2012

Effects of Elevated Glucocorticoid Levels on Dentate Gyrus Development

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EFFECTS OF ELEVATED GLUCOCORTICOID LEVELS

ON DENTATE GYRUS DEVELOPMENT

A thesis submitted in partial fulfillment of the

requirements for the degree of

master of science

By

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B.S., Wright State University, 2010

2012

Wright State University

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June 11, 2012

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ABSTRACT

Vallandingham, Zachary Dale. M.S., Anatomy Program, Department of Neuroscience, Cell Biology and Physiology, Wright State University, 2012. Effects of elevated glucocorticoids on dentate gyrus development.

Exogenous glucocorticoids are commonly used in modern medications and animal studies examining the effects of glucocorticoids on the developing brain report inconsistent results. Recent reports have questioned the reliability of available drug delivery methods in mice (Herrmann et al., 2009). In our laboratory, variable behavioral results using trace eyeblink conditioning (EBC) suggest that we may be having similar problems delivering glucoroticoids to developing rat pups (Claflin et al., 2005, 2011). Subcutaneous pellets and osmotic minipumps resulted in impaired learning during trace eyeblink conditioning whereas subcutaneous injection of corticosterone (CORT) resulted in facilitation of learning on the same task. One of the possible mechanisms for CORT-induced cognitive effects may be changes to hippocampal development, specifically neurogenesis. We examined potential differences in hippocampal neurogenesis for rats subjected to elevated CORT levels as in the aforementioned behavioral studies. For the present report, exogenous administration of CORT was achieved using subcutaneous pellets. CORT- treated males showed significantly less neurogenesis than the CORT treated females. Furthermore, males of the control group showed significantly more neurogenesis than the females.

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I. INTRODUCTION

The stress response in any vertebrate animal is an integral part of daily survival involving many complex events that serve to protect the body and eventually return it to homeostasis. The body's response to stress involves multiple systems including the nervous and endocrine systems. The nervous system is the main system involved with the immediate response (<1 minute post-stress) that coordinates what is known as the "fight or flight" response (Sapolsky, Romero, & Munck, 2000; Ulrich-Lai and Herman, 2009). During this time, an increase in catecholamines such as epinephrine and norepherine is released by the sympathetic nervous system in an effort to prepare the body for the task at hand. Increased heart rate, vasoconstriction, immune-activation, and energy mobilization are the body's primary actions to keep the organism alive. The endocrine system is also activated at this time by the release of corticotropin-releasing hormone (CRH) from the hypothalamus, and if the stress persists CRH will activate a new set of structures that coordinate the body's response to a more chronic stress by releasing steroid hormones (Sapolsky et al., 2000). There are three primary structures that integrate the endocrine stress response. The hypothalamus, the anterior portion of the pituitary gland, and the adrenal glands that reside on top of the kidneys, all of which act together to form what is known as the hypothalamo-pituitary-adrenal, or HPA, axis. The HPA axis is a focal point for regulatory and feedback mechanisms of the stress response over a longer period of time (>10 min). HPA axis activation induces hormone level changes that act to suppress the body's response to stress. A detailed account of HPA axis function starts with CRH release from the paraventricular nucleus of the hypothalamus (Figure 1). From there, CRH binds to receptors on the anterior pituitary

causing a release of adrenocorticotropic hormone (ACTH). ACTH is released into the bloodstream with its primary place of action at the zona fasciculata within the cortex of the adrenal glands, which in turn causes release of glucocorticoids into the bloodstream (As reviewed by Sapolsky et al., 2000; Ulrich-Lai and Herman, 2009). Glucocorticoids reach peak activation about one hour post stress. Glucocorticoids are an important class of steroid hormones whose partial list of actions includes anti-inflammatory mechanisms, mediation of the cardiovascular response, and fluid volume control in an effort to protect the body from the prolonged stress response (Sapolsky et al., 2000).

Exogenous glucocorticoid administration in the medical realm has been implemented for years in the treatment of ailments like allergies, asthma, and arthritis. However, protracted elevation of glucocorticoids has long been known to produce psychiatric and cognitive changes in animals and humans (Wolf, 2003). Patients undergoing chronic regimens with drugs such as prednisone have been shown to display depression-and mania-like symptoms (Naber, Sand, & Heigl, 1996). Studies have shown that elevated glucocorticoids, although providing relief for medical conditions also impair memory function in adults and children (Bender, Lerner, & Kollasch, 1988; Brown, Beard, Frol, & Rush, 2006). In one human study, healthy individuals given exogenous cortisol were asked to perform a declarative memory task. The results showed a significant decline in performance after administration of the drug (Newcomer et al., 1999). Alternatively, rats exposed to chronic restraint stress, which presumably raises circulating glucocorticoid levels, demonstrated spatial memory impairment during Y-maze testing, a hippocampal dependent task (Conrad, Galea, Kuroda, & McEwen, 1996). More recently, glucocorticoids have also been shown to have an enhancing effect within

the central nervous system. Elevated cortisol levels in response to stress and pharmacological manipulations have been shown to facilitate emotional as well as non-emotional memory formation in adults, respectively (Buchanan and Lovallo, 2001; Lupien, Wilkinson, Briere, Ng Ying Kin, Meaney, & Nair, 2002). Together, these results and others suggest an inverted U relationship between cognitive performance and glucocorticoid levels in mammals; very low or very high levels produce cognitive deficits whereas a moderate amount can enhance the learning process (Diamond, Bennett, Fleshner, & Rose, 1992; Mateo, 2008). Moreover, time of exposure to glucocorticoids is just as important as the concentration: acute exposure tends to be facilitative, whereas, chronic exposure to glucocorticoids typically produces cognitive impairment (Bodnoff, Humphreys, Lehman, Diamond, Rose, Meaney, 1995; Shors, 2001).

Clearly, glucocorticoids are necessary for proper modulation of a stress response but too much for too long can prove to be detrimental, especially for structures residing within the central nervous system. Within the brain, one particular structure is extremely sensitive to glucocorticoid elevations: the hippocampus. Studies have shown that hippocampal neurons (dentate granule cells and hippocampal proper cells) possess an abundance of mineralocoid (type I) and glucocorticoid (type II) receptors (Reul and de Kloet, 1985). Corticosteroids act on these intracellular receptors, which then elicit cellular changes through genomic mechanisms. The type I receptors display high affinity binding for corticosteroids, while the type II receptors exhibit opposite tendencies and bind corticosteroids with low affinity. It is postulated then that type I receptors regulate hormone level fluctuations coinciding with daily cyclic changes and hormonal variation relating to the animal's circadian rhythm.

