $RP3V^{Kiss}$ cells as a marker of neuronal activation. Sections from saline-treated animals had an average of 24.75 +/- 5.75 SEM labeled Kiss cells and average of 46.68 +/- SEM 6.38 % of Kiss cells labeled with c-Fos (Figure 2B and 2C). Sections from UCN2-treated animals had an average of 39.29 +/- SEM 7.41 labeled Kiss cells and average of 9.27 +/- SEM 5.45 % Kiss cells labeled with c-Fos. There was no significant difference in the number of Kiss labeled cells per section between saline and UCN2-treated animals (p-value=0.2); however, there was a significantly higher percentage of Kiss cells expressing c-Fos in the saline treated group versus the UCN2 treated group. (p-value=0.003)

Experiment 2: Chronic activation of the $A2^{NE}$ population disrupts estrous cyclicity

Animals were monitored for a 2 week pre period and two week CNO treatment period during which time Cre+ treatment animals had chronic activation of the $A2^{NE}$ population (Figure 3A). In the Cre- control animals, 8/8 showed normal cycling in the pre period and continued to cycle normally after CNO was added to drinking water. In contrast, 7/7 of the Cre+ treatment animals showed normal cycling in the pre period but showed disrupted cycles during CNO treatment. Many were stalled in diestrus (Figure 3B).

Ovaries and uteri were collected and weighed as a bioassay of upstream reproductive hormone support required to maintain tissue function and secretory activity (Figure 4A and B). At the time of collection, ovarian weight for Cre- controls was an average of 4.74 +/- SEM 0.17 mg but it was significantly diminished in treatment animals with an average of 2.96 +/- SEM 0.48 mg (p-value=0.02). Similarly, uterine weight was reduced in treatment animals. Cre- control animals had an average of 58.24+/-SEM 1.8m g while Cre+ treatment animals had an average of 34.93 +/-SEM 5.77 mg. The difference was again found to be significant (p-value=0.0002).

Considering that the $A2^{NE}$ population may be involved in other circuits that influence other physiological functions, we collected data on food intake and body weight throughout the duration of the experiment. We observed a reduction in both food intake and body weight in Cre+ treatment animals (Figure 5).

The data presented in this section may be included in future publications from the Breen Church Laboratory.

DISCUSSION

Our findings show that a UCN2 signaling pathway and $A2^{NE}$ activation mediate suppression of multiple aspects of reproductive function in females. First, we show that UCN2 can inhibit the LH surge via suppression of $RP3V^{Kiss}$ population. Then, we show that chronic activation of $A2^{NE}$ neurons inhibits cyclicity. This suppression in cyclicity is likely a suppression of gonadotropin secretion (LH and FSH) as ovarian and uterine weights are suppressed in chronically activated $A2^{NE}$ animals. This data suggests that both UCN2 signaling and $A2^{NE}$ activation are potential mediators of reproductive dysfunction and may be involved in a mechanism whereby stress inhibits normal reproductive functions including the LH surge and ovarian cyclicity.

The Breen Church Laboratory has preliminary data that chemogenetic activation of the $A2^{NE}$ population induces activation of the PVN^{UCN2} population. This suggests both players could potentially be involved in the same pathway (Figure 5) with the $A2^{NE}$ population as an upstream activator of the PVN^{UCN2} population. Additionally, previous research has found that $A2^{NE}$ neurons project directly to the PVN (Rinaman, 2011) providing an anatomical link between these two brain regions; however, we have not yet discovered if A2 signaling directly to the PVN, signaling to a mediator such as UCN2, is the pathway mediating suppression here. Future work is necessary to confirm the necessity of this A2 to UCN2 pathway as a mediator of LH suppression or determine whether A2 neurons act in a completely separate pathway to inhibit Kiss activation and downstream reproductive function during stress. Clearly, there is more research to be done to fully understand the bigger picture of what happens in the brain during stress that causes the suppression of reproductive function.

