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Effectiveness Of Different Corticosterone Administration Methods To Alter Corticosterone Levels In Serum And Depressive-Like Behavior In Female Rats

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EFFECTIVENESS OF DIFFERENT CORTICOSTERONE ADMINISTRATION METHODS TO ALTER CORTICOSTERONE LEVELS IN SERUM AND DEPRESSIVE-LIKE BEHAVIOR IN FEMALE RATS

by

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DEDICATION

This thesis is dedicated to my parents, Paul and Diane Kott for their love, support, and encouragement throughout my life and academic career, and to all of my little rats that were sacrificed for this study and the advancement of scientific research.

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1. INTRODUCTION

Stress is defined as a state that threatens or gives the perception of a threat to a person's physiological equilibrium (Weinstock, 2005). Stress is experienced when an individual encounters a perceived threat and undergoes the biological response that prepares the body for a reaction. A common term to describe this reaction is the "fight-or-flight" response. In this response, stress from the environment activates the hypothalamus to release corticotropin-releasing hormone (CRH). CRH then signals the pituitary gland to release adrenocorticotropic hormone (ACTH), which stimulates the adrenal gland to release stress hormones, most notably cortisol, into the bloodstream (see Figure 1). This network of structures that prepares the body to react to the perception of a threat is known as the hypothalamus-pituitary-adrenal (HPA) axis. It is through this network that stress, especially when experienced chronically, is able to exert its detrimental effects on the body. If there is an over-activation of this system, inflammatory markers may become elevated, due to what is known as stress-induced immunocompromise. Elevated levels of inflammatory markers are associated with pathophysiologic states such as an increased risk for contracting infectious diseases and poor ability to heal (Stix, 2007).

The involvement of these inflammatory markers in the experience of stress makes women especially vulnerable to the effects of stress, especially during pregnancy. This is particularly important, as there is high comorbidity between stress and depression in women, particularly during their reproductive years. Pregnant women undergoing high levels of stress or depression are increasingly vulnerable to effects such as preterm birth and lower birth weight of the infant. These results stem directly from the HPA axis activation in response to stress. Increased inflammatory response stimulates lymphocytes

to release cytokines, prostaglandins, and interleukins. Cytokines and prostaglandins are involved in controlling the contractions of uterine muscle, which may affect preterm labor and delivery, and cortisol released in response to stress is also closely linked to the process of childbirth. Most notably, cortisol is the most important modulator of the timing of normal parturition (Bulletins--Obstetrics, 2003). Cortisol is also involved in oxytocin release from the pituitary gland, which is a necessary process for the occurrence of the uterine contractions required for childbirth. Stress can complicate this experience by causing a premature release of cortisol, which could lead to preterm labor and delivery. This possibility depends on the stressor itself, the duration of pregnancy at the time of the stress, and other risk factors such as anxiety, depression, and other psychosocial factors (Cardwell, 2013). Due to the unique role of these hormones in women and the prevalence of stress and depression in women during pregnancy, it is necessary to study the effects of stress particularly in women and not just generalize findings from studies on the effects of stress in males.

Due to the subjective nature of stress in humans and the large variety of confounds incorporated in human research, a rodent model to study the effects of stress on pregnancy and the subsequent development of the offspring is essential. In the process of developing this model to study development, it is first necessary to determine how best to administer stress to female rodents to induce a depressive-like phenotype. This goal is hindered by variation in the results in current research, both based on administration of stress and differential results between stress administered to male rodents and stress administered to females, making it difficult to draw conclusions.

There are many commonly used methods for inducing stress in rodents. Most frequently, these involve paradigms such as restraint stress, chronic mild stress, or cold stress. Stress by these methods are subject to several confounds, including variation in techniques between labs and individual differences in rodents between stressful measures. For example, one animal may be particularly sensitive to the effects of cold stress but less so to restraint stress. There are also several different protocols for chronic mild stress paradigms and each method likely produces variability in the results. Furthermore, rats are readily habituate to predictable stressors which means these types of stressors eventually do not elicit elevated levels of CORT over time (De Boer et al., 1989; Herman, 2013). In order to avoid these pitfalls, we decided to employ an alternative method of stress by administering exogenous corticosterone (CORT). Corticosterone is the analog to cortisol, and both are the end-products of HPA activation in humans and rats, respectively. Previous research has given CORT through 3 different administration methods: a) time-release pellet implantation, b) in the drinking water, and c) subcutaneous injection.

These 3 administration methods are frequently used and well-established in the literature to induce a depressive-like phenotype in rodents. Interestingly, previous research has shown that these administration methods produce differential effects in females as compared to male rats (Dalla et al., 2010). While females appear to be more sensitive to the stressful effects of the forced-swim test than are males, females display less helplessness behavior in response to foot shock than do males (Dalla et al., 2008). The sex differences in stress response may result from the unique physiology of females and may be related to the female's role as a caregiver in many mammalian species.

Indeed it has been hypothesized that females may have developed a different behavioral response to stress than males that serves to be more adaptive during their vulnerable states during pregnancy and nursing. Further, females have naturally higher levels of circulating CORT as compared to males, which may contribute to the significant sex difference in stress sensitivity between males and females. Due to these differences, it is necessary to find a method to administer exogenous CORT that is effective to develop a depressive-like phenotype in female rats.

A depressive-like phenotype in rodents has been characterized by several features: elevated CORT levels in blood serum, decreased body weight, an increase in immobility in the forced swim test, and a decrease in hippocampal neurogenesis. It has been hypothesized that HPA axis dysregulation is incorporated in that pathology of depression, and as such, is able to modulate the behavioral effects of stress. This theory is supported in the human literature. One study in particular demonstrated that patients suffering from depression whose HPA axis regulation improved after one week of pharmacological treatment had lower depression scores and shorter admission times compared to patients that continued to display HPA axis dysregulation (Schule et al., 2009). Therefore, it is likely that the HPA axis serves to regulate the body's response to stress and is incorporated in its behavioral and physiological manifestations. As it may be impossible to achieve a true model of depression in rodents, the goal of this research was to mimic as many characteristics of the depressive-like phenotype of rodents as possible. To do this, this study used 3 administration routes (a) time-release pellet implantation, b) in the drinking water, and c) subcutaneous injection) to determine the efficacy of each in the development of a depressive-like phenotype in female rodents.

1.1. Administration by Subcutaneous Pellet

Time-release pellets have been used in the literature to administer CORT in rodents (Claflin et al., 2014; Claflin et al., 2005; Kraszpulski, et al., 2012; Murray et al., 2008). Briefly, the rat is placed under anesthesia and the pellet is surgically implanted in a pocket under the skin in the lateral neck area. The pellets are designed to release a specific dose of CORT over 21 days before dissolving completely. In addition to the surgical implantation and necessity of anesthesia, another factor that must be taken into account with the time-release pellets is that the dose cannot be adjusted after implantation, i.e., for weight loss or grain. However, there are no further invasive procedures after the surgery and CORT is delivered at a constant dose throughout the testing period. Research using these pellets has demonstrated their efficacy in increasing CORT levels in serum, increasing immobility in the forced swim test, and decreasing hippocampal neurogenesis in male rodents (Murray et al., 2008).

1.2. Administration by Drinking Water

While CORT has very limited water solubility, a related compound, corticosterone hemisuccinate, is readily water soluble and can be easily incorporated into a drinking water solution for administration to rodents, as demonstrated in previous research (Casolini et al., 207; Catalani et al., 2002; Catalani et al., 2000; Catalani et al., 1993). This method using CORT hemisuccinate has been frequently used to study the effects of prenatal stress on development due to the minimally invasive nature of its administration (Casolini et al., 207; Catalani et al., 2002; Catalani et al., 2000; Catalani et al., 1993). Administration of CORT via drinking water has been shown to elevate levels of CORT in serum in female rats (Catalani et al., 1993; Catalani et al., 2000; Catalani et

al., 2003) and increase immobility in the forced swim test in male rats (Donner et al., 2012). A consideration that needs to be made for administration via drinking water is that the solution will be consumed *ad libitum*, and as such, the dose or time of consumption cannot be directly controlled by the researcher.