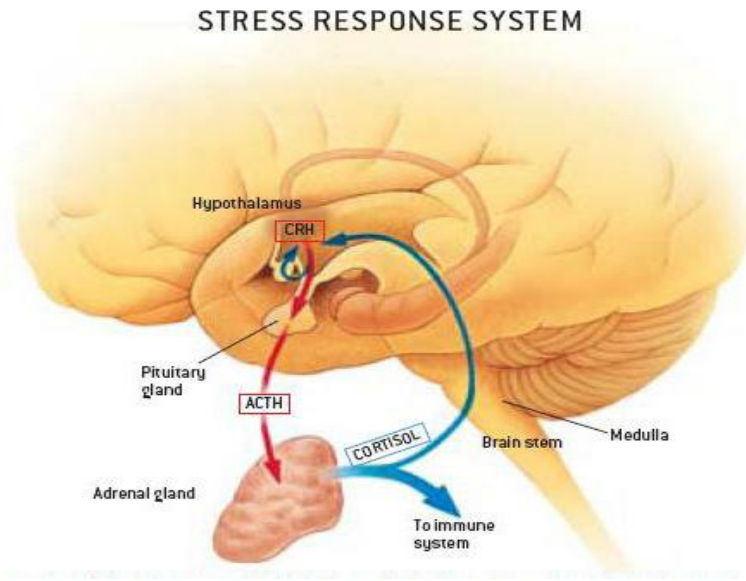

Figure 1. Overview of the hypothalamo-pituitarty-adrenal (HPA) axis. CRH is released from the hypothalamus onto the anterior pituitary causing a release of ACTH into the bloodstream. ACTH then acts on the adrenal glands causing the release of glucocorticoids (Cortisol) into the bloodstream. Taken from: http://www.homeopathyone.in/Psycho_somatic_stress.html

The type II receptors become occupied with a massive release of glucocorticoids into the bloodstream when a particular experience is extremely stressful (Beato and Sanchez-Pacheco, 1996; McEwan, Wright, & Gustafsson, 1997). These receptors act to provide negative feedback within the HPA axis at the level of the hippocampus and hypothalamus; neurons residing with the ventral subiculum (see Figure 2) as hippocampal-output to the hypothalamus, act to down-regulate CRH release (Herman and Mueller, 2006).

Developmentally, glucocorticoids have been found to play an important role in many processes involving proper brain formation from synaptic development to re-arranging cellular machinery (Reviewed by Matthews, 2001). Indeed, even in the adult, glucocorticoids are necessary for the continued development of the hippocampus (Gould, Woolley, & McEwen, 1990). However, prolonged elevation of glucocorticoids early in the developmental time period can cause lasting change to the hippocampus resulting in behavioral impairments later in life (Kikusui and Mori, 2009). Many animal studies have evaluated the developmental time frame known as the stress-hyporesponsive period (SHRP). Interestingly, during the SHRP, which lasts from PND 2-14 in rats, basal corticosterone (CORT), the main glucocorticoid in rats, levels are markedly low. Furthermore, in an effort to protect the developing nervous system, these basal CORT levels show minimal fluctuation in response to a stressor (Sapolsky and Meaney, 1986; Walker, Scribner, Cascio, & Dallman, 1991).

Many studies indicate that elevated glucocorticoids during this time period can cause brain malformation and behavioral impairments later in life. For example, neonatal maternal separation has been shown to increase HPA axis activity in response to stressors

later in life as well as impairing spatial learning and memory (Kalinichev, Easterling, Plotsky, & Holtzman, 2002; Uysal, Ozdemir, Dayi, Yalaz, Baltaci, & Bediz, 2005). Neonatal dexamethasone (a synthetic glucocorticoid) treatment in rats reduced volume in many brain regions including the hippocampus, and this was further substantiated by impairment in the Morris water maze task, a spatial learning task dependent on proper hippocampal function (Ferguson, Paule, & Holson, 2003).

Cellular changes have also been noted within the hippocampus in response to stress, as the structure is extremely sensitive to both endogenous and exogenous glucocorticoid elevations (Schoenfeld and Gould, 2011). One study subjected rats to unpredictable stressors over 21 days in an effort to chronically elevate glucocorticoid levels (Joels et al., 2005). The result was suppression of synaptic plasticity in both dentate gyrus and hippocampal proper regions. Moreover, this same study reported a decrease in cellular proliferation within the dentate gyrus. Brummelte and Galea (2010) demonstrated how chronic exogenous glucocorticoid administration through subcutaneous injections produces a similar reduction of neurogeneis within the dentate gyrus of adult rats. Acute stressors have also been shown to reduce neurogenesis in this structure as well (Gould, McEwen, Tanapat, Galea, & Fuchs, 1997).

The hippocampus, especially the dentate region, is one of the primary brain structures in which neurogenesis has been observed in a variety of mammalian species (Altman, 1963; Reviewed by Dranovsky and Leonardo, 2011). Neurogenesis consists of three stages, cell proliferation, neuronal differentiation and cell survival, but aside from this, hippocampal neurogenesis into the post natal period is poorly understood. Studies have been going on for decades postulating the reasons for and examining the

mechanisms by which hippocampal neurogenesis occurs, in an attempt to better understand this phenomena (Reviewed by Schoenfeld and Gould, 2011). Evidence suggests that neuronal proliferation in the hippocampus into adulthood may help regulate the stress response as well as help the animal adjust to different stressors (Dranovsky and Leonardo, 2011). Suppression of adult neurogenesis has been applied in animals to examine the HPA axis response in the absence of new neurons. One study used transgenic mice that contained a gene called Herpes-Simplex Virus Thymidine Kinase (HSV-tk). The mice were fed valganciclovir, which is phosphorylated by the protein produced by the HSV-tk gene, resulting in a product that is toxic to proliferating cells in the S-phase of mitosis. After neurogenesis was decreased, the mice were subjected to a mild environmental stressor. The mild stressor resulted in a potentiated coticosteroid response suggesting negative feedback disruption to the HPA axis in the absence of new neurons (Schloesser, Manju, & Martinowich, 2009). Furthermore, studies evaluating the type I and type II receptors located in new neurons within the dentate gyrus suggest adaptational effects during the stress response. Type II receptors mature before type I receptors on immature neurons. Although both receptors are active when corticosteroid levels are high, type II receptor activation has been correlated with increased calcium influx resulting in up-regulation of parasympathetic activity. Their data suggest a role for immature neurons in stress adaptation (Hesen et al., 1996; Parischa, Joels, Karst, 2011).

In order to study neurogenesis, a tool for marking cellular proliferation must be used. DNA is the only consistent cellular material that is handed down through generation after generation in every cell making it an apropos target for labeling cellular proliferation.