Another question raised by the current data set is regarding the source of UCN2. Although we have shown that UCN2 is sufficient to inhibit activation of the LH surge, we have not yet established that the signal is coming from the PVN. The PVN is a good candidate because of its known activation during stress; however there are other areas of the brain, such as the preoptic area, the locus coeruleus, etc. that contain UCN2 neurons (Tanaka et al., 2003). More work needs to be done to confirm that the UCN 2 signal is coming from the PVN. This could include examining the connectivity between UCN2 cells in the PVN and Kiss neurons in both the RP3V and ARC regions and ruling out other UCN2 populations as potential mediators.

Finally, this data confirmed the function of the $A2^{NE}$ population in physiologic processes other than circuits controlling reproduction. The decreased food intake and body weight phenotype is evidence to suggest that activation of the $A2^{NE}$ population may be activating multiple different pathways including feeding behavior. Given that undernutrition has been shown to inhibit cyclicity (Kreisman et al., 2021), restrictive feeding behaviors and subsequent loss of body weight could also be contributing to the loss of reproductive function in these animals. Emerging evidence suggests that brainstem NE circuits are vitally important for physiologic function and behavior, and these data add to that evidence suggesting many different connectivity and signaling pathways to understand.

This data makes a strong case for both the PVN^{UCN2} and $A2^{NE}$ population as potential mediators of the suppression of the LH surge and ovarian cyclicity during stress. There is more to uncover to fully understand the mechanisms and pathways responsible for stress induced reduction of reproductive function. This research opens the door to better understanding stress and the physiological implications of stress and in the future may be useful for treating disorders of ovulatory function and infertility in adults.

