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Short-Term Administration of Corticosterone has Lasting Effects on Learning in Young Rats

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SHORT-TERM ADMINISTRATION OF CORTICOSTERONE HAS LASTING EFFECTS ON LEARNING IN YOUNG RATS

A thesis submitted in partial fulfillment
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degree of Master of Science

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Christine Lynn Wentworth-Eidsaune ENTITLED Short-Term Administration of Corticosterone has Lasting Effects on Learning in Young Rats BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science

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ABSTRACT

Wentworth-Eidsaune, Christine Lynn. M.S., Anatomy Program, Department of Neuroscience, Cell Biology and Physiology, Wright State University, 2010. Short-term administration of corticosterone has lasting effects on learning in young rats.

Corticosterone is a glucocorticoid released as part of the body's response to stress and is known to affect cognitive function, presumably via effects on the hippocampus. Trace classical eyeblink conditioning depends on the hippocampus, and has been used to examine the development of learning processes in young organisms. Experiment 1 was a dosing study, in which time course of effect of corticosterone was followed in 15-day-old Long-Evans rat pups over 24 hours for 4 different concentrations (high: 0.02 mg/g body weight (b.w.), medium: 0.01mg/g b.w., low: 0.005 mg/g b.w. and a vehicle control). In Experiment 2, two subcutaneous injections (0.02 mg/g b.w., 0.005 mg/g b.w., or vehicle control) were administered over a 3-day period, starting at PND 15. Ten days after injections, animals underwent trace classical eyeblink conditioning to examine the possible lasting effects of the elevated corticosterone levels on learning and memory. Eyeblink conditioning was affected by corticosterone treatments, but only for males, and only very early in acquisition. Males receiving the high dose of corticosterone exhibited facilitation of learning relative to controls.

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I. INTRODUCTION

It is well understood that elevated levels of glucocorticoids, as part of a prolonged natural stress response, have been associated with various cognitive deficits in humans and laboratory animals (Alderson & Novack, 2002). Administration of glucocorticoids as medical treatments to pregnant mothers (e.g. to increase fetal lung maturation prior to a premature birth), to young children (e.g. for asthma), as well as to older adults (e.g. respiratory illnesses) can lead to deficits presenting in the form of learning and memory impairment (Bender, Lerner, & Kollasch, 1998; Bender, Lerner, & Poland, 1991). Studies show that when humans and animals alike are under significant amounts of stress, cognitive functions decline and the ability to recall long-term and short-term memories is impaired (for review, see Lupien, Maheu, Tu, Fiocco, & Shramek, 2007).

Not all stress or glucocorticoid exposure is bad, however. Glucocorticoids have an inverted U-shaped relationship with cognition in humans and animals; very high and very low levels of glucocorticoids impair cognitive abilities, while moderate levels tend to facilitate the learning process (Mateo, 2008; Pavlides, Watanabe, & McEwen, 2004). Along with varying glucocorticoid concentrations, the length of exposure to glucocorticoids has an effect on cognition: acute stress tends to facilitate learning processes, whereas the opposite effect is produced with chronic stress (De Kloet, Vreugdenhil, Oitzl, & Joels, 1998; De Kloet, Oitzl, & Joels, 1999; Sandi, Loscertales, & Guaza, 1997).

As a result of the body's stress response, the hypothalamic-pituitary-adrenal (HPA) axis is activated, which in turn initiates the secretion of glucocorticoids in order to reduce inflammatory responses and to protect the organism under stress (Wolf, 2003).

Although these same glucocorticoids are an essential component of normal brain function, too much secretion or secretion for too long can cause long-term structural damage (McEwen & Steller, 1993). Within the brain, the hippocampus is exceptionally sensitive to endogenous and exogenous glucocorticoids (Bohn, 1984; Weinstock, 1997) likely because of the large number of glucocorticoid receptors present (Sapolsky, Meaney, & McEwen, 1985). The hippocampus is also of particular interest because of its critical involvement in many types of learning and memory, including associative learning, spatial learning, and complex learning tasks such as story-recall. For example, Kirschbaum, Wolf, May, Wippich, & Hellhammer (1996) treated human subjects with the glucocorticoid hydrocortisone and then asked them to perform a declarative memory task. The results showed significant impairment of performance for this hippocampal-dependent task. In another study, women who were asked to perform spatial learning tasks showed the need for additional acquisition training when cortisol levels were increased (Schwabe, Oitzl, Richter, & Schachinger, 2008). These studies have provided evidence that glucocorticoid elevations in adults generally impair performance on hippocampal-dependent tasks.

Along with cognitive deficits, a reduction in hippocampal neuronal volume has also been associated with increased glucocorticoid levels as shown in research with monkeys (Uno et al., 1994). Cortisol pellets implanted near the hippocampus produced abnormal shrinkage and condensation of hippocampal neurons. Long-term glucocorticoid elevations were found to have neurotoxic effects on the adult hippocampus in humans and in other animals, including rats (for review, see Lupien & McEwen, 1997; Lupien et al., 1998; Sousa, Madeira, & Paula-Barbosa, 1998). The

neurotoxic effects largely result in the inhibition of cell production in the hippocampus, affecting hippocampal function both in early postnatal development and adulthood (Gould, 1999).

Another task used to study hippocampal function is trace eyeblink conditioning. In rabbits and humans, eyeblink conditioning has become a model system for studying the behavioral and neural properties of simple associative learning (Gormezano, Kehoe, & Marshall, 1983; Lavond, Kim & Thompson, 1993). This type of training involves the formation of an association between an initially neutral tone stimulus and either an air puff to the cornea, or a mild electrical shock to the face, that elicits an eyeblink response. Over time, the pairing of these two stimuli will result in a reaction to the tone in the absence of the shock. Specifically, trace eyeblink conditioning is defined by a time separation between the tone and the shock stimulus, creating a gap during which a memory trace of the conditioned stimulus must be maintained in order for the association to occur. Several studies have demonstrated that the hippocampus is an essential brain region involved in acquiring the conditioned response in trace eyeblink conditioning (Kim, Clark, & Thompson, 1995; Moyer, Deyo, & Disterhoft, 1990; Solomon, Vander Schaaf, Thompson, & Weisz, 1986). To mention just two examples, adult rabbits that received hippocampal lesions were significantly impaired relative to the controls during trace eyeblink conditioning (Weiss, Bouwmeester, Power, & Disterhoff, 1999), and hippocampal stimulation has been shown to improve acquisition of trace eyeblink conditioning (Prokasy, Kesner, & Calder, 1983).