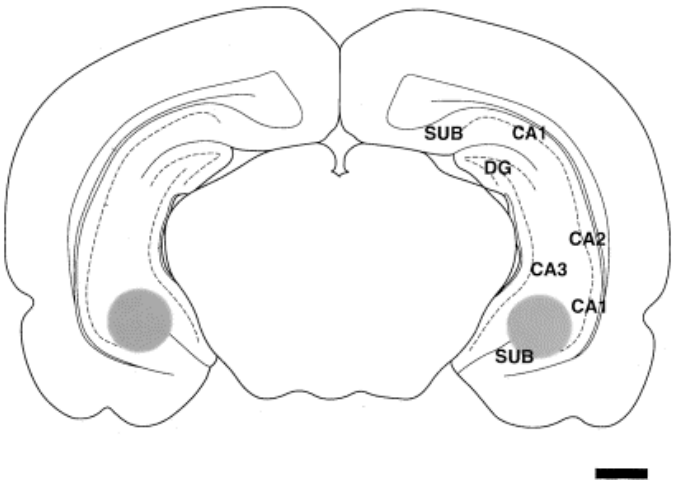

Figure 2. General arrangement of the hippocampus showing the dentate gyrus (DG), hippocampal proper region (CA1, CA2, CA3) including the dorsal (top) and ventral (bottom) subiculi (SUB; Gimenez-Llort, Wang, Ogren, Ferre, 2002).

Historically, tritiated thymidine was used along with autoradiography for detection of cellular proliferation. Thymidine analogs make a perfect choice for marking DNA synthesis only, as the other nucleotides guanine, adenine, and cytosine, are found in RNA which is present in many other cellular processes. One popular thymidine analog that allows for detection of cellular proliferation is called bromodeoxyuridine (BrdU). BrdU inserts into single stranded DNA of dividing cells during the S-phase of the cell cycle.

BrdU is easy to use, and in combination with appropriate immunohistochemical co-labeling methodology for appropriate localization of neurons, BrdU has the ability to be a potent marker for neurogenesis (Kuhn and Kuhn, 2007).

Interestingly, neurogenesis can be enhanced through associative learning. Survival of newly generated neurons after exposure to associative learning tasks was significantly increased in a couple of novel studies (Anderson, Sisti, Curlik, & Shors, 2011; Gould, Beylin, Tanapat, Reeves, & Shors, 1999). Furthermore, the hippocampus is critical in many forms of learning and memory such as spatial and associative memory in animals, and declarative memory in humans (Conrad et al., 1996; Ivkovich, Paczkowski, & Stanton, 2000; Squire, 1992). One form of associative learning that has been correlated with hippocampal neurogenesis is classical eyeblink conditioning. Classical eyeblink conditioning involves the pairing of a conditioned stimulus, often a sound or visual cue, with an unconditioned stimulus, such as a shock or air puff, that elicits an eyeblink response. Over time the animal learns to associate the tone with the shock, and blinks before the shock is delivered. "Trace" eyeblink conditioning involves a separation between the conditioned stimulus and unconditioned stimulus, such that a memory "trace" of the tone must be maintained until the shock is delivered for proper acquisition of an association between the two stimuli. Many hippocampal lesioning, recording, and stimulation studies across the species have proven that the hippocampus is critically involved during acquisition of the conditioned response using a trace eyeblink paradigm (McGlinchey-Berroth, Carrillo, Gabrieli, Brawn, & Diesterhoft, 1997; Moyer, Deyo, & Diesterhoft, 1990; Port, Romano, Steinmetz, Mikhail, & Patterson, 1986; Walker and Steinmetz, 2008; Weiss, Bouwmeester, Power, & Diesterhoft, 1999). Hippocampal

stimulation has been shown to improve learning of trace eyeblink conditioning (Prokasy, Kesner, & Calder, 1983).

Recently, Claflin and colleagues have been examining the effects of different exogenous glucocorticoid administration methods immediately post-SHRP (PND 15) on plasma CORT levels as well as the effects on later behavioral testing (Claflin, Hennessy, & Jensen, 2005; Greenfield, Hennessy, & Claflin, 2009; Claflin, Wentworth-Eidsaune, & Hennessy, 2010). For example, subcutaneous CORT pellets were placed subcutaneously at the back of the neck immediately following the SHRP on PND 15. Blood CORT levels were evaluated at different time points with peak CORT levels reaching 800 ng/mL at PND 18, significantly above normal physiological range (< 30 µg/mL). However, these CORT levels returned to normal six days post-implant. The rats were tested for hippocampal-dependant learning impairments using both trace and delay eyblink conditioning on PND 28. Impairment was found in neither group for delay eyeblink conditioning as this task relies primarily upon primitive brain circuitry within the cerebellum and brainstem (Ivkovich et al., 2000). However, trace eyeblink conditioning was impaired, but in males only. Unpublished data from the Claflin lab (Greenfield et al., 2009; Claflin et al., 2010) further investigated CORT elevations post-SHRP using injections and osmotic mini-pumps for delivery. Plasma CORT elevations for injection groups were observed to peak at 900 ng/ml but returned to baseline levels six hours post-injection. A lower but steady elevation of plasma CORT above the normal physiological range was noted for the osmotic mini-pump group, resulting in peak CORT levels of 120 ng/mL. Behavioral data from the same studies indicate variable results in terms of acquisition of trace eyeblink conditioning. The osmotic mini-pumps produced

results similar to that of Claflin et al. (2005) with pellets producing significant impairment of trace eyeblink acquisition on PND 28. Impairments were observed in both male and females following use of mini-pumps, but more-so in the males. Interestingly, the injection study yielded different results from the previous two methods of exogenous CORT delivery: facilitation of trace eyeblink acquisition, and only in males. These studies demonstrate the effects of CORT delivery after the SHRP and also suggest sexual dimorphism in response to elevated CORT levels post-SHRP and possibly in hippocampal development.

The present study focuses on the effects of elevated CORT levels immediately post SHRP on neurogenesis within the hippocampus. Given that trace eyeblink conditioning is a hippocampal dependent task, and significant conditioning deficits were observed after exposure to CORT, we were curious to see if the behavioral result might be mediated by decreased neurogenesis within the hippocampus. We administered CORT on PND 15, in order to avoid the SHRP, and all three exogenous delivery methods were used. Bromodeoxyuridine (BrdU) was administered shortly thereafter to label proliferating cells. Animals were sacrificed on PND 28, the same day trace eyeblink acquisition was examined in the previous studies. Brains were harvested and processed immunohistochemically to examine neurogenesis within the dorsal dentate gyrus of the hippocampus.