FIGURES

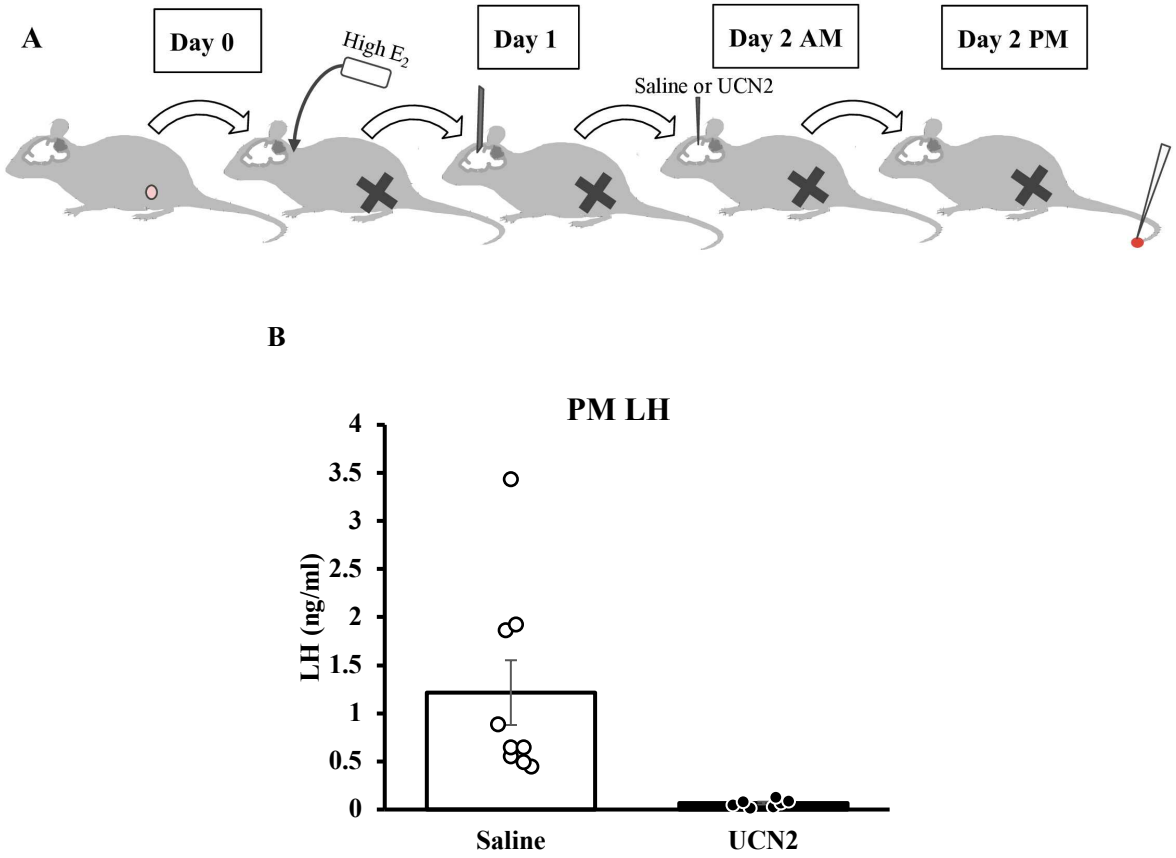

Figure 1: Central Administration of UCN2 was sufficient to block an induced LH surge. A) Animals were OVX+E then given an ICV injection of saline or UCN2 the day of the expected surge. An LH surge was defined as a PM LH concentration >0.5 ng/ml and 8/9 control animals met the criteria for a surge while 0/7 UCN2 treated animals met the criteria expression an LH surge. B) PM blood samples revealed saline treated animals had an average of 1.21 ng/ml while UCN2 treated animals had an average of 0.07 ng/ml in UCN2 treated animals than in saline controls (p-value = 0.009). Error bars represent standard error of mean.