1.3. Administration by Subcutaneous Injection

Administering CORT through a subcutaneous injection has the advantage of being under a great deal of control by the investigator. The experimenter is able to ensure the exact dose is given and is able to adjust the dose in the event of weight loss or weight gain throughout the course of the experiment. This method has been shown effective in increasing immobility in the forced swim test in male rats (Hill et al., 2003) and alter levels of hippocampal neurogenesis (Brummelte & Galea, 2010; Mirescu & Gould, 2006; Wong & Herbert, 2006). A limitation of administration by injection, however, is that the full dose of CORT is administered at the time of the injection and is not steadily increased throughout the day.

This study aimed to evaluate the effectiveness of these 3 administration methods to generate a depressive-like phenotype in female rats by chronic CORT administration (21 days) at a dose of 40 mg/kg as used in the literature. As the majority of the literature on this topic is in male rats, it is critical to elucidate the most effective method for CORT administration in female rats. The goal of this experiment is to provide data for the future investigation of the effects of stress before pregnancy on development, so in addition to the efficacy of these methods, the levels of their invasiveness were also considered for future use. The measures taken were: CORT levels in serum, body weight, immobility in the forced swim test, and reduced levels of hippocampal neurogenesis using doublecortin

(DCX), which taken together are indicative of a depressive-like phenotype. To our knowledge, this is the first study to compare across CORT administration methods in female rodents on their ability to produce a depressive-like phenotype.

2. METHOD

2.1. Subjects

Thirty-six female rats (Sprague-Dawley, age 77-84 days) were purchased from Charles River Laboratory (Portage, MI, USA) and were housed in a 12:12 hour light:dark cycle (lights on at 06:00) with food (Rodent Lab Diet 5001; PMI, Nutrition International, Inc., Brentwood, MO) and water available *ad libitum*. Animals were group-housed upon arrival and single-housed after a 3-day acclimation period. CORT was administered via 3 different routes, each with a corresponding control group for which the female rats were randomly assigned to 1 of 6 total groups: CORT pellet (n=6), control pellet (n=6), CORT injection (n=6), vehicle injection (n=6), CORT in drinking water (n=6), and control drinking water (n=6). All animals were weighed every 3 days.

2.2. Hormone Preparation and Administration

CORT or vehicle was administered for 23 days with a wean-off period from days 21 to 23 (see Fig. 2). The wean-off period for the injection and drinking water groups was to mimic the potential decrease in CORT release in the implanted pellets. The dosages for each administration group were calculated to reflect ~40 mg/kg/day (i.e., ~10 mg/day for a 250 g female rat). Thus, the injection group received 40 mg/kg/day via injection. The pellets contained 200 mg, which was designed to be released over 21 days (200 mg/21 d = 9.52 mg/day; highest dose available). The dose for the drinking water (200 μg/mL) was based on previous studies using CORT in the drinking water with the same concentration

which has previously been reported to result in 13.5 ± 1.5 mg daily CORT intake (Catalani et al., 2002; Catalani et al., 2000; Catalani et al., 1993). An average daily water consumption of 30-50 mL in rats would reflect ~10 mg/day of CORT exposure (200 µg/mL * 50 mL/day = 10mg/day; Ray & Behari, 1990; Stricker et al., 1997).

2.2.1. Pellet Implantation

Both CORT (#G-111, 200 mg, 21-day release) and placebo pellets (#C-111) were purchased from Innovative Research of America (Sarasota, FL, USA) and implanted as instructed by the manufacturer. Briefly, animals were anesthetized with isoflurane and the flow rate was maintained at a rate of 2.5-3% throughout the procedure. While the use of anesthesia may have been a potential limitation in these groups, it was required for the implantation surgery. Once deeply anesthetized, eye-lubricant was applied and animals received a subcutaneous injection of the non-steroidal anti-inflammatory analgesic, Carprofen (5 mg/kg). The surgical site was shaved and disinfected and a small incision (~2.5-3 cm) was made in the left shoulder area using aseptic techniques. The pellet was placed in a subcutaneous pocket in the lateral neck area in accordance with the manufacturer's instructions. The incision site was sealed using sterile wound clips. Animals were weighed before being returned to a clean cage. Post-surgical monitoring was conducted every 5 minutes for 45-60 minutes until the animal was mobile and alert. Monitoring continued at less frequent intervals after the animal was returned to the standard housing cage and once daily for 3 weeks after surgery. Triple antibiotic ointment was applied to the incision site daily for 3 days following surgery and as needed after the third day. Wound clips were removed 7 to 10 days after the surgical procedure under

brief isoflurane anesthesia. As the pellets should have been fully dissolved after the 23-day period, there was no attempt to surgically remove them at the end of this period.

2.2.2. Subcutaneous Injection

CORT (C2505, Sigma-Aldrich, St. Louis, MO, USA) was administered daily subcutaneously at a dose of 40 mg/kg. CORT was prepared fresh every 2 to 3 days by suspending 40 mg/ml CORT in sesame oil containing 10% ethanol. The control group received an injection of sesame oil vehicle alone. Ethanol was deemed unnecessary to incorporate into the control group, as it acts only to assist in dissolving the CORT into solution and would only evaporate if incorporated into the sesame oil vehicle. Injections were performed between 08:00 and 10:00 for 23 days. The dose of corticosterone was decreased over the last injection days (days 21-d23) so that animals received 30 mg/kg on day 21, 20 mg/kg on day 22, and 10 mg/kg on day 23 to mimic the wean-off period that would be experienced with the pellet implantation.

2.2.3. Drinking Water

CORT hemisuccinate (#Q1562-000, Steraloids, Newport, RI, USA) was dissolved at a dose of 200 µg/mL in drinking water. The use of CORT hemisuccinate was necessary as CORT is difficult to dissolve in water. The added salt group in CORT hemisuccinate makes it readily soluble in water and its efficacy has been demonstrated previously (Catalani et al., 2002; Catalani et al., 2000; Catalani et al., 1993). The CORT-water was administered in standard drinking bottles that were wrapped in aluminum foil due to the potential light sensitivity of the solution. The control group received regular tap water, also in bottles wrapped in aluminum foil to control for any effects of the novelty of aluminum foil impacting drinking behavior. All bottles were weighed daily and refilled

with fresh CORT or control water every 4 days or as needed. After 20 days, the dose of the CORT water was reduced to 150 µg/mL on day 21, 100 µg/mL on day 22, and 50 µg/mL on day 23 to mimic the wean-off period that would be expected with the pellet administration. On day 24, all injections were stopped and all animals were returned to normal tap water. We confirmed that all pellets were fully dissolved on the day of sacrifice.

2.3. Vaginal Lavage

All animals were lavaged on the days of testing to determine the estrus cycle phases. This data was used as a covariate in the analysis of the behavioral data to ensure that the phase of the estrus cycle did not affect behavior in the tasks. Vaginal lavage was performed by administering 3 to 4 drops of water into the vagina with an eyedropper and immediately reabsorbing the fluid with the same eyedropper. The sample was then placed on a glass microscope slide and stained with Cresyl Violet before the sample was dry. Once completely dry, each sample was examined under a microscope and the estrous cycle phase (metestrus, diestrus, proestrus, estrus) was determined based on the type of cells present as previously described (Marcondes et al., 2002; McLean et al., 2012). This data was later used to control for effects of phase of the estrous cycle on behavior in the data analysis.