II. METHODS

Subjects and Design

Untimed-pregnant Long-Evans rats were received from Harlan Laboratories and typically gave birth within a week of arrival. Born litters were culled to 10 pups (5 male and 5 female pups whenever possible) on post-natal day (PND) 5. Animals were housed in a colony room approved by the AAALAC and all procedures were approved by the Laboratory Animal Care and Use Committee of Wright State University in Dayton, Ohio, with the colony room maintained on a 12:12 h light-dark cycle with lights on at 07:00 h. Ad libitum access to food and water was provided. On PND 15 animals were randomly assigned to 1 of 3 delivery groups (described below) balancing experimental and control conditions as well as males and females, for each delivery method along the way. The animals were weighed and handled every other day to monitor any potential negative health effects of CORT. BrdU was administered on PND 16 – 18 as described below. After experimental procedures, on PND 21, rat pups were weaned into same sex littermate housing. Transcardial perfusions were performed on PND 28. All pups were anesthetized with a single intraperitoneal euthasol injection (58.5 mg/mL) at a dose of 0.25mL/100g and perfused with 0.1M phosphate buffered saline (PBS) followed by 4% paraformaldehyde. The brains were harvested and kept in 4% paraformaldehyde for 24 hrs before being switched to 0.1M phosphate buffer.

Corticosterone Administration

Three different types of exogenous CORT administration were tested for this project: subcutaneous injection, slow release pellets, and micro osmotic mini-pumps. Animals were assigned to their experimental groups on PND 15 with no more than 1 male and 1 female being assigned to a particular experimental condition. For the injection group (n = 8), CORT was dissolved in sesame oil (0.04 g/10 mL) and administered based on body weight (0.5mL/100g). Injections were administered subcutaneously twice a day at 9 a.m. and 5 p.m., for three days starting on PND 15. Control animals (n = 8) received equal volume sesame oil injections. Pellets and osmotic mini-pumps required brief surgical procedures. Pellet implantation surgeries occurred on PND 15 at which time the back of the neck was shaved and sterilized with 70% ethanol and betadine. Under CO_2 anesthesia (< 2 min), a small incision was made at the back of the neck and either a 35 mg CORT pellet (21 day release, Innovative Research of America); (n = 6) or an inert 35 mg placebo pellet (n = 8) was inserted subcutaneously. The incision was closed with sterile staples. After wound closure, animals were administered buprenorphine (0.3 mg/mL) at a dose of 0.1 mL/30 g and allowed to recover for approximately 30 minutes on a heating pad before being returned to their mother as a group. Osmotic mini-pump surgeries also occurred on PND 15. Prior to surgery the mini-pumps (Alzet Micro-Osmotic Pumps, model 1003D) with a pump rate of 0.1 µL/hour were loaded with CORT dissolved in polyethylene glycol (PEG 400) at 50 mg/mL. These were implanted subcutaneously (n = 9) using the same surgical procedures as for pellet implantation, while control animals received pumps loaded with PEG 400 only (n = 9). The mini-pump CORT was highly concentrated and therefore

required heat and constant stirring to keep in suspension. All pumps were primed by soaking in sterile saline at 37^{o}C for 24 hours prior to implantation

Bromodeoxyuridine (BrdU) Administration

BrdU is a thymidine analog that is a marker of cell proliferation. Its ability to insert into DNA and then be labeled immunohistochemically allows for visualization of newly proliferating cells. In the context of our study, for appropriate visualization of new neurons born within the dorsal dentate gyrus (DDG), all rat pups received an intraperitoneal BrdU injection once a day for three days starting on PND 16 and continuing through PND 18. The BrdU injections were administered at a concentration of 10mg/ml and .5mL/100g rat pup. Because of concerns that BrdU is a potential developmental neurotoxin, special precautions were taken during its administration. All procedures on days 16-21 were conducted under a fume hood. The rats were housed in special filter top cages, for fear of possible aerosolization of BrdU contaminated excreta until at least 3 days after the last BrdU injections. Weight was monitored and bedding was changed every other day as well.

Histological Procedures

For analysis, the rat brains were sliced 80-µm-thick rostro-caudally in the coronal plane through the entire region of the dorsal hippocampus (slicing was halted when dorsal and ventral hippocampi were connected) using a vibratome. Out of the first four

sections, two sections were randomly chosen (a primary set and a secondary set) and from then on every fourth section was collected and put into vials containing PBS (approximately 7-9 sections per brain). For staining, the sections were rinsed with 0.01M PBS/0.3% Triton (PBS/Tx), washed in cold (4^0C) 1N HCl, immersed in 2N HCl (room temperature), incubated in 2N HCl (37^oC), rinsed with PBS/Tx, then blocked with normal donkey serum (1:10) in PBS/Tx. After 30 minutes, the serum was pipetted off and the primary antibodies, anti-BrdU rat (Abcam Inc., 1:4000), a marker of proliferating cells, anti-NeuN mouse (EMD Millipore, 1:500), a marker of mature neurons, anti-GFAP rabbit (Promega Corporation, 1:1000), a marker of astroglia and Sox-10 goat (Santa Cruz Biotechnology, Inc., 1:100), a marker of oligodendrocytes, in PBS/Tx were added and the sections were placed on a shaker (4^oC) overnight with gentle agitation. The next day, the primary antibodies were pipetted off and the sections washed with PBS/Tx. The secondary antibodies with attached fluorochromes (Jackson ImmunoResearch Laboratories, Inc.), Cy3 donkey anti-mouse (1:100), FITC donkey anti-rat (1:40), DyLight649 donkey anti-rabbit (1:100) and DyLight649 donkey anti-goat (1:100) in PBS/Tx were then added to the vials. The sections were placed under a light protected cover and put on a shaker with gentle agitation for 3 hours. Following agitation, the sections were rinsed again with PBS/Tx and finally rinsed with plain PBS. Sections were then mounted in rostral to caudal order onto subbed slides (4-5 sections per slide) with Vectashield (Vector Laboraties, non-hardening), coverslipped and stored at 4^oC until microscopic analysis could take place.

Microscopic Analysis

Microscopic analysis of stained tissue sections was done using an Olympus FV1000 confocal microscope. The FV1000 confocal microscope consists of different lasers used to excite the various fluorochromes attached to the secondary antibodies. We used red, green, and blue excitation lines at wavelengths of 568, 488, and 405 nm for visual analysis of the different neurons within the dorsal dentate gyrus. Each scan was conducted at a magnification of 20x, at 1 micron intervals through the Z-axis. This yielded approximately 40 layers on average for each section, indicating about 50% shrinkage from the staining procedures. These images were then stored digitally for composite analysis using stereological techniques.

Stereological Analysis

The three quantifications that were analyzed separately were total number of new neurons (cell number), total volume of each dorsal dentate gyrus, and density of new neurons. Volume and cell number were measured directly, whereas density was calculated as cell number/volume. Volume was obtained by combining the digitized images of a brain section in the z-plane and tracing the perimeter. New neurons were identified as cells that were co-labeled by BrdU (green) and NeuN (red) or BrdU only. NeuN is a marker for mature neurons. On the other hand, co-labeling of BrdU and GFAP/Sox-10 (blue) marked proliferating glial cells, so by default BrdU-only labeled cells were assumed to be immature neurons (see Figure 3). We counted both mature and immature neurons.