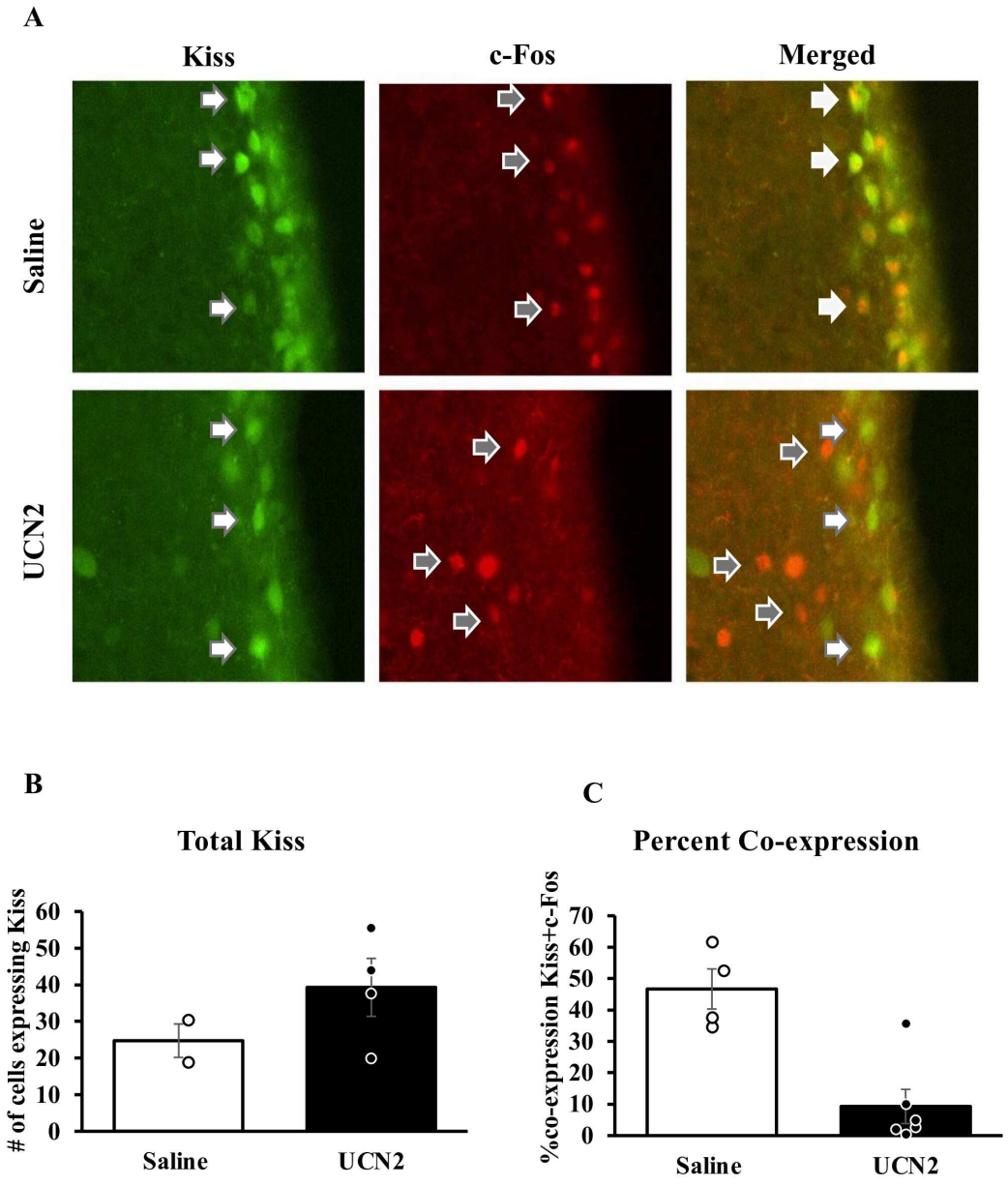

Figure 2: A) Central Administration of UCN2 was sufficient to reduce RP3V^{Kiss} activity. B) There was no significant difference in the number of Kiss labeled cells per section between saline and UCN2 treated animals (p-value=0.2). Saline treated animals had an average of 24.75 Kiss labeled cells per section UCN2 treated animals had an average of 39.29 Kiss labeled cells per section. C) There was a significantly higher percentage of Kiss cells expressing c-Fos in the saline treated group versus the UCN2 treated group. (p-value=0.003) Saline treated animals had an average of 46.68% of Kiss cells expressing c-Fos and UCN2 treated animals had an average of 9.27% of Kiss cells expressing c-Fos.