2.4. Blood Collection and CORT Assay

Blood samples were collected early during the CORT administration on day 3 (d3), near the end of the administration period (d18), and a full day after being weaned off of the corticosterone (d25). Further, a blood sample was collected right after the first forced swim test session on day 20 to measure the CORT levels evoked by the test as an

acute stressor. Blood was collected by poking the saphenous vein with a sterile 25-gauge needle and allowing the sample to fall into a blood collection tube. The collection area was shaved the day before collection to ensure rapid blood collection that lasted less than 3 minutes from the time the experimenter first touched the animal's cage. CORT levels quickly rise in response to stress, so the 3-minute time limit ensures consistent collection points across animals with the stressful experience being the experimenter touching the cage. All basal samples were collected in the morning between 08:00 and 10:00 to ensure consistency in the CORT levels by being taken at the trough of the diurnal CORT rhythm and kept on ice. Blood samples were allowed to clot overnight and spun down at 8,000G for 10 minutes the next day. Serum was extracted and stored at -20C until further processing. Serum CORT was analyzed using a standard corticosterone EIA kit (Arbor Assays; Cat. # K014-H5, Ann Arbor, MI, USA) following the manufacturer's instruction.

2.5. Behavioral Testing

2.5.1. Forced Swim Test

Depressive-like behavior was assessed in the Porsolt Forced Swim Test (FST) on days 20, 21, and 29 as previously described (Porsolt et al., 1978). Briefly, animals were placed individually into a Plexiglas container (44cm deep, 30cm in diameter, Noldus Information Technology, Leesburg, VA, USA) filled with tap water at 25 ± 1C. The animal was placed in the tank for 15 minutes during the first session (d20) for habituation to the task, and 10 minutes for sessions 2 (d21) and 3 (d29). Session 2 was conducted at the end of the CORT administration period and session 3 was conducted after the washout from the CORT administration. All sessions were videotaped and later manually scored by an investigator blind to the animals' conditions for swimming, struggling, or

immobile behavior. The investigator would press a key on the keyboard at the start of each behavior and the Noldus computer software would total the duration of time spent in each behavior. Behaviors were defined as (1) swimming – movement of the forelimbs or hind limbs in a paddling fashion; (2) struggling – quick movements of the forelimbs with the front paws breaking the surface of the water; or (3) immobility – floating with no additional movement than what is required to stay afloat.

2.5.2. Open Field Test

The Open Field Test (OFT) was conducted on d19 to assess activity level of the animals. This was conducted in a circular arena with a diameter of 180cm and 50cm high walls (Jain et al., 2011). Movement was tracked during a 5-minute session using Noldus Ethovision® XT Version 7.0 software (Leesburg, VA, USA). The arena was cleaned with 70% ethanol between animals. Measures taken were central entries, defined as entries into the inner circle (30cm from the wall) and overall distance travelled.

2.6. Immunohistochemistry

To probe for effects of the different delivery methods on hippocampal neurogenesis, we collected brains from all animals on d30. To do this, animals were deeply anesthetized with sodium pentobarbital and perfused transcardially. Following extraction, brains were post-fixed in 4% paraformaldehyde for 24 hours and then transferred to 30% sucrose in phosphate buffered saline (PBS until sectioning. Coronal sections (40µm) were obtained using a freezing microtome (Microm HM 450, Thermo Scientific, Ann Arbor, MI, USA) and stored in antifreeze until staining. Every 10^{th} slice was processed for Doublecortin (DCX), an endogenous marker of new neurons. Slices were rinsed in 0.1M PBS overnight to remove traces of antifreeze. If not stated otherwise,

tissue was rinsed 3 times for 8 minutes each in 0.1M PBS between each step. Tissue was incubated in 0.3% hydrogen peroxide (H_2O_2) for 30 minutes, before being incubated in goat anti-DCX (0.1 M PBS, 0.5% Triton-X, 1:1000 goat anti-DCX) for 24 hours on a rotator at room temperature.

The following day, tissue was incubated in biotinylated rabbit anti-goat in 0.1M PBS (1:200 Vector Laboratories, Burlingame, CA, USA) for 90 minutes on a rotator. Afterward, tissue was incubated in ABC Complex (Vector Laboratories, Burlingame, CA, USA) for an hour on a rotator at room temperature, before reacting with 3,3'-daminobenzidine (DAB kit, Vector Laboratories, Burlingame, CA, USA) for 4 minutes using the provided Nickel from the DAB kit to enhance the staining. Tissue was mounted on glass microscope slides. Once dry, slides were dehydrated and then cover-slipped using Permount.

2.7. Cell Counting

Counting was performed by an experimenter blind to the animal's group designation. DCX-positive cells were counted at 400x magnification. Due to the potential functional differences between the dorsal and ventral hippocampus, we examined the density of cells in both the dorsal and ventral regions separately For this, all slices on which the ventral portion of the dentate gyrus (DG) at the bottom of the slice was obviously present (-5.2 mm to -6.7 mm below Bregma according to the dorso–ventral coordinates of Paxinos and Watson (2005)) were considered "ventral," while all slices rostral to this section were considered "dorsal," (-1.8 mm to -5.2 mm below Bregma) as previously described (Brummelte & Galea, 2010). The area of the dentate gyrus was measured using image analysis software (ImageJ, NIH, Bethesda, MD, USA), and the

total volume for the dorsal and ventral portion of the DG was estimated using Cavalieri's principle (Gundersen et al., 1978), i.e. by multiplying the total area measured by the distance between measured sections (400 μm). Densities were calculated by dividing the total number of cells for each area (estimated by multiplying the total number counted by 10, as we counted every 10th slice) by the volume of that area (dorsal or ventral).

2.8. Data Analysis

Repeated measures analysis of variance (ANOVA) were used to analyze body weight (d1, d6, d13, d20, and d23 of CORT administration as within-subject factors), immobility in the FST (sessions 2 and 3 as within subject factors), volume of the dentate gyrus, and density of DCX-positive cells (dorsal and ventral as within subject factors). Treatment (control, CORT) and administration route (injection, pellet, drinking water) were treated as the between-subjects factors. Further, factorial ANOVAs with treatment (control, CORT) and administration route (injection, pellet, drinking water) as the between-subjects factors were performed to analyze body weight on d30, serum CORT levels taken on d3, d18, d25, and after the FST on d20, and open field test behaviors for anxiety-like behavior. After initial analysis, estrous-cycle phase was added as a covariate to the analysis to test whether it had an impact on the reported group differences. All post-hoc tests utilized Fisher LSD. Data was analyzed using STATISTICA (Statsoft, Tulsa, OK, USA).

3. RESULTS

3.1 Body Weight

A factorial repeated-measures ANOVA revealed no main effect of CORT treatment or route ($p > 0.11$, for both). There was, however, a significant interaction of

CORT treatment and administration route ($F(2,26) = 3.95$, $p < 0.03$), as well as an interaction of days and CORT treatment ($p < 0.001$), and days and administration route ($p < 0.04$). An LSD post-hoc test further revealed that these interaction effects were due to the CORT injection group, which had significantly lower body weight compared to their control group ($p < 0.004$) and compared to the other 2 CORT treatment groups ($p < 0.04$). Looking at the post-hoc results by day revealed that this lower body weight began on d6 ($p < 0.04$) of CORT administration and continued throughout the rest of the experiment with the difference between the groups increasing towards d23 ($p < 0.001$) (see Fig. 3A).

Body weight on the day of perfusions (d30) was analyzed with a factorial ANOVA, as the wash-out period between d24 and d30 would disqualify this measure from being incorporated in the repeated-measures ANOVA. This analysis specifically assessed whether or not the difference in body weight remained constant between groups after the wash-out period. The factorial ANOVA for body weight on d30 revealed a significant CORT by administration route interaction effect ($F(2,23) = 4.84$, $p < 0.02$). A LSD post-hoc test revealed that this effect was driven by the CORT injection group, which still had lower body weight compared to all other groups ($p < 0.04$), except the water control group ($p < 0.06$) (see Fig. 3B).