More recently, eyeblink conditioning has been used in young rats in order to examine the development of the hippocampus. To best understand cognitive and neural

development, trace eyeblink conditioning is often compared to delay eyeblink conditioning. In delay eyeblink conditioning, known to involve the cerebellum and brainstem (Lavond et al., 1993), the tone and the shock stimulus overlap for a brief period of time. In 1992, Stanton, Freeman, & Skelton found that associative delay eyeblink conditioning emerged around 20 – 24 days of age with the learning rate increasing over this age range. In a study directly comparing delay and trace eyeblink paradigms, Ivkovich, Paczkowski, & Stanton (1999) found that between postnatal days (PND) 19 and 23, delay conditioning emerged and peaked, whereas trace conditioning emerged more gradually and peaked between PND 28 and 31, due to greater task difficulty and perhaps further development of the hippocampus. Further evidence that the hippocampus is required during trace conditioning was demonstrated in rat pups with hippocampal lesions created at PND 10. These animals showed impairment of trace eyeblink conditioning, but not delay eyeblink conditioning, when tested on PND 25 (Ivkovich & Stanton, 2001).

Developmentally, glucocorticoids play a significant role in brain growth and functions in humans and animals, but chronic elevated exposure can be detrimental later in life. Prenatal studies have indicated that developing animals exposed to increased glucocorticoid levels are at risk for functional changes in the hippocampus resulting in long-term behavioral alterations. For example, prenatally stressed offspring have heightened levels of emotionality and HPA sensitivity (Takahashi, 1997). About 7% of human pregnancies, at a high risk of early delivery, are treated with synthetic glucocorticoids, such as dexamethosone, to promote fetal lung maturation. Fetal exposure can permanently alter HPA activity in young children, adolescents, and even

aging adults, leading to a modification in behavior and brain morphology (Owen, Andrews, & Matthews, 2005).

Interestingly, there is a critical period early in rat development during which the organism is protected from elevated corticosterone (CORT) and its detrimental effects. This period of time occurs between about PND 2 – PND 14 and is called the stress-hyporesponsive period (SHRP). During the SHRP basal levels of CORT, the endogenous stress hormone in rats, are dramatically reduced and stress-induced increases are typically minimal (Sapolsky & Meaney, 1986; Walker, Scribner, Cascio, & Dallman, 1991). The SHRP is followed by a peak of endogenous CORT around PND 15, and a continual rise into at least the fourth week of life, aiding in development (Walker, Perrin, Vale & Rivier, 1986).

Many of the previous developmental studies of glucocorticoid sensitivity have focused on fetal exposure and exposure during the SHRP. In a study by Ordyan, Pivina, Rakitskaya, & Shalyapina (2002), administration of exogenous CORT during and after the SHRP produced a decrease in baseline CORT levels at PND 30. In the same study, the animals treated with CORT just after the SHRP showed a prolonged duration of secretion of CORT in adulthood when challenged with restraint stress. This was not observed for animals given CORT during SHRP. This study reinforces the belief that rats exhibit a decreased sensitivity to stress during the SHRP, but experience additional enduring effects of elevated glucocorticoids during post-SHRP administration.

Recently, Claflin and colleagues (Claflin, Hennessy, & Jensen, 2005; Greenfield, Hennessey, & Claflin, 2009) have begun examining the lasting effects of post-SHRP CORT administration on learning and memory, while many of the earlier studies

reporting learning and memory deficits with CORT exposure focused on administration during SHRP. In one study (Claflin et al., 2005) 35 mg, 21-day timed-release CORT pellets were implanted subcutaneously in the rat on PND 15, followed by trace and delay eyeblink conditioning on PND 28. A pharmacologically high level of CORT was measured in the blood on Day 3 post-implant (80μg/dl), but this elevation returned to control levels by Day 6 post-implant. This manipulation produced no significant impairment in delay conditioning, but acquisition of trace eyeblink conditioning was impaired, and interestingly only in males. These data suggested differences in vulnerability to CORT at this stage in development, and in order to further examine these findings, Greenfield et al. (2009) attempted a similar study using osmotic mini-pumps as a means of delivering a more physiologically relevant elevation of CORT. This time, a mild elevation of CORT (12 μg/dl at 24 hours after implant) impaired trace acquisition for all animals, again with a trend of greater impairment in the males.

The possibility that developmental sex differences are occurring in trace eyeblink conditioning as a result of CORT treatment post-SHRP is intriguing. The research on developmental sex differences and various forms of stress have yielded conflicting information that bears further study. Oomen et al. (2009) showed that 24 hours of maternal separation during the first week of life significantly increased neurogenesis in male rats, but significantly decreased neurogenesis in female rats. In contrast, Spivey et al. (2009) demonstrated that repeated maternal separation in young rats had a negative effect on males, but females were found to be more resistant to the same stressor. Human research has shown a negative correlation between the cortisol elevation induced by a psychosocial stressor and performance on a verbal recall task for young adult males

(Wolf, Schommer, Hellhammer, McEwen, & Kirshbaum, 2001). It has been suggested that such sex differences may be due to differences in adrenal hormones during development (Wood, Shors, & Beylin, 2001), or possibly differences in glucocorticoid receptor expression (Owen & Matthews, 2003). Developmental differences in vulnerability to glucocorticoid exposure are not yet understood, but may provide a valuable link to a better understanding of developmental learning disorders. Moreover, some psychiatric diseases and disorders seem to have a higher prevalence in males than females, suggesting sex differences in vulnerability to injury in the developing brain.

In order to build on previous knowledge, the present study focuses on the effects of CORT during development immediately after the SHRP. We were particularly interested in the possibility of sex differences in hippocampal learning in response to CORT. Because the previously used CORT pellets and osmotic mini-pumps yielded extremely different blood levels, subcutaneous injections of CORT were used in this study to try to obtain an intermediate effect. CORT was administered starting on PND 15 in order to avoid the SHRP. The animals underwent trace eyeblink conditioning 10 days later to examine the possible lasting effects of CORT exposure on memory processes in the young rat.