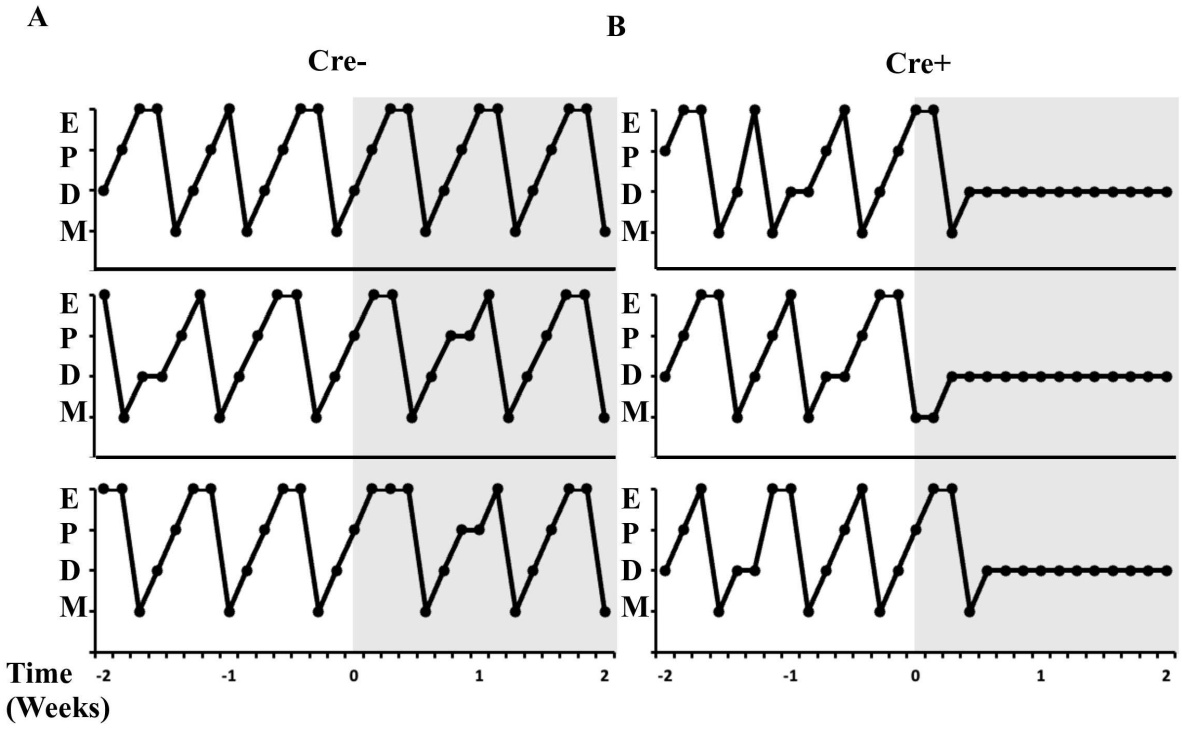

Figure 3: Chronic activation of the A2^{NE} population inhibited normal reproductive function. A) Stereotaxic injection of a stimulatory DREADD virus into the A2 region of the NTS was performed on WT (Cre-) and DBH Cre (Cre+) mice. After a period of recovery from neurosurgery, food, body weight, and estrus cycle stage was monitored daily was recorded for a two week pre-period then CNO was added to drinking water and monitoring continued for a two week treatment period. CNO activates the A2^{NE} population in Cre+ animals. B) Cre- animals showed normal cycling in the pre-period and continued normal cycling when CNO was added to drinking water. Cre+ animals showed normal cycles in the pre period and a reduction of cyclicity during CNO treatment.

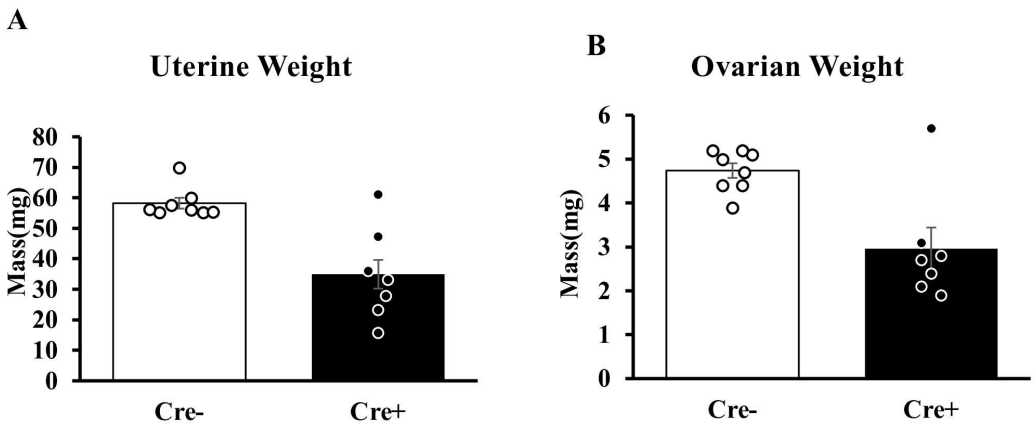

Figure 4: Chronic activation of the $A2^{NE}$ population reduced ovarian and uterine weight. After two weeks of treatment animals were sacrificed and ovaries and uteruses were collected. A) Ovarian weight was significantly reduced in treatment animals at the time of collection (p-value=0.02). Average ovarian weight of Cre- at the time of collection was 4.74 mg while average ovarian weight of Cre+ animals was 2.96 mg. Error bars represent standard error of mean. B) Uterine weight was significantly reduced in Cre+ (p-value=0.0002). Cre- control animals had an average uterine weight of 58.24 mg at the time of collection and Cre+ $A2^{NE}$ activated animals had an average uterine weight of 34.93 mg at the time of collection. Error bars represent standard error of mean.