3.1.1. Water Intake

There was no difference in average daily water intake between the 2 groups receiving either normal tap water or CORT in their drinking water ($p < 0.38$, No CORT: 39.06mL ± 1.5mL; CORT: 40.97mL ± 1.4mL). This amount of daily water consumption means that animals received only ~8mg of CORT daily (40ml* 200µg/ml = 8mg).

3.2 Serum CORT Levels

Serum CORT levels were analyzed separately for each collection time point (d3, d18, and d25) with factorial ANOVAs, as day 25 was after the wash-out period, and due to the fact that CORT levels can vary substantially between days due to environmental circumstances. The use of factorial ANOVAs aided in reducing any confounds created by intermittent behavioral testing on CORT serum levels. On d3 there was a significant main effect of treatment ($F(1,28) = 150.2$; $p < 0.001$) and route of administration ($F(2,28) = 85.1$, $p < 0.001$), and an interaction effect of CORT and route ($F(2,28) = 80.8$, $p < 0.001$). The post-hoc test revealed that the CORT pellet and injection groups had significantly elevated levels compared to their own and all other vehicle groups (p's < 0.001). Interestingly, the CORT-water group had similar levels to all the control groups ($p < 0.37$).

On d18 there was a significant effect of route ($F(2,22) = 11.76$, $p < 0.001$) and treatment ($F(1, 22) = 9.23$, $p < 0.006$), and an interaction effect of route and CORT treatment ($F(2,22) = 14.53$, $p < 0.001$). The post-hoc test showed that only the injection group had significantly elevated CORT levels compared to all control groups (p's < 0.001).

On d25, one full day after the last reduced dose CORT administration, there was a significant interaction effect of route and CORT treatment ($F(2,23) = 4.22$, $p < 0.027$), but no significant main effect. The post-hoc test revealed that the injection group had still slightly higher CORT levels compared to their control group ($p < 0.01$) (See Fig. 4).

CORT levels were analyzed in response to the stress of the first swim test session (d20). The factorial ANOVA revealed a significant main effect of treatment ($F(1,26) = 19.5$, $p < 0.001$) and route of administration ($F(2,26) = 4.1$, $p < 0.028$), but no interaction

effect. Post-hoc tests showed that CORT-treated animals had significantly lower stress CORT levels than their controls (pellet: ($p < 0.04$), water: ($p < 0.01$), injection: ($p < 0.007$)). We repeated the analysis and included the estrous cycle phase for the first FST session to control for potential variation in stress CORT levels due to the estrous phase, however, the estrous cycle phase was a non-significant covariate ($p < 0.87$) and did not impact the significant effects (See Fig. 5).

3.3 Forced Swim Test Behavior

Animals were tested in the FST at the end of the CORT administration period (d20/21) and again 6 days after weaning off the CORT (d29). A repeated measures ANOVA with time spent immobile in FST 2 and FST 3 as the within subject factor revealed a significant CORT by route of administration interaction effect ($F(2,26) = 9.5$, $p < 0.001$) and a significant effect of the test session ($F(1,26) = 11.3$, $p < 0.002$), with animals being generally more immobile in the third compared to the second FST session. The post-hoc test further showed that CORT injection animals spent significantly more time immobile compared to their vehicle control ($p < 0.001$). Further, the CORT in drinking water group spent significantly less time immobile compared to their control group ($p < 0.02$). However, this may be an alpha error as the water control group had significantly higher immobility scores compared to the other 2 control groups ($p < 0.04$ and 0.006, respectively; See Fig. 6).

To account for variability in FST performance due to estrous-cycle phase, we repeated the analysis separately for each session while including the estrous-phase as a co-variant. The estrous cycle was a non-significant contributor to FST 3 performance ($p <$

0.98). However, estrous cycle was a significant co-variable in the FST 2 immobility scores analysis ($p < 0.002$), but with no effect on the main outcomes.

3.4. Open Field Test

There was no significant difference for treatment or route of administration for the distance travelled in the open field between the groups (all p's > 0.19), nor was there a difference in the time spent in the center (all p's > 0.07; See Table 1).

3.5. Neurogenesis in the Dentate Gyrus

A repeated-measures ANOVA (controlling for the estrous cycle) revealed no significant main effects of treatment ($p < 0.90$), route ($p < 0.69$), or their interaction ($p < 0.21$), and no effect by area (dorsal versus ventral; all p's > 0.21) for the density of DCX-positive cells (Table 2). Also, the estrous cycle did not significantly impact these results ($p < 0.68$) as almost 80% of the animals were either in estrus or pro-estrus at the time of sacrifice.

4. DISCUSSION

The present study is the first to compare methods of administering exogenous CORT in female rodents on their efficacy in producing a depressive-like phenotype. It was found that CORT via injection alone was able to produce an increased level of CORT in serum, decreased body weight, and increased immobility in the forced swim test. None of the methods investigated impacted hippocampal neurogenesis. Results from the present study indicate that body weight, CORT serum levels, and FST behavior were very different depending on the method used for CORT administration. Female rats that were administered CORT through injections weighed less, were more immobile in the FST, and had the highest CORT serum levels, which is consistent with previous

publications (Brummelte & Galea, 2010; Gregus et al., 2005). Conversely, animals that received CORT in their drinking water or through subcutaneously inserted 21-day release pellets showed no significant weight loss or increased immobility in the FST. Only the pellet group had transiently increased serum levels of CORT that returned back to normal on day 18.

4.1 Route of CORT Administration Affects Body Weight

Starting on the 6th day of CORT administration, injection animals weighed less than their controls and this difference remained significant until the last day of the experiment, even after the animals were weaned off the CORT. This effect is consistent with previous reports on reduced body weight after CORT injections (Brummelte et al., 2006; Gregus et al., 2005). However, we did not observe significant reductions in body weight in the CORT pellet or drinking water group. Previous studies using CORT in the drinking water found that male animals receiving CORT via drinking water at 40 and 100 μg/mL displayed significantly less weight gain as compared to control groups while animals receiving 400 μg/mL showed significant weight loss compared to control animals (Donner et al., 2012; Pekary et al., 2008). Similarly, several previous studies using CORT pellets have reported some reductions in body weight (Claflin et al., 2005). This apparent discrepancy with our results may be due to the fact that we used only females in our study. Females are known to have naturally higher circulating CORT levels and adult females do not show the continued significant weight increases observed in males. Thus, CORT delivered by drinking water to the female rats in our study may not have been as effective in altering body weight as it is males. For the pellet group, there was a trend for reduced body weight on day 6 which became significant if the pellet

group was analyzed separately without the injection group included in the analysis. It is not surprising that this effect disappears after day 6, as it seems that all CORT may have been released from the pellet too early.

The larger effect of CORT on body weight when given via injection correlates with the higher serum levels observed in this group. The ability of stress to exert physiological effects through the HPA axis could result in this observed reduced body weight and decreased weight gain. Interestingly, s.c. injections of 10mg/kg led to significantly elevated CORT serum levels and reduced body weight in males in a previous investigation, but non-significant changes in females in the present investigation, again suggesting that females are less sensitive to lower levels of CORT (Brummelte & Galea, 2010). This highlights the importance of taking sex differences into account as many studies have investigated the effects of chronic CORT exposure in only males (Bush et al., 2003; Gregus et al., 2005).