II. EXPERIMENT 1: Corticosterone dose-response study

The purpose of this study was to evaluate the time course of effect for injections of CORT at four different concentrations. CORT was delivered by subcutaneous injection, and blood samples were collected at 6 time points over a 24-hour period in

order to evaluate how long circulating levels of CORT were elevated above control levels. The information gathered from this experiment determined dose concentrations to be used in Experiment 2.

METHODS

Subjects

Timed-pregnant female Long-Evans rats were received from Charles River Laboratories (Raleigh, NC) around E15. On PND 4 or 5, litters were culled to 10 pups, 5 male and 5 female whenever possible. Animals were housed with dams in a colony room accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC). Animals were maintained on a 12:12 hr light:dark cycle with lights on at 0700. *Ad libitum* access to food and water was provided. On PND 15, animals were randomly assigned to one of 4 dose groups roughly balanced for sex: high, medium, low and vehicle control (details under *Injections*, below). One injection was administered at 0730 hrs on PND 15, and terminal blood samples were collected at various time points throughout the next 24 hours, one sample per animal.

The final data set included 134 Long-Evans rat pups, 65 females (f) and 69 males (m), sampled from 14 litters. No more than 1 female and 1 male from the same litter were assigned to a particular condition to control for litter effects. The high dose group consisted of 37 pups (18 f, 19 m), medium dose group consisted of 35 pups (17 f, 18 m), low dose group consisted of 38 pups (18 f, 20 m), and the control dose group consisted of 24 pups (12 f, 12 m).

Procedures

Injections. Corticosterone (*Sigma* C2505) was dissolved in sesame oil (*Sigma* S-3547), vortexed and placed in warm water bath 24 hours prior to injections and maintained at 37°C until injections were completed. The concentration of CORT for the high dose was 0.02 mg/g body weight (b.w.), the medium dose was 0.01 mg/g b.w., and the low dose was 0.005 mg/g b.w. At 0730 hrs on PND 15, CORT was administered via subcutaneous injection. The control animals received an equal volume by body weight of sesame oil (0.005 ml/g). Animals were then returned to dams until blood collection.

Blood collection. Within each dose and sex group, animals were randomly assigned to have blood samples collected at 1, 2, 4, 8, 16 or 24 hours. Animals were anesthetized with CO_2 prior to blood sampling. Blood samples were collected via decapitation to ensure adequate plasma volume for later assays (approx. 1 ml). Blood was collected within less than 4 minutes of disturbance in order to minimize any effect of the procedure on CORT levels in the samples obtained. Blood was centrifuged to separate plasma, which was then frozen until assayed at a later date. Assays were run in duplicate with a radioimmunoassay kit (Siemens, ^{125}I Rat Corticosterone). Intra- and Inter-assay variability was calculated to be less than 5%.

Data Analysis. Plasma concentrations of CORT were analyzed using a 4 (Dose) x 2 (Sex) x 6 (Hour) between-groups analysis of variance (ANOVA). Select one-way ANOVAs were performed for the first 4 time points of blood sampling to determine how long significant dose differences were present. Post-hoc Tukey tests for multiple comparisons were used for time points where a significant main effect between doses was observed. Data were analyzed using SPSS statistical software.

RESULTS

There was a significant main effect of dose [F (3, 86) = 12.6, p = 0.001] and a significant main effect of hour [F (5, 86) = 17.2, p = 0.001], and there were no significant main effects or interactions involving sex. There was a significant Dose x Hour interaction, F (15, 86) = 3.4, p = 0.001 (see Figure 1). Post-hoc one-way ANOVAs were used to evaluate dose differences during the first 4 time points. A significant difference between doses was found at 1 hour, 2 hours and 4 hours, but not at 8 hours [F (3, 19) = 4.9, p = 0.013; F (3, 21) = 3.9, p = 0.026; F (3, 21) = 8.7, p = 0.001; F (3, 24) = 0.3, p = 0.254, respectively]. Post-hoc Tukey tests for multiple comparisons revealed that the significant group effect at 1, 2 and 4 hours was due to a difference between the high and control dose groups. The high dose group was significantly elevated relative to the controls at those times. The elevations for the medium and low doses were not significantly above control levels. The CORT concentration at 1 hour was 103.8 ± 27.6 s.e.m. μg/dl for high dose group, 61.5 ± 10.9 s.e.m. μg/dl for the medium dose group, 44.2 ± 8.7 s.e.m. μg/dl for the low dose group, and 6.5 ± 0.9 s.e.m. μg/dl for the control dose group.

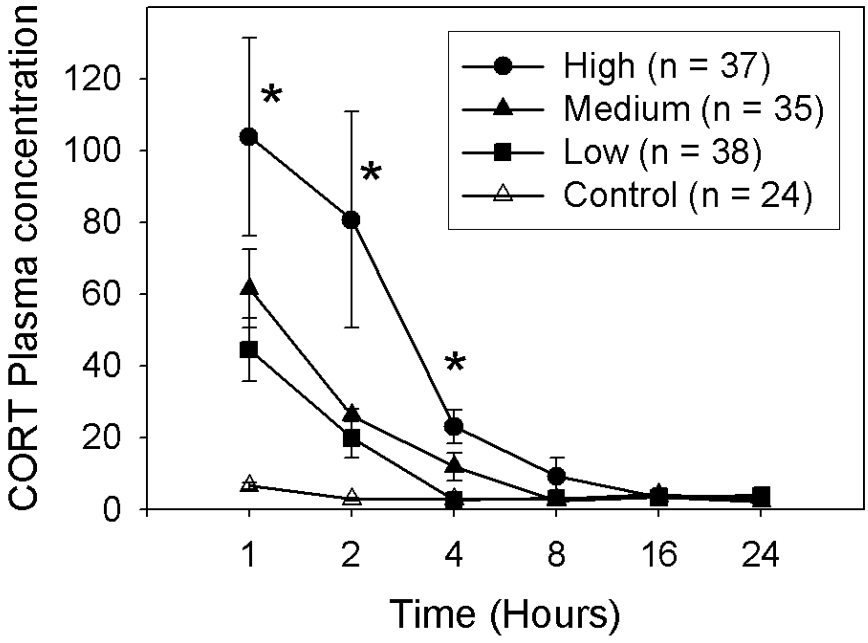

Figure 1. Plasma levels of corticosterone at 1, 2, 4, 8, 16, and 24 hours (x-axis) after injection. Values represent mean ± s.e.m. plasma concentrations for 4 dose groups. Plasma concentration was measured in μg/dl.