Stereology combines mathematical statistics with two dimensional observations to give quantitative data about the actual three dimensional structure. Through stereology, it is possible to provide quantitative analyses about the size, shape, and number of any objects residing within a structure. Early works of Cavalieri, Buffon, Delesse, Weibel and many others resulted in model based stereology laying the framework for modern, design-based stereology which has been applied across multiple disciplines (Kamp et al, 2009; Aherne & Dunnill, 1982). More recently, a bias free method of stereological analysis has been created called the optical dissector, and has proven extremely useful in neuroscience research (Sterio DC, 1984). For this project, analysis of BrdU labeled cells within the dorsal dentate gyrus (DDG) was done using a modified version of the optical dissector method known as the rare event protocol (REP; Mouton, 2011, pp. 90-95). The typical optical dissector method involves a systematic, random placement of probes throughout the section. When the number of objects becomes low, so as to call them "rare events," the typical dissector method is less efficient because many of the randomly placed dissector probes would not cross paths with the objects of interest. The solution then, using typical dissector methods, would be to decrease the spacing between the dissector probes resulting in an increase in the number of overall dissector probes further causing an increase in the overall time required to analyze such probes. Instead of using random placement of dissector probes, the REP utilizes manual scanning of the object throughout the x-y-z planes. Defining N as the total number of objects, the mathematical approach to define N for the optical dissector principle is as follows: Total $N = \sum Q^- \times S \times A \times T$. Where $\sum Q^-$ = the total number of objects with their topmost point within the volume of sampled sections, S = 1/section sampling fraction, A = 1/area

sampling fraction, and T = 1/thickness sampling fraction. For the REP, the differences are that the total volume of the sections are being used so the area sampling fraction and thickness sampling fraction become equal to 1, therefore, A and T become 1. To make sure the REP was more efficient for our purpose, we conducted preliminary studies comparing the efficiency of the original optical dissector method with the REP in three different subjects. The REP was found to be at least twice as efficient in each case in terms of the time used to properly count the number of proliferating neurons.

Statistical Analyses

Because the data reported here focused only on pellet administration, the design of this study was 2 (Sex) by 2 (Condition): CORT vs. control. Data were analyzed separately using simple two-way ANOVAs for significant effects of sex or condition. Significant 2-way interactions were further analyzed using pairwise comparisons with a Bonferroni adjustment. Data were analyzed using SPSS software.

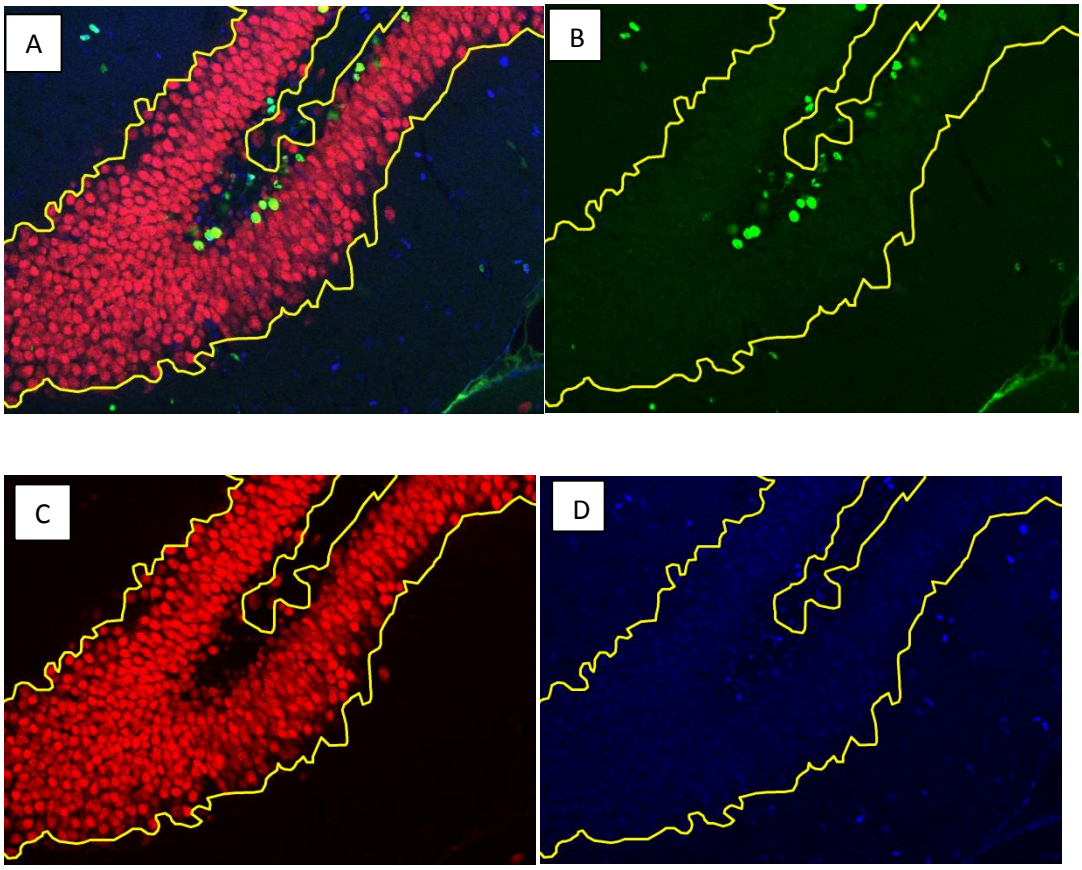

Figure 3a-d. Representative photos of (a) combined fluorochromes, (b) BrdU labled cells, (c) NeuN labeled neurons, and (d) GFAP/Sox-10 labeled glia. A combination of green and red, or green by itself was used to identify neuronal cell proliferation within the dorsal dentate gyrus (DDG). Also note the yellow perimeter tracing, used for calculating the volume of the DDG.

II. RESULTS

For the purposes of this study, only results from the pellet group will be discussed due to the extensive time necessary for tissue processing. I was, however, involved with tissue collection for all of the treatment methods and the data is continuing to be processed. In addition, there was originally a third non-surgical control group but due to the few animals within that group yielding similar numbers as the pellet placebo group, the non-surgical controls and placebo groups were combined into one control group. Because of this, the final number of animals in the dataset is as follows: CORT – 3 males and 3 females, Control – 4 males and 4 females.