4.2 Different Administration Methods Lead to Varying CORT Serum Levels

Rats in the injection group had elevated CORT levels on days 3 and 18 of the experiment, while animals in the drinking water group did not reveal any alterations in basal CORT levels during the administration period. Animals that received CORT pellets had higher CORT serum levels 3 days after the implantation, but levels were back to baseline on day 18. All doses were originally calculated to reflect ~10mg of CORT per day; however, there were certain limitations on ensuring that all groups received exactly the same amount of CORT. The pellet animals received ~9.5mg per day if the pellet released CORT at a constant rate, while the drinking water group ended up taking in ~8mg a day based on ~40mL of water intake per day. Further, with the 21-day release

pellets and CORT in the drinking water, it is impossible to adjust the dose of CORT given for weight changes in the animals during the experiment. All but the CORT injection group continued to gain weight throughout the experiment, so that by the last day of CORT administration animals in these 2 groups may have received less CORT per body weight than they received in the beginning. These slightly lower doses in the pellet and water group and the unintentional but unavoidable tapering off of the dose due to increasing body weight, may partly explain why these animals had lower serum concentrations and seemed better adjusted to the CORT treatment.

Our study was designed to compare the different administration methods of CORT exposure as well as compare our results to previously used doses in other studies investigating the developmental effect of CORT exposure (Catalani et al., 2002; Catalani et al., 2000). These papers reported serum levels of 4.3 ± 0.5 to 9.5 ±1.8µg/100mL (equivalent to 43-95ng/mL) after 200µgl/mL in the drinking water in postpartum female rats (Catalani et al., 1993). These levels are within the normal range of basal CORT serum levels for adult females, and thus in line with our findings in the present study of similar CORT levels in control and CORT water females. This effect was also displayed in male rats at doses varying from 25µg/L to 400µg/L, where CORT levels in serum were only elevated at the 400µg/L, but not at 25µg/L (Pekary et al., 2008). Other studies using 400µg/mL of CORT in drinking water for 21 days in males reported significant increases in morning CORT levels, but not beyond the natural evening peak in CORT (Magarinos et al., 1998). These studies collectively are in agreement with our finding in the present study that CORT administered via drinking water did not significantly increase CORT levels in serum. It should be noted that the drinking water group in the present study

received CORT hemisuccinate, instead of CORT, which does not go into a water-based solution as easily. The use of CORT hemisuccinate versus CORT may have also contributed to the lower serum levels observed in the present group. Also, the timing of blood collection (early morning, in this study) compared to the peak drinking time of the animals (beginning of dark cycle) may explain why increases in CORT serum levels are not consistently found across studies using water drinking administration.

The pellet group displays elevated CORT serum levels on day 3, but these levels were less than half the CORT serum levels of the injection group. The fact that the CORT serum levels were back to normal by day 18 indicates that the pellets may have released its contents earlier than the 21-day period. This idea is in line with previous studies that have found similar effects with these pellets releasing most of their CORT within a few days rather than over the period of 21 days (Claflin et al., 2014; Claflin et al., 2005; Kraszpulski, et al., 2012; LaPlante & Tomaszyki, 2012). However, it is possible that these results may be specific to the brand of pellet that was used and other pellets may be more reliable in their delivery.

Interestingly, all 3 CORT groups revealed lower stress-induced CORT levels after the FST on day 20 as compared to the control groups. This indicates that even the groups that did not show measurable increases in baseline CORT levels experienced alterations in HPA axis functioning in response to the exogenous CORT administrations. The fact that CORT levels were lower may suggest that the animals down-regulated their endogenous CORT production as a consequence of the excess amount of exogenous CORT circulating in their systems. All groups were still able to mount a stress-induced response, though the magnitude was lower in the injection group, probably due to the

already high baseline levels. These results agree with previous research showing that high levels of CORT are able to alter HPA axis function (Dallman et al., 1994). For instance, exposing rats to a series of acute stressors for 14 days resulted in reduced HPA response following acute immobilization stress on day 15 (Roth et al., 2012).

One limitation of our study is that we did not measure levels of Corticosteroid-binding globulin (CBG). While only free CORT is biologically active and can induce effects in the body, serum CORT levels reflect the total amount of CORT (i.e., free and CBG-bound CORT). We cannot exclude the possibility that the CORT-treated animals up-regulated their CBG levels to bind most of the excessive CORT. However, the fact that we did observe behavioral (FST) and body weight changes in at least the injection group, suggests that the elevated total levels of CORT did have an impact on the animals' brain and behavior. Another potential limitation is that only one dose of CORT was used in this study. However, this dose has been frequently and successfully used in the literature, so we don't think this is a major limitation in this study. Another limitation in this study is the inconsistent delivery of CORT in the CORT pellet group. It seems that the pellets released too much CORT too soon and had stopped working before the end of the experiment, as evidenced by the CORT levels in serum which were only significant at the first time point. It is also noteworthy that the rats receiving the CORT placebo pellets developed fluid-filled seromas around the site of the pellet which may have impacted their body weight. A valuable future direction may include repeating the study with the inclusion of males or a different brand of pellets to test the theory that some of these methods are effective in males at similar doses, but less so in females.

4.3. Only Injections of CORT Resulted in Increased Immobility in the Forced Swim Test

High levels of CORT have repeatedly been shown to induce depressive-like behavior in the FST in males and females (Galea et al., 2008; Gregus et al., 2005; Hill et al., 2003). However, this was only evident if using sufficiently high doses. For instance, 10mg/kg of CORT s.c. injections into postpartum dams did not increase immobility levels in female rats (Brummelte et al., 2012) whereas 20mg/kg per day for 20 days increased immobility in males, but not female rats (Hill et al., 2003).

Similar results to this study have been previously reported for chronic CORT administered via pellet implantation. Male mice implanted with CORT pellets showed an increase in immobility in the FST 7 days after implantation, but this effect was abolished by days 14 and 21 (Murray et al., 2008). Studies administering CORT via drinking water demonstrated an increase in immobility in the FST at doses of 100 and 400μg/mL after 18 days of CORT treatment. However, this study utilized exclusively male rats and these results may reflect sex specific differences in CORT-sensitivity and behavior in the FST (Donner et al., 2012). One study using 80ug/ml CORT in drinking water of female mice failed to demonstrate differences in immobility in the FST based on CORT administration, further emphasizing the importance of assessing females in the FST (Zoratto et al., 2011). Thus, it is conceivable that females need higher levels of circulating CORT before their FST performance is affected, which may explain why we only saw an effect in our injection group that had significantly higher serum concentrations than the other groups.

4.4. Chronic CORT Administration Did Not Alter Neurogenesis Levels

A growing body of research indicates that heightened levels of stress or CORT in rodents can change levels of neurogenesis in the brain (Brummelte & Galea, 2010; Mirescu & Gould, 2006; Wong & Herbert, 2006). For example, previous work has shown that 40mg/kg of CORT led to slightly fewer Ki67-positive and DCX-positive cells in the ventral dentate gyrus of females rats, while the same dose led to fewer DCX-positive cells in the ventral and dorsal hippocampus of male rats (Brummelte & Galea, 2010). Surprisingly, none of the CORT-treated groups in the current study showed any alterations in the number of DCX-positive cells in the dentate gyrus. However, it should be mentioned that the previous study measures were taken immediately after the last day of injections while in the current study, females were weaned off the CORT for 3 days and sacrificed a week after the last reduced dose injection. The wean-off period in the present study was deemed necessary as the subcutaneous pellet would have a slow decay in CORT release and this effect needed to be mimicked in the other groups for consistency. Thus, neurogenesis levels may have recovered and returned to normal by the point we took measurements. In a previous study a lower dose of CORT via s.c. injection (10mg/kg) did not result in altered cell proliferation or neurogenesis levels (Brummelte & Galea, 2010) consistent with our current finding of no difference in the water or pellet group. However, there may be other changes to the hippocampal structure as others found that 21 days of 400μg/mL of CORT in the drinking water led to reduced apical dendrites in the CA3 region and reduced number of DCX-positive cells in the piriform cortex layer II (Magarinos et al., 1998; Nacher et al., 2004).