DISCUSSION EXPERIMENT 1

The findings of this experiment show that administration of a high concentration by injection caused a significant increase in CORT levels 1, 2 and 4 hours after delivery. The only statistically significant elevation observed was for the high dose group, and only during the first 4 hours after injection. By 8 hours after injection CORT levels had returned to control levels, consistent with recently reported data from Hermann et al. (2009). Because the low and the medium doses in this experiment did not significantly elevate circulating hormone levels, the high dose was selected as the main dose of

interest for Experiment 2. Based on the assay results, however, the high dose produced a pharmacologically high (Sapolsky et al., 1985; Claflin et al., 2005) level of CORT initially (103.8 μg/dl), whereas the low dose produced a more physiologically relevant elevation of CORT (16.5 μg/dl). For this reason, the low dose was also included in Experiment 2.

III. EXPERIMENT 2: Trace Eyeblink Conditioning and Corticosterone

In order to examine the early post-SHRP effects of CORT on learning and memory, injections on PND 15-17 were followed by trace eyeblink conditioning on PND 28. Because CORT levels in Experiment 1 dropped significantly after 4 hours, we chose to give two CORT injections each day for a 3-day period in this experiment. We hypothesized that the prolonged elevation of CORT levels would significantly impair acquisition of trace eyeblink conditioning. Based on previous work, we expected the impairment to be observed specifically in males.

METHODS

Subjects

Animals were ordered and housed just as in Experiment 1. On PND 15, animals were randomly assigned to one of 3 dose groups, roughly balanced for sex: high, low and control. Injections were given twice a day for 3 days. Animal health and weight were monitored every other day throughout the experiment. Pups were housed with dams until weaning on PND 21, at which time they were housed with same-sex littermates until

surgery on PND 26. On PND 26, pups underwent a surgery to implant electrodes used during eyeblink conditioning (details below). After surgery, and during behavioral training, animals were housed individually.

The sample size consisted of 57 Long-Evans rat pups, 29 female and 28 male sampled from 15 litters. As in Experiment 1, no more than 1 female and 1 male from the same litter were assigned to a particular condition to control for litter effects. The high dose group consisted of 20 pups (12 f, 8 m), the low dose group consisted of 20 pups (9 f, 11 m) and the control group consisted 17 pups (8 f, 9 m).

Procedures.

Injections. Starting on PND 15, two subcutaneous injections were given daily for 3 days, one at 0900 and one at 1700 hrs. The CORT solution was prepared as described in Experiment 1: the high dose was 0.02 mg/g b.w., the low dose was 0.005 mg/g, and the control group received an equal volume by weight of sesame oil injections (0.005 ml/g).

Eyeblink Electrode Surgery. On PND 26, two days before conditioning procedures began, stimulating and recording electrodes were implanted according to procedures described by Stanton et al. (1992). Differential electromyographic (EMG) recording electrodes were implanted in the upper eyelid muscle (*orbicularis oculi)* to monitor eyelid activity. A bipolar stimulating electrode (*Plastics One,* Roanoke, VA) was placed subcutaneously for delivery of a mild periocular shock to elicit the eyeblink reflex.

Prior to surgery, each animal was anesthetized with a ketamine and xylazine cocktail (0.075 mg/g b.w. ketamine, and 0.005 mg/g b.w. xylazine) administered intraperitoneally. If during surgery a pup began to show signs of awakening, an

additional half-dose of the ketamine/xylazine cocktail was given. Once the pup was fully anesthetized, as determined by a toe pinch, the hair on top of the skull was shaved and the exposed skin was disinfected with a betadine scrub solution and alcohol. Ophthalmic ointment was administered on the eyes of the animals to keep the eyes lubricated.

A midline incision, approximately 0.5 inches in length, was made on the scalp. One to three drops of bupivicaine was applied to the exposed skull as a topical/local analgesic. Connective tissue was cleared away from the surface of the skull using a scalpel to scrape the periosteum. A 26-guage needle was then used to drill 4 small holes into the soft skull: two bilateral, immediately posterior to the bregma, and two bilateral, anterior to lambda. Two triangular-shaped, sterile wire skull hooks were inserted into the holes located at the front and back of the skull to serve as anchors for the electrodes and dental acrylic.

The EMG electrode was threaded through the upper eyelid muscle of the left eye, with the end of the wire terminating at the outer surface of the eyelid with a 1-2 mm portion bent up away from the eye. Bipolar electrodes were placed subcutaneously with the tips in a v-shape immediately caudal to the left eye. Electrode connectors were secured to the skull with dental acrylic. Immediately following surgery, subcutaneous injections of buprenorphine (0.003 mg/g b.w.) were given to manage post-operative discomfort. Animals were placed in a recovery chamber on a heating pad and monitored during recovery from anesthesia. Subjects were then returned to individual housing and allowed to recover one more day prior to the start of behavioral testing. They remained individually housed for the remainder of the study.

Eyeblink conditioning apparatus. Animals were allowed to move freely in a Plexiglas test chamber (28 x 24 x 30 cm) with a stainless steel grid floor contained within a sound-attenuating chamber (Med Associates, Inc., St. Albans, VT). The chamber was equipped with a fan (background noise level 65-70 dB), a dim light (15W), and two speakers (2-12 Hz range) that were used to deliver the conditioned stimulus (CS). The shock, or unconditioned, stimulus (US) was produced by a constant-current, 60 Hz square wave stimulator (World Precision Instruments, Sarasota, FL) set to deliver a 1.5 mA shock lasting 100 ms. During conditioning sessions, the electrodes were secured to the animals' skull via wire leads that passed through an opening in the chamber to a commutator suspended above the chamber to allow maximum mobility. A custom-built Eyeblink Conditioning System (JSA Designs, Raleigh, NC) controlled stimulus presentations and recorded EMG activity from the eyelid.