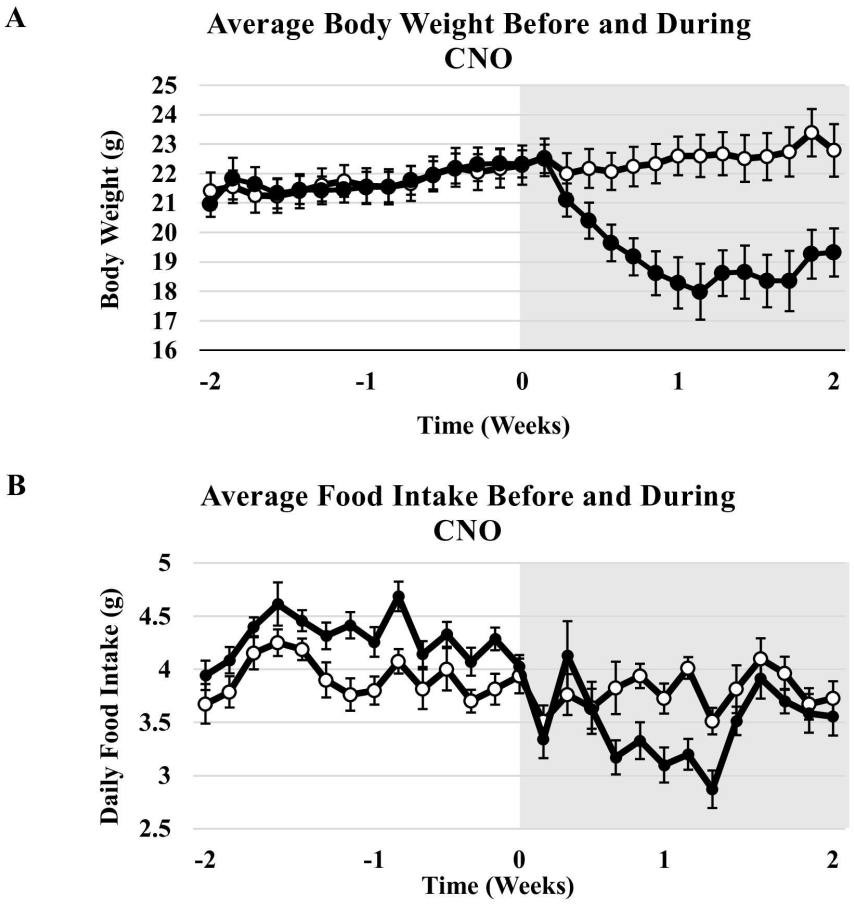

Figure 5: Chronic activation of the A2^{NE} population reduced A) food intake and B) body weight. Open circles represent Cre- control animals. Closed circles represent Cre + treatment animals.

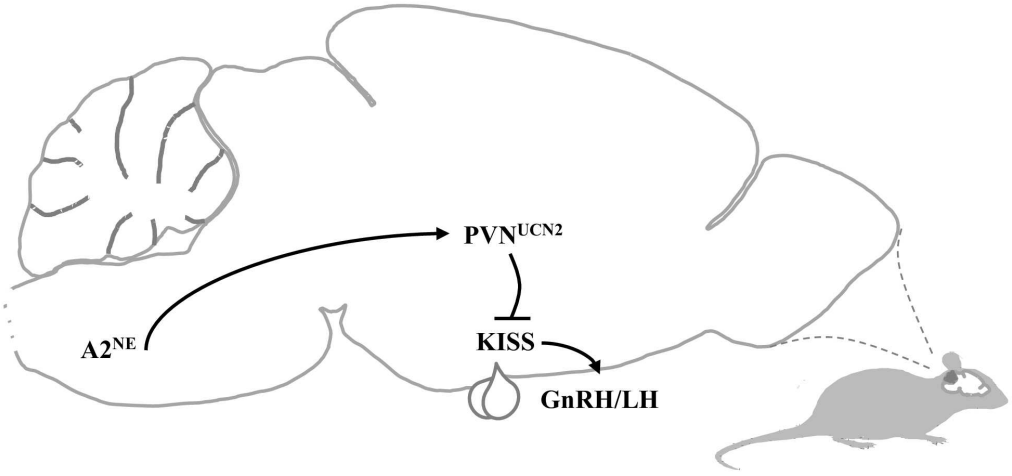

Figure 6: A proposed pathway for the stress induced reduction of reproductive function.

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