Other studies focusing on the effects of CORT administration via pellet implantation on hippocampal neurogenesis have demonstrated that a 100mg CORT pellet was able to significantly reduce hippocampal neurogenesis after 21 days of implantation, which remained significant after a 7-day washout on day 28 (Murray et al., 2008). This study did measure CORT levels in serum at day 21, and saw a significant effect of CORT pellet implantation on CORT in serum, and also measured CORT levels at day 28 after a seven-day washout period where this effect was abolished. These results may differ from those of the present study due to the fact that in our study, CORT serum levels were not significantly elevated 18 days after pellet implantation. Thus, our animals had plenty of time to regain normal levels of neurogenesis by day 30 when they were sacrificed. The study (Murray et al., 2008) demonstrating significant differences in levels of neurogenesis may have utilized pellets that did not release their content entirely before the end of the 21-day period, as is theorized ours may have.

Significant sex differences exist in the effects of CORT administration or stress on neurogenesis levels. For example, adult males show diminished cell survival in response to chronic stress, whereas the same stressors in females demonstrate enhanced cell survival, further demonstrating the sex-specific effects of stress on hippocampal neurogenesis in adulthood (Kuipers et al., 2006). These studies suggest that we cannot generalize findings from males to females and need to pay particular attention to the unique physiology of females that may result in different or opposite effects of stress and corticosterone.

4.5 Considerations for Choosing a CORT Administration Method

Overall, CORT pellets and water administration were less controllable methods in regards to managing the CORT dose, but even if the final dose in these 2 groups was slightly lower than anticipated (due to less water intake or weight gain), it does not account for the drastic difference in CORT serum levels between these groups and the CORT injection group. More likely, the different pharmacokinetics involved with each administration method may play a role. The absorption and distribution of CORT from an oral route will be much slower and probably less effective compared to subcutaneous administrations, which may explain why the water group seems to be the least affected by the CORT treatment. Further, the timing and the natural diurnal rhythm of CORT are essential considerations. While the pellets provide a supposedly constant supply of exogenous CORT, the injections deliver one acute burst in increased CORT levels per day, and the drinking water dose results in varying amounts depending on the water consumption during a given day. Considering that the artificially increased amount of CORT in the system will lead to some form of HPA axis reprogramming or adjustment, it is conceivable that the HPA axis reacts to each delivery method differently.

Taken together, our least preferred administration method were the subcutaneous pellets, as they were apparently not reliable in their release pattern. This could however have been specific to the brand of pellet we used, as others have reported no such issues (Claflin et al., 2014, Claflin et al., 2005). We also had some trouble with wound healing in 3 animals of the CORT pellet group, and all of the placebo pellet rats developed a fluid-filled pocket around the implant several days after the implantation, which led to the exclusion of some of these animals from the study. None of the animals had infected incision sites or any indication that this was caused by the surgical procedure according

the veterinarian consult, who further confirmed that the complications were unlikely due to surgical complications, as only the placebo animals developed seromas, while the CORT pellet group had trouble with wound healing. The extra fluid may also explain the temporary weight gain seen in the placebo pellet animals between days 6 and 9. Besides that, the placebo pellet animals looked very similar to the other 2 control groups in most measures, suggesting no significant impact of these complications on our measures.

To our knowledge, this is the first study to compare CORT levels by administration route in female rodents. The results confirm previous studies that only high CORT levels in serum reduce FST behavior and body weight, but neurogenesis levels seem to recover after a washout period. This study enables us to better understand how CORT levels are impacted by administration route, and we plan to use this to further investigate the effects of CORT administration during pregnancy on the development of the offspring. This study also indicates the necessity of verifying CORT levels in the system after administration regardless of administration route.

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ABSTRACT

EFFECTIVENESS OF DIFFERENT CORTICOSTERONE ADMINISTRATION METHODS TO ALTER CORTICOSTERONE LEVELS IN SERUM AND DEPRESSIVE-LIKE BEHAVIOR IN FEMALE RATS

by

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August 2015

Advisor: Dr. Susanne Brummelte

Major: Psychology -- Behavioral and Cognitive Neuroscience

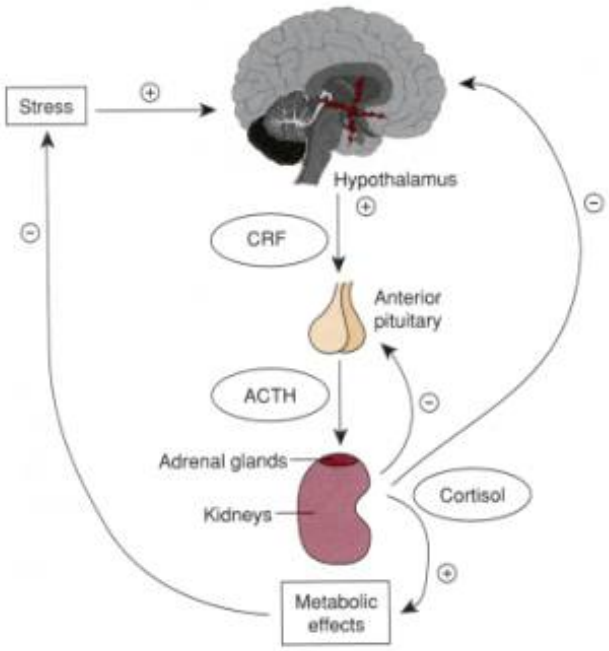
Degree: Master of Arts

There is wide variation in the current literature on rodent models of high levels of chronic stress. This study aims to reduce these discrepancies by investigating the effects of 3 different administration methods of corticosterone (CORT; the endogenous stress hormone in rodents) and the differential effects it has in the female sex. The majority of studies utilize male rodents, while research in female rodents is largely under-investigated. This study will utilize female rats and 3 different administration routes: a) subcutaneous implantation of a CORT pellet, b) CORT in the drinking water, and c) CORT by a daily subcutaneous injection, and assess its impacts on CORT levels in serum, depressive-like behavior, and levels of hippocampal neurogenesis. This study will enable researchers to better understand the effects of chronic CORT exposure in female rodents, as well as reduce the variation in current literature on the effects of CORT based on administration route.

AUTOBIOGRAPHICAL STATEMENT

Jennifer Kott is currently a student in Wayne State University's Psychology department with a major in Behavioral and Cognitive Neuroscience. She is minoring in Psychopharmacology. She got her Bachelor of Science degree in Psychology from Michigan State University in 2011. She is currently working under the supervision of Dr. Susanne Brummelte to further her research and academic career studying the effects of adverse early life experience on development.

Figure 1



Source: http://soundersleep.com/hpa.php

Figure 1. Diagram of the hypothalamic-adrenal-pituitary (HPA) axis.