Trace Eyeblink Conditioning procedures. The procedures for eyeblink conditioning involved presentation of a 2.8 kHz, 90 dB tone CS for 380 ms, followed by a 500 ms stimulus-free trace period, and then a 100 ms, 1.5 mA periocular shock US creating an 880 ms interstimulus interval (see Figure 2). The training protocol consisted of 6 conditioning sessions administered over 2 days (three sessions/day at 4-5 hour intervals). Each session consisted of 100 trials, 90 paired CS-US trials and 10 CS-alone test trials. The intertrial interval averaged 30 s.

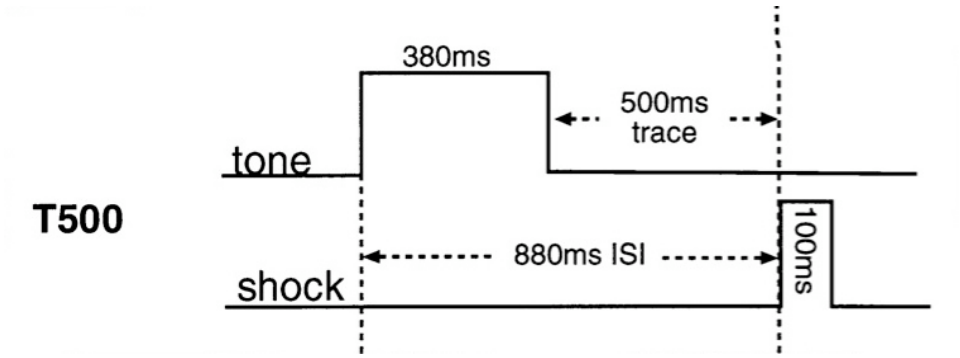

Figure 2. Trace eyeblink conditioning paradigm.

Behavioral recordings

Eyeblink behavior was monitored using EMG. EMG signals were sampled in 3.5 ms bins during the 1400 ms trial epoch. The raw signal was amplified (5 K), rectified (bandwidth 500 Hz to 5 kHz) and integrated (20 ms time constant) for quantitative analysis. The threshold for registering an EMG response was set at 0.4 arbitrary EMG units above the average baseline amplitude calculated during the pre-CS period (280 ms prior to CS onset).

Measures of learning. Learning was measured with a focus first on a subset of conditioned responses called "adaptive" conditioned responses (CRs). These eyeblinks occurred within the 200 ms just prior to the US onset, or 680 ms after tone onset. Overall total CRs, any responses occurring after an initial 80 ms startle period (see *Control Measures,* below), were also analyzed. CRs were defined as any muscle response exceeding a threshold of 0.4 V- EMG units. CR percentage (CRP) and CR amplitude (CRA) were calculated using paired CS-US trials that make up 90% of the training sessions in order to capture the acquisition process. These measures reflect the frequency and strength, respectively, of anticipatory responses of the US. The adaptive CR measure

is more sensitive to well-timed responses whereas the total CR measure captures any anticipatory response. An increase in CRP or CRA over the course of training is an indication of learning. Other measures that further characterize the conditioned or learned response include onset latency (CL) and maximum peak latency (CML). CL and CML were calculated using tone-alone trials on which a CR occurred (10% or less of training data) so that response timing would not be contaminated by a US occurrence. Changes in latency reflect anticipation or preparation for the US in its absence.

Control measures. Differences in sensitivity to the CS or US can influence acquisition of eyeblink conditioning, and are therefore compared to the pattern of behavioral differences in acquisition. The startle response (SR) is used to measure sensitivity to the tone, and the unconditioned response (UR) is the standard measure of sensitivity to the US. Possible SRs were measured during the first 80 ms after the CS, indicating a reflexive/orienting response to the sound. The UR was measured 140 ms after the US, indicating a reflexive response to the shock. The percentage of startle responses (SRP) and maximum amplitude of unconditioned responses (UMA) were analyzed as measures of stimulus effectiveness and subject sensitivity.

Data Analysis. The design of this study was 3 (Dose) x 2 (Sex) x 6 (Session). Data were analyzed using repeated-measures ANOVAs. Data were analyzed separately for different measures of learning (percentage, amplitude and latency of conditioned responses, as described above), as well as for control measures of sensory processing (percentage of startle responses and amplitude of unconditioned responses). For all repeated-measures ANOVAs, where assumptions of sphericity were violated, Huynh-Feldt corrected degrees of freedom and *F*-values are reported. Post-hoc Tukey tests for

multiple comparisons were performed on data that demonstrated a significant main effect on a between-groups variable. Significant 3-way interactions were further analyzed using simple ANOVAs followed by simple means analysis with Bonferroni's adjustment, where applicable. Data were analyzed using SPSS statistical software.

RESULTS

Eyeblink conditioning was affected by CORT treatments, but only in males, and only during Session 1 of acquisition. Significant differences were found for CRP between the high dose and control treatments. The high dose males produced significantly more CRs than the control males. There were no significant differences found for females. For each measure there was a significant main effect of session, indicative of acquisition of the CR in trace eyeblink conditioning for all groups across training.

CR Percentage

Adaptive CRs. There were no main effects of dose or sex. There was a significant main effect of session, as expected, reflecting learning and an increased percentage of adaptive CRs over the conditioning period, F (2.6, 133.5) = 82.4, p = 0.001. A significant three-way interaction of Dose x Sex x Session was also observed, F (5.2, 133.5) = 2.3, p = 0.048. To follow up, separate ANOVAs (Dose x Session) were performed for males and females. There were no significant differences found for the females. Females, therefore, were not analyzed further. However, a significant Dose x Session interaction was found for males, F (5.3, 66.6) = 2.4, p = 0.045 (See Figure 3A). Post-hoc analysis revealed that the effect was due to group differences in Session 1 only