Cell Number

A 2-way ANOVA yielded a marginally significant main effect for sex, $F(1, 10) = 4.84$, $p = 0.052$, with the males averaging more neurogenesis overall than the females ($14{,}057.3 \pm 732.8 > 12{,}441.3 \pm 732.8$). The main effect of condition was not significant, $F(1, 10) = .018$, $p = .896$. The 2-way ANOVA did reveal a significant Sex by Condition interaction, $F(1, 10) = 34.83$, $p < 0.001$. Post-hoc analyses revealed a significant decrease in neurogenesis for males given CORT, relative to the control animals ($p < 0.05$) and significant increases in neurogenesis for the female CORT group, relative to controls ($p < 0.05$). Also of importance, males and females were different in the "baseline" control condition with males having a higher rate of nuerogenesis than females ($p < 0.001$). This magnifies the change seen for males given CORT (see Figure 4).

Volume

Volume data are presented in Figure 5. Statistical analyses revealed no significant main effects for volume across sex or condition.

Density

No significant main effects were observed for sex or condition for the density value. Post-hoc pairwise analyses approached but did not reach significance for males, CORT vs. control ($p = 0.06$), but not for females, CORT vs. control ($p = .1$).

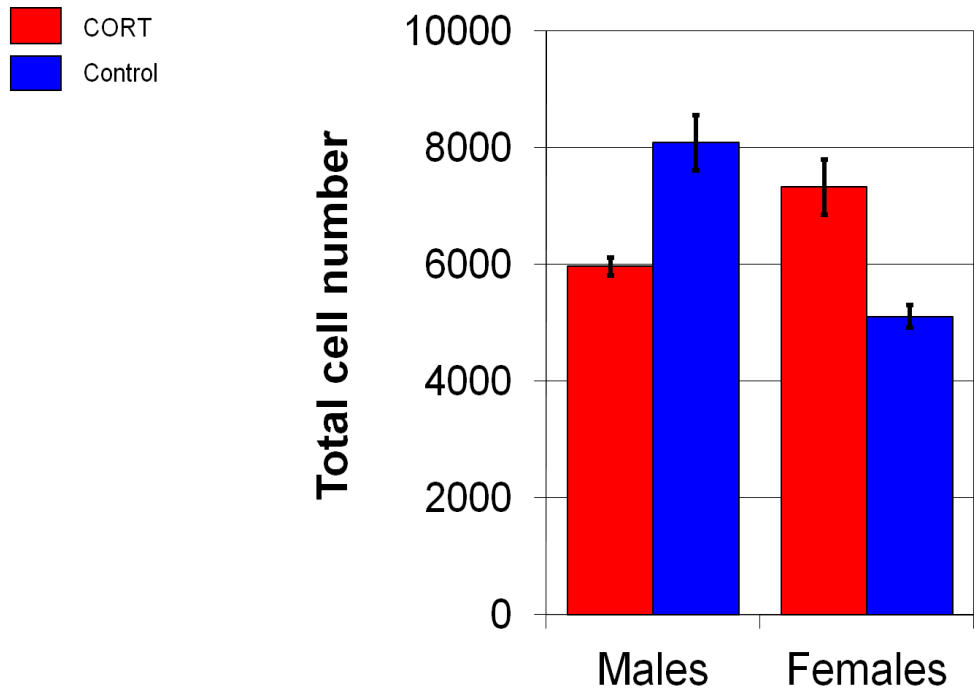

Figure 4. The interaction of Sex x Condition is evident in this figure where CORT-treated males show a *reduction* in neurogenesis while CORT- treated females show an *increase* in neurogenesis relative to control groups. There was also a significant difference in neurogenesis for the control condition with males showing more neurogenesis than females. Bars indicate standard error.

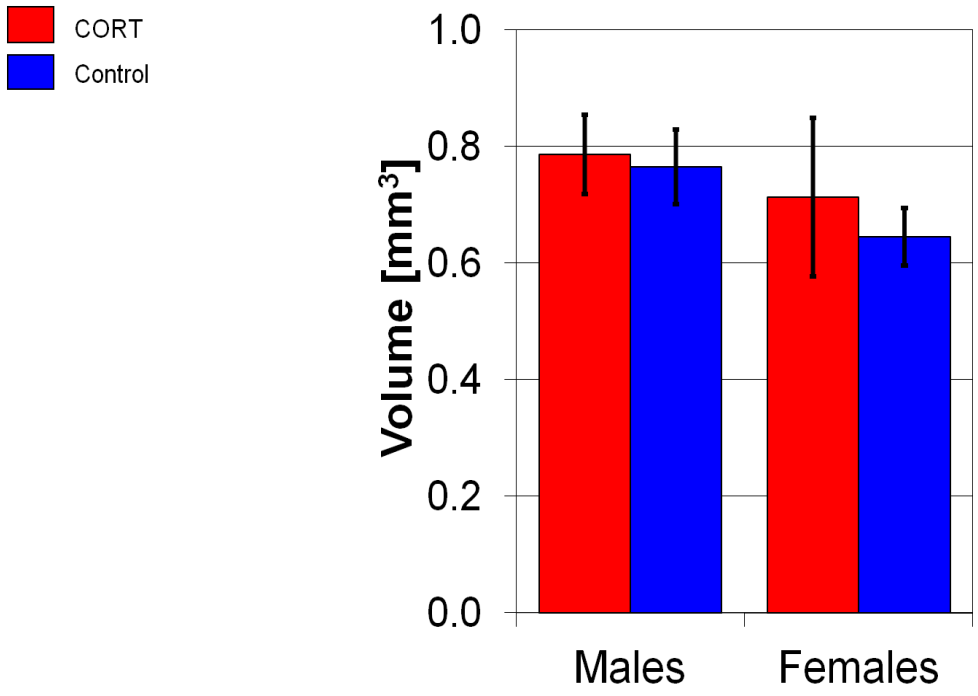

Figure 5. No statistical differences were found for the average total volume of the DDG between CORT and control groups of males and females. Bars indicate standard error.