Figure 2

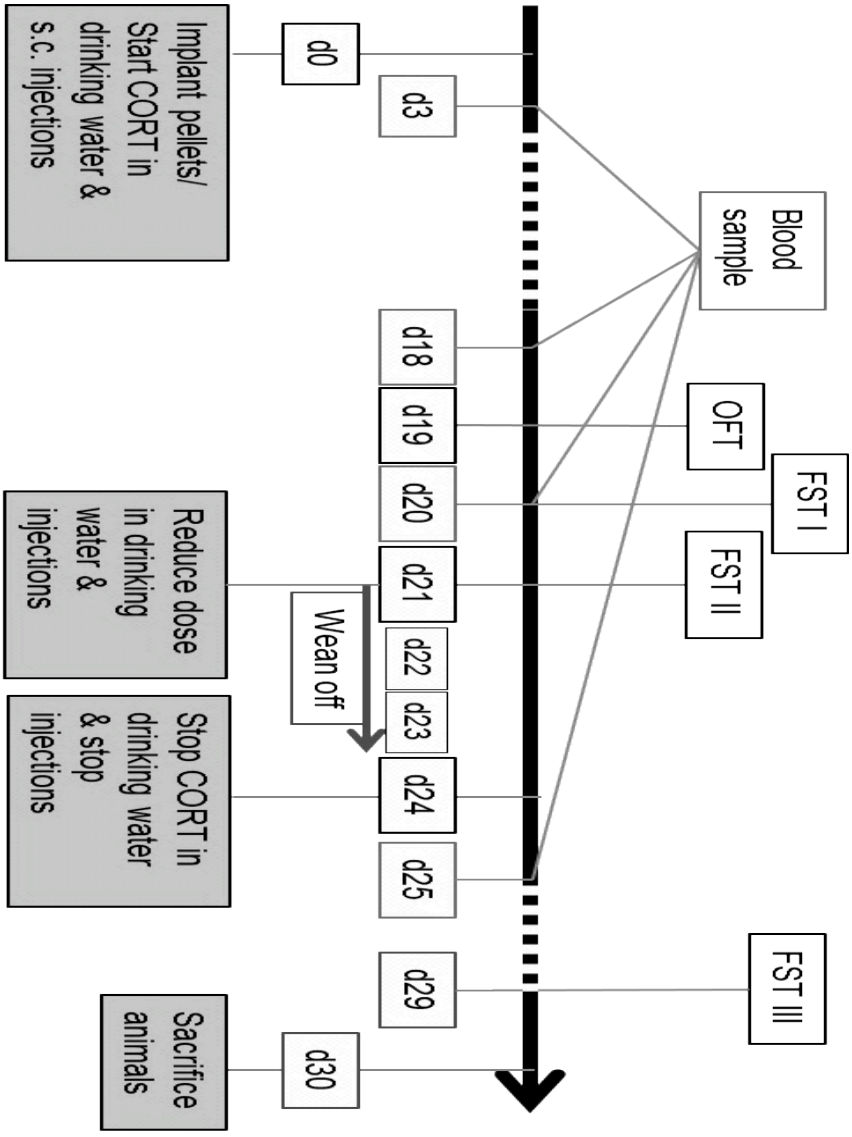


Figure 2. Overview of the experimental procedures. CORT will be administered for 21 days followed by a wean-off period before sacrifice. Behavioral testing including the forced swim test and the open field test will occur throughout the experiment, and blood will be collected at several time points. OFT: Open Field Test, FST: Forced Swim Test, CORT: corticosterone

Figure 3

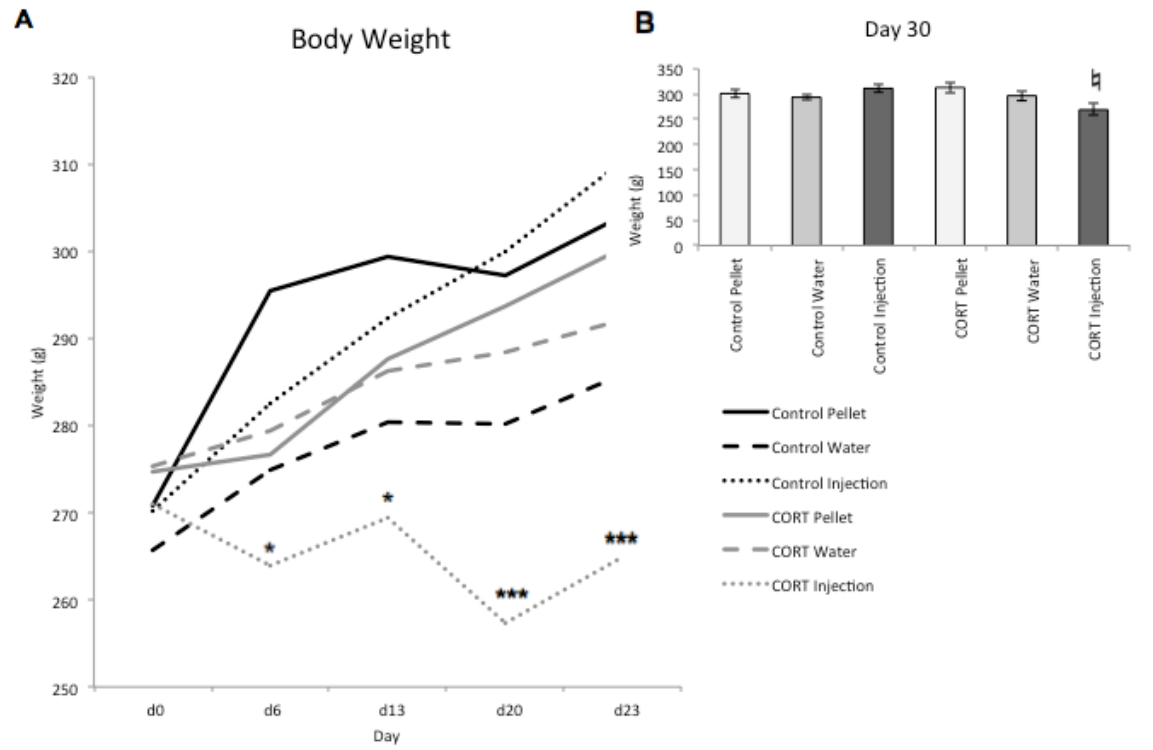


Figure 3. (A) Animals receiving CORT injections had significantly reduced body weight compared to all other groups beginning on the 6th day of treatment and continuing throughout the experiment. (B) Animals receiving CORT injections still had significantly reduced body weight compared to all groups except for the water control group on the day of perfusions $*p < 0.05$, $***p < 0.001$ compared to all other groups. $\natural p < 0.05$ compared to all groups except water control group.

Figure 4

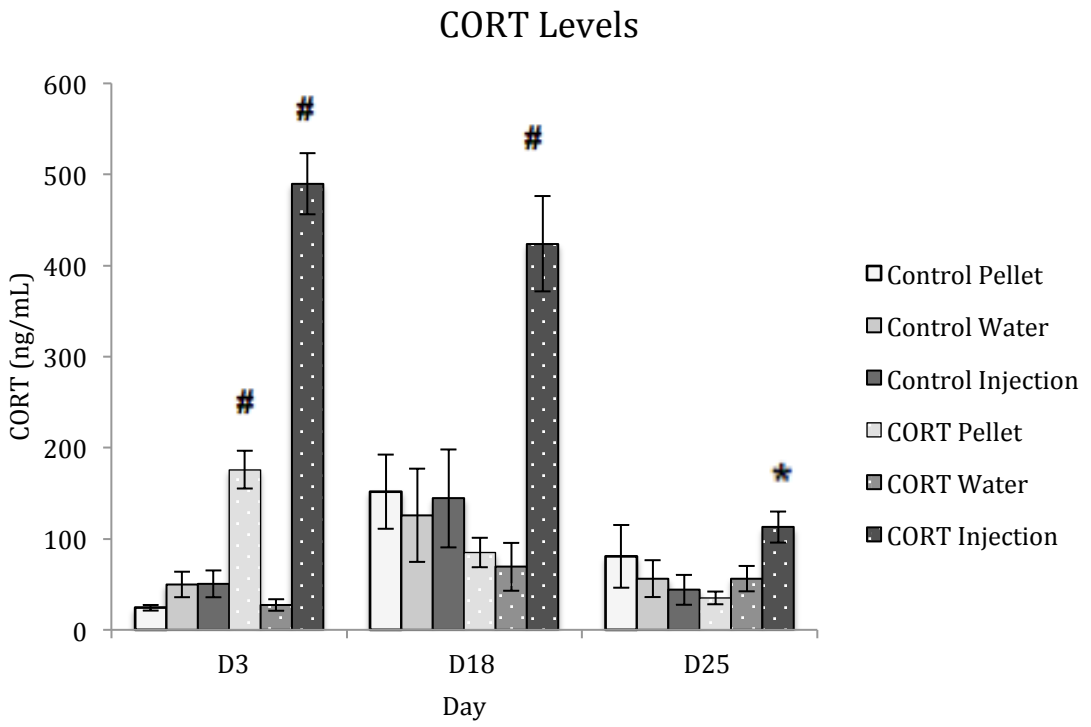


Figure 4. Serum CORT levels at different time points throughout the experiment

On day 3 of CORT administration, the CORT pellet and CORT injection group had significantly elevated levels of CORT compared to all vehicle groups. On day 18, this effect held true for only the CORT injection group. On day 25 of CORT administration, only the CORT injection group displayed significantly elevated levels of CORT compared to its own control group. #$p < 0.001$, *$p < 0.01$ compared to its own control group.