($p = 0.043$). A post-hoc Tukey test showed a significant difference between the high dose males and the control males in Session 1 ($p = 0.029$). The males in the high dose treatment group produced significantly more adaptive CRs (58.6 ± 8.0% s.e.m.) than the control-treated males (28.8 ± 7.2% s.e.m.), indicating that the high dose treatment facilitated the acquisition of trace conditioning in males during Session 1 only. For comparison, the low dose group produced 45.8 ± 7.6% s.e.m. CRs. It is important to note that all the facilitation occurred in Session 1 and that all groups started out the same (see Figure 3B). Analysis of Session 1 alone [3 (Dose) x 10 (Blocks)] confirmed that there was a main effect of group [$F(2, 25) = 3.8$, $p = 0.036$], and a main effect of block [$F(9, 225) = 17.2$, $p < 0.001$], but no Dose x Block interaction. High dose males started at 27.5% but increased to 76.5% over the 10 blocks in Session 1. This confirms that learning was occurring across the first session for all groups, and these animals were not starting training at 58.6%. Subsequently, across Sessions 1- 6, the high dose group showed no significant changes in CRs (Session (S) 1 vs. S2, n.s.; S1 vs. S3, n.s.; S1 vs. S6, $p < 0.001$). The low dose group showed significant changes in CRs occurring in Sessions 3-6 (S1 vs. S2, n.s.; S1 vs. S3, $p < 0.001$; S1 vs. S6, $p < 0.001$), and the control group showed significant changes in CRs occurring in Sessions 3-6 (S1 vs. S2, n.s.; S1 vs. S3, $p < 0.001$; S1 vs. S6, $p < 0.001$).

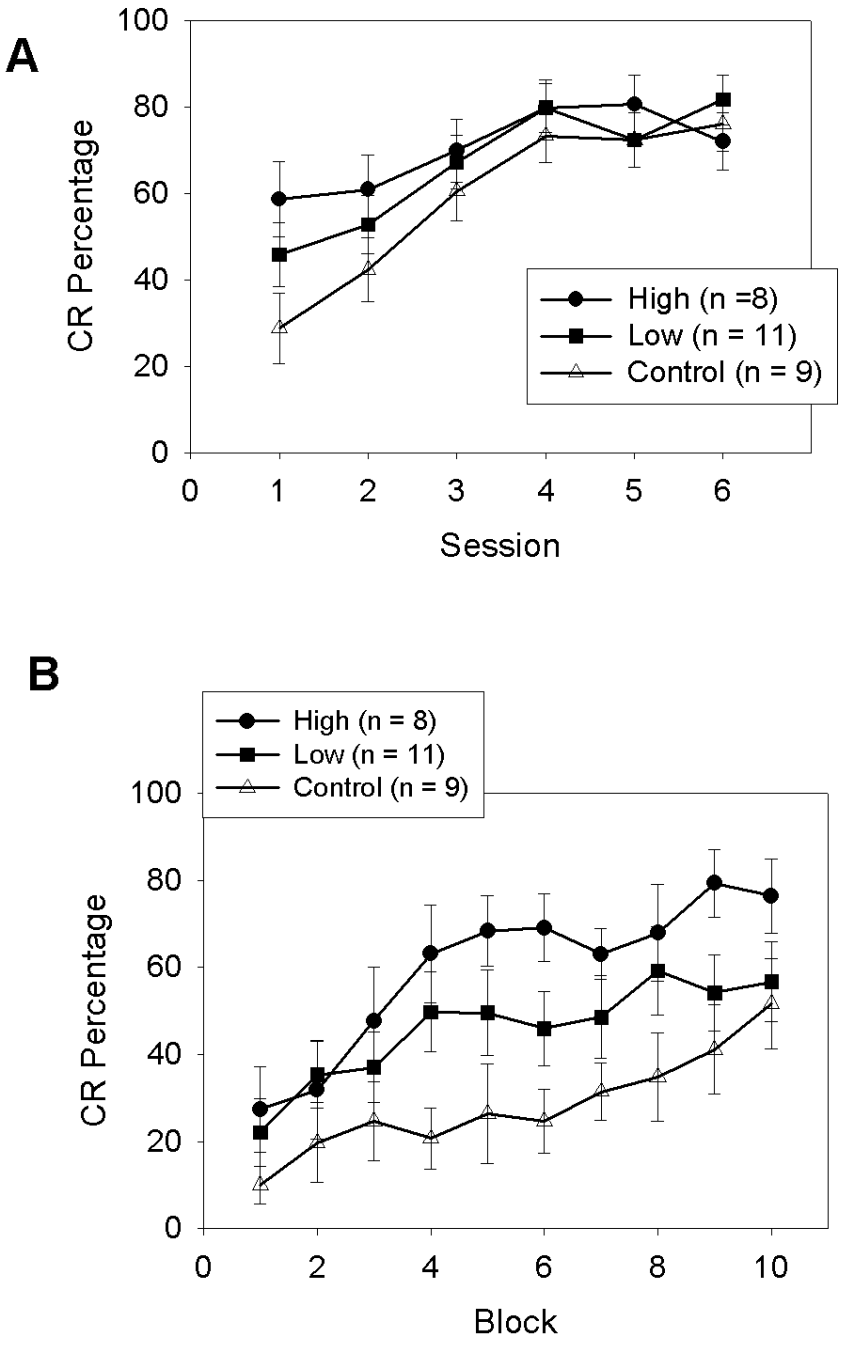

Figure 3. Mean (± s.e.m.) percentage of adaptive CRs over 6 training sessions (A) and over 10 blocks of paired CS-US trials in Session 1 (B) for males only.

Total CRs. A similar pattern of effects was observed for the total number of CRS occurring anywhere within the defined anticipatory window. There were no main effects for dose or sex. There was a significant main effect of session [F (2.8, 142.6) = 28.1; p = 0.001]. A significant three-way interaction of 3 (Dose) x 2 (Sex) x 6 (Session) was observed for percentage of total CRs, F (5.6, 142.6) = 2.5, p = 0.026, so separate ANOVAs (Dose x Session) were performed for males and females. Again, there were no significant differences for females, but a significant Dose x Session interaction for males, F (5.1, 64.7) = 3.1, p = 0.013 (see Figure 4A). Post-hoc multiple comparisons, however, revealed no significant dose differences within any particular session, but rather different learning curves across the sessions. The high dose animals did not demonstrate significant changes in total CR responses across sessions (S1 vs. S6, n.s.), whereas the low dose animals began to increase total CRs in Session 4 relative to Session 1 (S1 vs. S2, n.s.; S1 vs. S4, $p < 0.05$; S1 vs. S6, $p < 0.05$), and control animals increased gradually across all sessions (S1 vs. S2, n.s.; S1 vs. S3, $p < 0.05$; S1 vs. S6, $p < 0.001$). Visual inspection of Session 1 (see Figure 4B) revealed that both CORT groups appeared to produce more anticipatory responses overall, despite the lack of statistical significance.