IV. DISCUSSION

The present study demonstrated different effects of CORT for males versus females in terms of total number of proliferating cells within the dorsal dentate gyrus (DDG). CORT-treated males demonstrated a reduction in neurogenesis while CORT-treated females demonstrated an increase in neurogenesis, consistent with our expectations given the learning impairments reported by our lab previously on hippocampal-dependent learning specific to males (Claflin et al., 2005; Greenfield et al., 2009; Claflin et al., 2010). Unexpectedly, there were significant differences in males versus females in the baseline condition. The control males demonstrated significantly greater cellular proliferation than the control females. However, significant differences in volume were not observed. Our calculation of density reflected the ratio of proliferating cells/DDG volume, so density followed the pattern of differences seen with cellular proliferation because of this common factor between the two values. Moreover, this density calculation based on neurogenesis rather than overall neuronal density, may not be able to capture apoptosis, an alternative cellular change that may be taking place in hippocampus following CORT exposure. Although our results showed no decrease in hippocampal volume due to CORT elevations, it has been documented in other studies (Sapolsky, Uno, Rebert, and Finch, 1990; Uno et al., 1994). For our study, further investigation of total number of neurons within the dentate gyrus will enable us to better assess overall density and possibly apoptosis as opposed to neurogenesis, in order to paint a more complete picture of the effects of elevated CORT levels. Elevations in glucocorticoids that cause reduction of hippocampal density and volume have been associated with diseases like schizophrenia and posttraumatic stress disorder (Hughes and

Shin, 2011; Peralson and Marsh, 1999). Furthermore, out of a study of 34 adult patients with hippocampal sclerosis, about half of those patients reported having a history of major depression throughout their life time (Breillmann, Hopwood, & Jackson, 2007). It is interesting to consider whether severe changes to the hippocampus result in certain neuropsychiatric diseases, or whether the diseases may cause the hippocampal changes to occur.

Considering the parallel experimental protocol used in this study and previous studies from this lab, we assumed elevations in plasma CORT from the pellet administration were similar: peaking on the first day, dropping to 800 ng/mL at day three and back to control levels after day five (Claflin et al, 2005; Figure 6). Subcutaneous pellets demonstrated the largest behavioral impairments of all the methods used and researchers noted that CORT pellets resulted in: (a) less weight gain during subsequent development and (b) brains that appeared significantly smaller upon removal though brain weight was never quantified (Claflin et al., 2005; Claflin, personal communication). Furthermore, the behavioral data from all the previous studies suggested sexual dimorphism with males being more responsive to changes in plasma CORT. In the case of pellet administration, the males were significantly impaired on acquisition of trace eyeblink conditioning.

Sex related differences in response to stress or exogenous glucocorticoids are not uncommon (Vicedomini, Nonneman, Dekosky, & Scheff, 1986; Wood and Shors., 1998). Our results, indicating that CORT-treated males showed significantly less neurogenesis than CORT-treated females, demonstrate that sexually dimorphic vulnerabilities are present at a young age. Although the reduced neurogenesis for males was expected given

behavioral data, it is more difficult to reconcile with recently collected blood sample data charting the time course of CORT elevations for males and females with pellet implants. As shown in figure 6, the pellet-treated females demonstrate significantly *higher* plasma CORT levels than the males, even at 3 days post-implant. It seems the CORT-treated females should have had significantly less neurogenesis than the males if such high CORT levels were the reasons for reduced neurogenesis. If CORT elevation instead was beneficial, it could also explain the differences within the placebo group where males had marginally higher rates of circulating CORT and higher rates of neurogenesis. However, it does not explain why CORT implants decrease neurogenesis for males but increase it for females. The answer to this must lie in the organizational effects of the sex hormones. It seems that something truly different does reside within the developing physiology of male and female rats.

Other studies of chronic stress or elevated CORT levels and their effects on neurogenesis demonstrate a wide range of results, and these results depend on the experimental designs (Falconer and Galea., 2003; Westonbroek, Denboer, Veenhuis, & Terhorst, 2004). While neurogenesis in males was shown to decrease in our study in response to CORT, one study showed no decreases in neurogenesis after a stress response. Animals were subjected to multiple, mild stressors and then injected with BrdU immediately after for analysis of newly generated cells. No significant differences were found in any of the experimental groups compared to control animals. In contrast, another recent study demonstrated the exact opposite: significant changes in both male and female neurogenesis within the dentate gyrus, in response to 21 days of high-dose (40 mg/kg), subcutaneous CORT injections (Brummelte and Galea, 2010). It seems the

experimental model used, either stress induced, or exogenous administration as well as the concentration of CORT can cause lasting effects within the hippocampus of adult animals, or cause no effects at all. It was mentioned previously that CORT affects cognitive performance in concordance with an inverted U shape (Diamond et al., 1992; Mateo, 2008). Placing the CORT-delivery methods onto the inverted U paradigm relating concentration of CORT and cognitive performance, it is possible that the mini-pumps provide no change or very low elevations of CORT, injections provide a moderate amount of CORT, and the pellets provide a high concentration of CORT (Figure 7). Further investigations are needed to characterize possible changes in neurogenesis and apoptosis following the other drug administration methods and to decide what experimental methods might produce the most natural, and lasting deficits on hippocampal structure and function for proper application into human models.

Microglia also play a huge role in developmental processes of neurons and their development has been shown to coincide with neurogenesis in many brain structures (Rice and Barone, 2000). Stress and depression are being increasingly linked to inflammatory mechanisms similar to immune system challenges, and the chemical messengers of these inflammatory processes, known as cytokines, act specifically on glial cells and have been shown to cause cognitive change (reviewed by Bilbo and Schwarz, 2009). Therefore, we cannot discount possible changes of glial cell populations in response to CORT during the course of our study. However, most of our BrdU labeled cells were found within the subgranular zone of the dentate gyrus and fewer were co-labeled as glia. Also, changes within the glial cell population may have been present in other hippocampal areas not examined for this paper.

We also do not know how long it may take for CORT effects to be measurable at the cellular and molecular levels. We allowed approximately one day between CORT administration and the start of BrdU administration so that CORT may have some time to elicit its effects on the hippocampus. The proper timing of CORT and cellular proliferation markers is essential. Effects of acute stress early in life have been shown to be reversible through certain behaviors that return the animal to a normal state (van Oers, de Kloet, and Levine, 1999). If a normal state can be achieved days after a stressor occurs, it makes sense to administer BrdU early so that immediate effects of CORT may be observed. Alternatively, if the stress is chronic and lasts many days much like the pellets of our study then the timing between CORT and BrdU may yield different results. It may be that later outcomes would be either more or less severe. Alternatively, stress (elevated glucocorticoids) at different developmental stages may produce different effects on neurogenesis. There is some evidence that the hippocampus may be undergoing a growth spurt around post-natal day 14 in rats and therefore, may be more vulnerable to injury at this time (Towfighi, Mauger, Vannucci, &Vanucci, 1997). An interesting route of further investigation would be to stress animals at different developmental ages and then administer immunohistochemical markers weeks later to observe the lasting effects, if any, on neurogenesis and neuronal morphology.