Figure 5

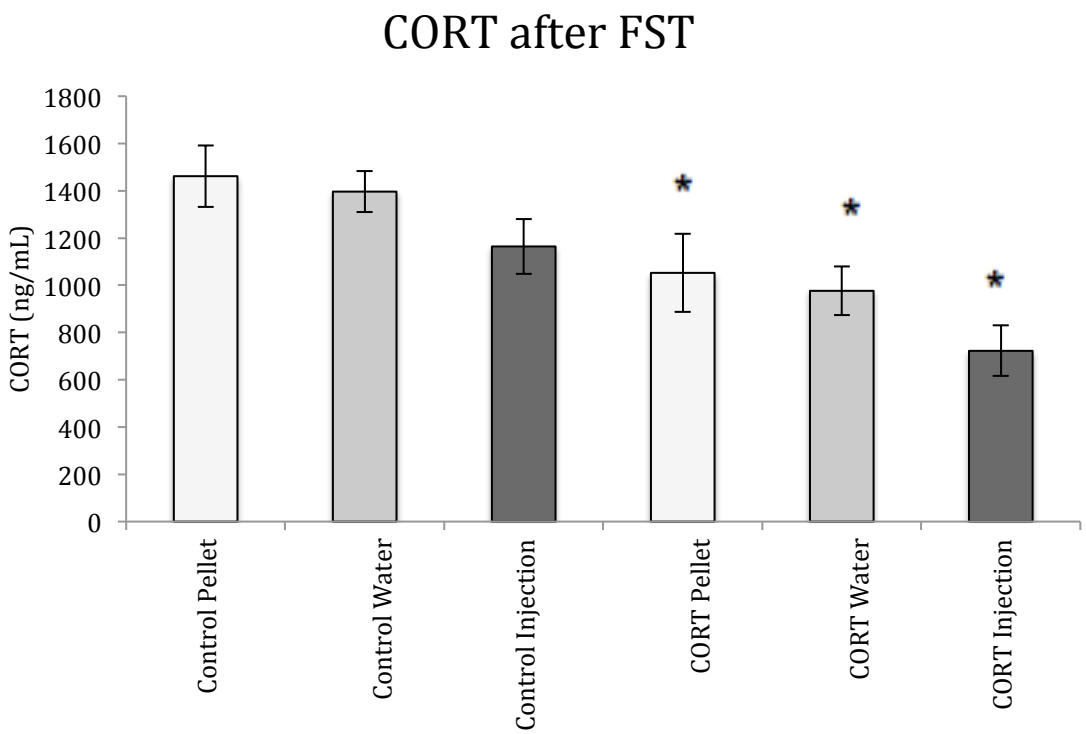


Figure 5. Shown are the CORT levels on d20 after the FST. CORT levels were significantly lower in each CORT-treated group compared to its respective control group *$p < 0.05$.

Figure 6

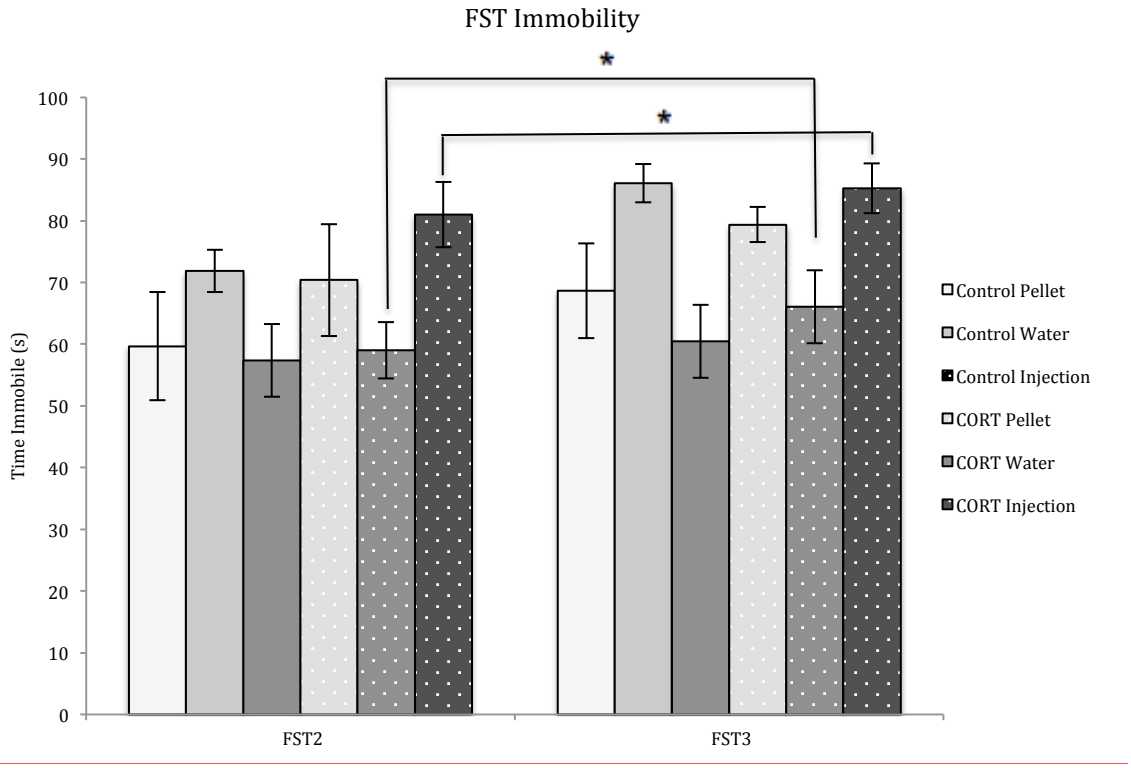


Figure 6. In both FST sessions, animals that received CORT via injection spent significantly more time immobile compared to their control group. Animals that were administered CORT in the drinking water spent significantly less time immobile compared to their control group. *$p < 0.05$ compared to their own control groups.

Table 1

Open Field Test

	Total Distance (cm)	Duration in Center (s)
Control Pellet	4440.26 ± 504.21	38.17 ± 11.37
Control Water	4128.36 ± 461.29	33.23 ± 8.58
Control Injection	4473.52 ± 292.03	28.35 ± 4.56
CORT Pellet	5141.64 ± 355.83	55.73 ± 13.53
CORT Water	4701.78 ± 352.49	28.06 ± 5.73
CORT Injection	4545.02 ± 345.66	25.64 ± 6.15

Mean ± S.E.M.

Table 1. Shown are the effects of CORT on the open field test. No significant differences were observed in the distance travelled or time spent in center between treatment groups or route of transmission.

Table 2

Hippocampal Neurogenesis (DCX Density)

	Dorsal	Ventral
Control Pellet	8199.29 ± 924.252	6255.391 ± 972.212
Control Water	8989.381 ± 1044.423	6694.054 ± 944.081
Control Injection	7693.381 ± 760.394	5777.204 ± 591.506
CORT Pellet	8757.175 ± 668.111	8398.541 ± 475.372
CORT Water	7518.803 ± 702.397	5191.190 ± 1078.961
CORT Injection	8519.247 ± 769.592	5303.460 ± 582.210

Mean ± S.E.M.

Table 2. Shown are the effects of CORT on hippocampal neurogenesis. No significant differences were observed by treatment, route, the interaction of treatment and route, or difference between areas in hippocampal neurogenesis.