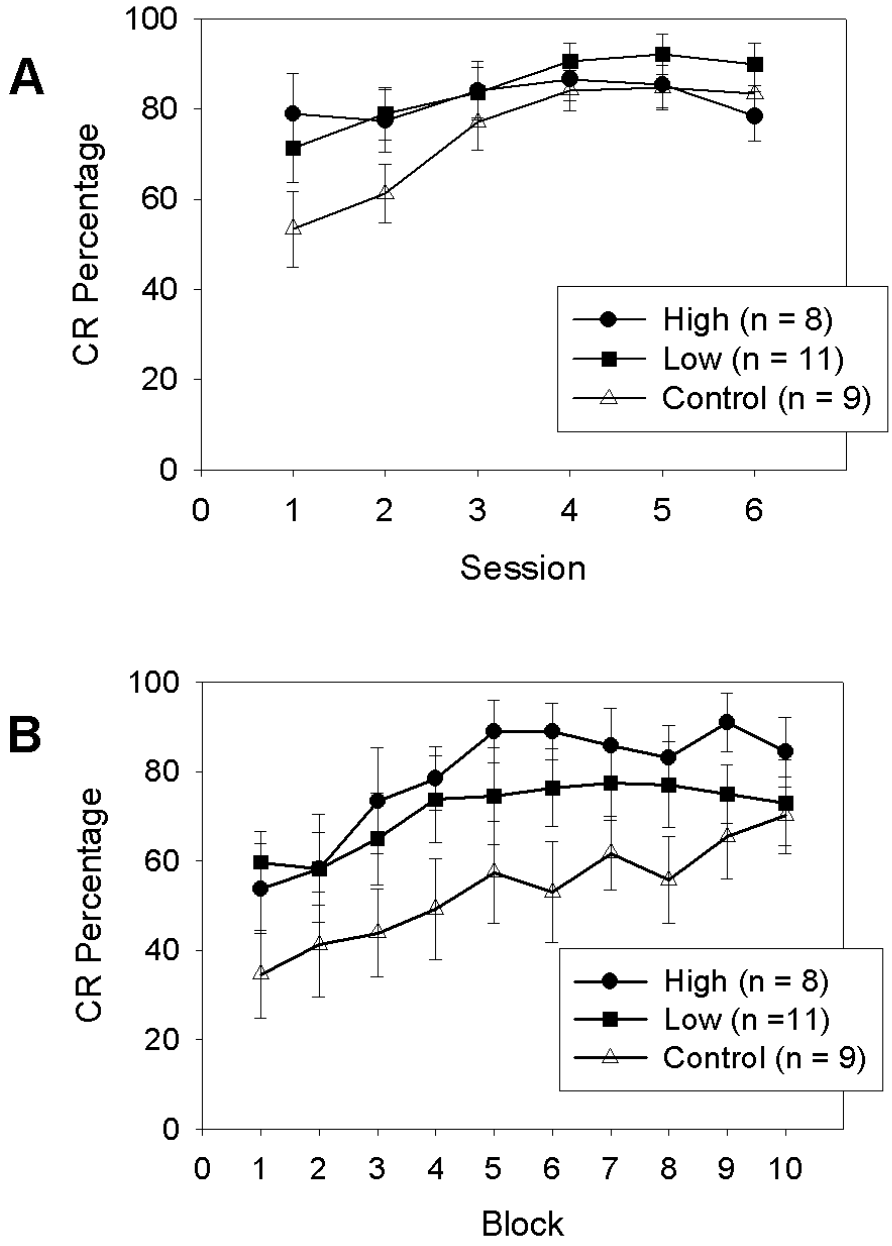

Figure 4. Mean (± s.e.m.) percentage of total CRs over 6 training sessions (A) and over 10 blocks of paired CS-US trials in Session 1 (B) for males only.

CR Amplitude (CRA)

Adaptive and Total CR Amplitude. There was a significant main effect of session in adaptive and total CR amplitude [F (3.0, 152.6) = 42.9, p = 0.001 and F (2.9, 149.6) = 26.7, p = 0.001, respectively], reflecting increasing CR amplitudes over the conditioning period, but there were no main effects of dose or sex in these measures. Also, there were no significant two-way or three-way interactions using the same design [3 (Dose) x 2 (Sex) x 6 (Session)].

Onset Latency (CL) and Peak Latency (CML)

There was a significant main effect of session for both onset and peak latency reflecting responses gradually moving later in the trial period and closer to the US onset [F (4.0, 203.3) = 25.7, p = 0.001 and F (2.2, 113.9) = 66.9, p = 0.001, respectively]. There were no main effects of dose or sex in these measures. There were no significant three-way or two-way Dose x Sex x Session differences.

Control measures

There were no significant differences found in UR amplitude or SR percentage between groups. Therefore, differences in sensitivity to the CS and US did not contribute to differences in learning.

DISCUSSION: EXPERIMENT 2

The only measure of trace eyeblink conditioning affected by CORT was the percentage of CRs, in both adaptive and total measures. Interestingly, this effect was only seen in males, consistent with earlier studies (Claflin et al., 2005; Greenfield et al., 2009); however, CORT *increased* CR production rather than impairing it. The

facilitation of learning occurred dramatically very early in acquisition. Although improvement of learning was not expected, such behavior is consistent with an acute or low dose elevation of glucocorticoids (Mateo, 2008; Pavlides et al., 2004). Below we discuss further the broader implications of these data and the importance of certain methodological details.