Exogenous glucocorticoid delivery in animal models remains a challenge. Our study was an effort to produce chronic elevation of glucocorticoids, and based off of previous blood sample data, the pellet administration method demonstrated the most consistent elevation over the greatest amount of time. Other studies have demonstrated similar problems administering glucocorticoids to animals. In one study conducted on

mice, Herrmann et al. (2009) compared the same three exogenous delivery methods employed in our current studies. The pellets produced peak elevations of CORT around the 24 hour mark, but these levels decreased back to baseline within 7 days (Herrmann et al, 2009), similar to Claflin et al. (2005) and Figure 6 (Claflin et al., 2011). It should be noted that the pellets used in both studies were advertised as 21-day release. The osmotic mini-pumps in the Herrmann study produced no significant CORT elevation whereas the mini-pumps from Claflin et al. (2009) resulted in smaller, but physiologically relevant elevations of plasma CORT over a period of time. Injections from Herrmann et al. (2009) produced cyclic elevation resulting in peak CORT at 1 hour and returning to baseline within 4 hours post-injection, similar to Claflin et al. (2010). Herrmann et al. (2009) looked at measures of bone density in response to hypercorticosteronemia and only repeated implantation (every week) of the pellets produced significant changes. Many other studies use similar prefabricated methods of CORT delivery, and try to measure effects of elevated CORT on many different bodily systems, but do not post blood sample data. It is imperative that researchers using any exogenous glucocorticoid delivery method show plasma CORT levels for proof of function of the device before conducting any behavioral studies on the effects of glucocorticoids.

Many other aspects of neuronal development are also affected by stress or exogenously elevated glucocorticoid levels. Brain-derived neurotrophic factor (BDNF) is a secreted protein necessary for the development of neurons within the central nervous system. Recently, altered BDNF expression has been linked with decreases in neurogenesis after exposure to elevated glucocorticoid levels. Furthermore, given that young rats who have been weaned early show altered glucocorticoid receptor expression

within the hippocampus, the suppression of neurogenesis and BDNF levels within the structure may be a result of increased sensitivity to glucocorticoids. In agreement with our study, these changes in BDNF expression appear to be sexually dimorphic as well (males showing greater vulnerability). Altered BDNF levels, neurogenesis, and altered hippocampal function have all been tied to some cognitive change (Reviewed by Kunugi, Hori, Adachi, & Numakawa 2010; Kikusui and Mori, 2009). Further investigation is needed to try and tie everything together in an effort to better help the patients afflicted with these impairments.

What remains unknown from many of the studies discussed previously is the effect of a developmental time frame where glucocorticoids may have lasting effects. Premature babies are given glucocorticoids to promote lung development and many young children stricken with asthma at a young age are given synthetic glucocorticoid inhalers. These children have been shown to develop certain cognitive changes later in life (Bender, Lerner, & Poland, 1991; Bender et al., 1988). Further studies are needed to evaluate the effects of glucocorticoids at certain developmental time frames that may produce detrimental neuropsychological changes in adulthood.

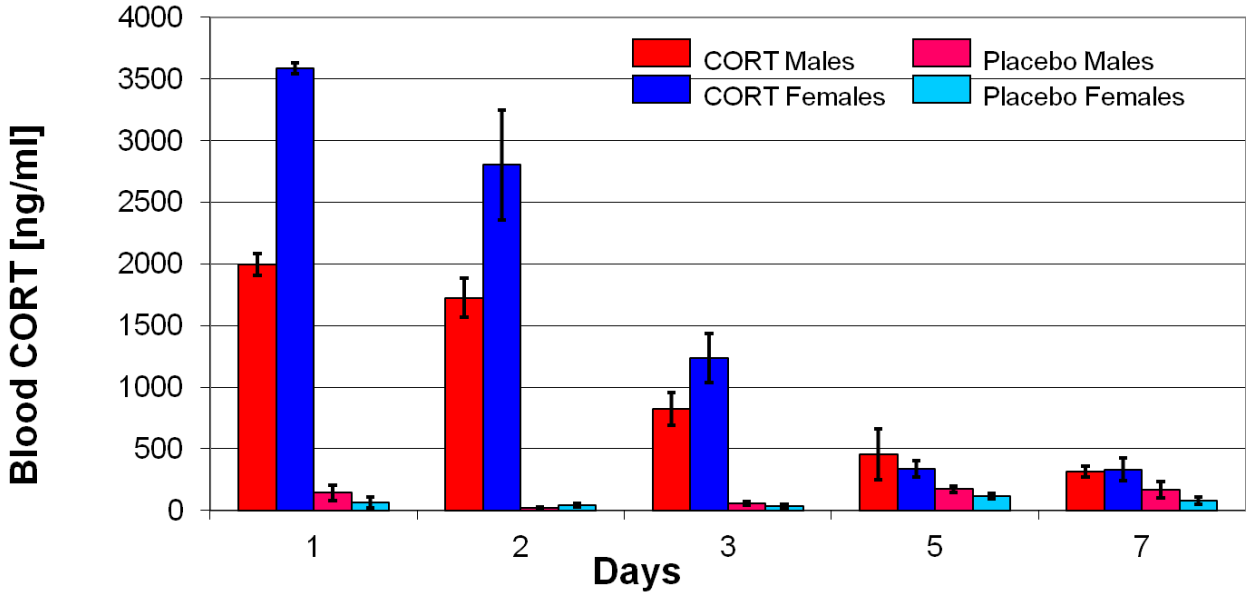

Figure 6. Previous blood sample data demonstrating higher plasma CORT elevations in the females than males. Furthermore, the placebo controls are also visible and in comparison to the CORT treated group it is clear how truly elevated the plasma CORT levels were. Bars indicate standard error.

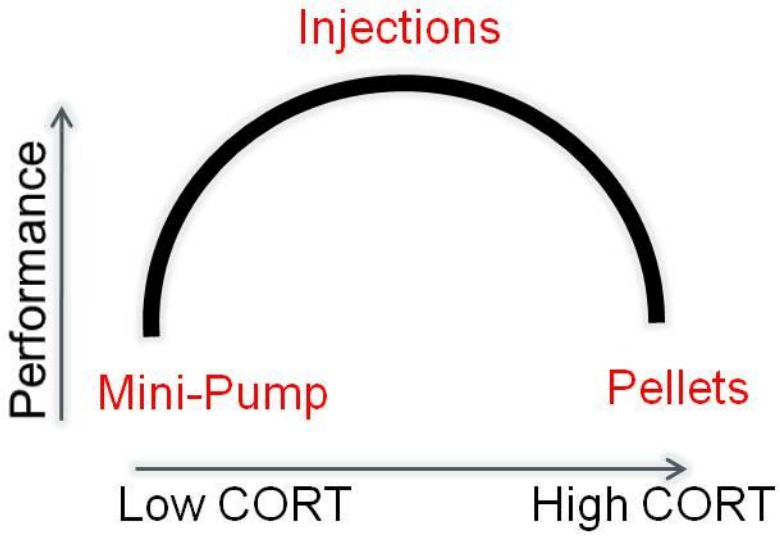

Figure 7. The different glucocorticoid administration methods mapped over the inverted U shape paradigm relating CORT levels with cognitive performance (Claflin, Wentworth-Eidsaune, and Hennessy, 2010).

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