IV. GENERAL DISCUSSION

In Experiment 1, we determined that plasma concentrations of the high dose of CORT remained elevated for at least 4 hours. Therefore, Experiment 2 followed with a study of CORT effects on later trace eyeblink conditioning. We thought that by giving two CORT injections per day over 3 days, we would see elevated circulating CORT levels in the pharmacological range long enough to potentially produce significant lasting impairment on acquisition of trace eyeblink conditioning. Based on previous studies, we expected to see a significant impairment in learning, especially for males. Whereas the present study demonstrated no effects of CORT for females during later eyeblink conditioning, there was *improvement* seen for males, but only those treated with the high dose of CORT, and only during Session 1. These findings are inconsistent with previous CORT administration studies from this lab showing impairment for males given high doses of CORT via pellets (~ 80 μg/dl; Claflin et al., 2005) and osmotic mini-pumps (~ 12 μg/dl; Greenfield et al., 2009).

One reason for the discrepancy in findings may have to do with the variability in CORT elevation. Hermann et al. (2009) recently demonstrated that the consistency of

plasma elevation with a variety of delivery methods was lacking in terms of both level and duration. Specifically, they reported that pellets delivered high levels of CORT by 24 hours, but dropped dramatically to zero at 7 days instead of the advertised 21 days, similar to data reported in Claflin et al. (2005). Osmotic mini-pumps failed to consistently alter plasma levels, and injections only elevated CORT levels for about 4 hours, consistent with our data in Experiment 1. It is interesting to realize that many researchers use prefabricated pellets and pumps to deliver drugs and measure the effects of the drug on behavior without carefully monitoring circulating drug levels in the plasma.

As previously mentioned, there is thought to be an inverted U-shaped relationship between glucocorticoids and cognition: very high and very low levels impair the acquisition and retention of memories, and moderate levels facilitate acquisition and retention (Mateo, 2008; Pavlides et al., 2004). Regardless of methodological differences in administering CORT, a behavioral difference was observed in the present study. Although the effect was small and only apparent during the first training session, a distinct improvement was seen for CORT-treated males. If we consider the U-shaped function mentioned above and the behavior observed, the acute nature of the dosing of CORT (rapid elevations and declines twice a day for 3 days) may have produced similar effects to a low or moderate but longer lasting CORT elevation that could actually contribute to the improved cognitive performance, even on a task as basic as classical eyeblink conditioning.

In other studies, acute stress activated by short-term CORT injections was shown to cause antidepressant-like effects in rats (Sandi et al., 1997). Similarly in mice, Keeney

et al. (2006), reported that exposure to an acute social defeat stress elicited an increase in serotonin levels in the hippocampus, possibly reducing detrimental effects of the stress-induced increase in CORT levels. In this study, it is possible that we have delivered a moderate, acute, physiological dose of CORT sufficient to produce facilitation of the conditioned response for males along one of these mechanisms. It is important to remember, however, that CORT was administered 10 days prior to conditioning, and so the effects were lasting. More research is needed to understand the mechanisms underlying any change in brain function resulting in improvement of learning. Perhaps in order to get a sustained, long-term damaging effect, CORT must be delivered over a substantially longer period of time. As shown by Sapolsky, Krey, & McEwen (1985), significant hippocampal degeneration was present following 90 days of injections in adult rats. In contrast, Zhao, Weidon, Dai, Wang, & Huang (2009) delivered CORT injections for 6 days and found no behavioral differences. However, in our experience such a long administration period is not necessary for deleterious effects in young developing organisms. CORT exposure for 1-7 days after the SHRP (osmotic mini-pump vs. pellets) produced measurable declines in trace eyeblink conditioning for male rats (Claflin et al., 2005; Greenfield et al., 2009).

Sex-related differences in vulnerability to glucocorticoid effects have been reported in many studies. These differences are very complicated and depend on the duration of the stressor (acute or chronic), and the age of the subject. Acute stress, as argued in the current study, has a facilitating effect on adolescent males during trace eyeblink conditioning. Similarly, acute stress facilitated performance in adult males in the Morris water maze (Sandi et al., 1997). Adolescent female rats appear to be more

resistant to acute stressors in an open field test (Spivey et al., 2009). While acute stress may facilitate performance for young rats, an opposite effect may appear for adults. Adult female rats that experienced acute stress displayed impaired learning during trace eyeblink conditioning (Hodes & Shors, 2005; Wood et al., 2001; Wood & Shors, 1998). Alternatively, experiencing prolonged, chronic stress may produce negative effects rather than facilitation. Conrad, Grote, Hobbs, & Ferayorni (2003) reported that in adult rats, chronic stress impaired the performance of both males and females in the first minute of testing in a Y-maze (spatial learning task), but stressed females quickly recovered after the first minute and males continued to perform poorly. Again, Claflin et al. (2005) found that adolescent male rats were impaired during trace eyeblink conditioning when CORT was delivered via subcutaneous pellets lasting about one week. These sex-specific differences are likely due to adrenal and reproductive hormones and differences in glucocorticoid receptor expression throughout development (Wood et al., 2001; Matthews, 2001; Owen & Matthews, 2003).

The implications of this research and other similar studies are important for medical treatments using glucocorticoids and people suffering from acute vs. chronic stress. It is important for us to gain an understanding of the glucocorticoid levels and exposure durations that might be beneficial versus detrimental. This knowledge could prevent devastating stress-related psychiatric disorders showing a higher prevalence in males versus females, such as attention deficit hyperactivity disorder (ADHD) and schizophrenia (Cuffe, Moore, & McKeon, 2005; Iacono & Beiser, 1992). Also, diseases such as schizophrenia and posttraumatic stress disorder (PTSD) have been associated with a reduction in hippocampal volume (Findling et al., 1995; Shin et al., 2004). Patients

with PTSD have a lower hippocampal volume and altered function relative to healthy controls, supporting the notion that increased glucocorticoids are detrimental to the hippocampus, even as an adult (Bremmer, 2006). What is unclear from these studies, but is an emerging line of research, is the possibility that vulnerability is established early in development and that adult experiences trigger disease onset (Brunson et al., 2005). Because of a lack of long-term follow up on the administration of neonatal glucocorticoids, we cannot know for certain if early glucocorticoid treatments create a sex-specific vulnerability to neurological diseases. Further studies are needed to examine critical periods in the developmental timeline during which stress or elevated glucocorticoids may produce lasting detrimental neurophysiological effects.

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