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SHORT-TERM, HIGH-DOSE ADMINISTRATION OF CORTICOSTERONE BY INJECTION FACILITATES TRACE EYEBLINK CONDITIONING IN YOUNG MALE RATS

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Abstract

Glucocorticoids released as part of the physiological response to stress are known to affect cognitive function, presumably via effects on the hippocampus. Trace classical eyeblink conditioning is an associative learning task which depends on the hippocampus and has been used to examine the development of learning processes in young mammals. Previously, we demonstrated deficits in trace eyeblink conditioning associated with postnatal administration of the glucocorticoid corticosterone by creating a sustained elevation with methods such as subcutaneous timed-release pellets and osmotic mini-pumps which were active over several days. In the present study, we examined the effects of an oscillating pattern of corticosterone elevation on subsequent trace eyeblink conditioning. Twice daily corticosterone injections (high, low, or vehicle) were administered over a 3-day period, starting at postnatal day 15. Then, on postnatal day 28, animals underwent trace classical eyeblink conditioning to examine the possible influence of earlier corticosterone elevations on the development of 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. These data demonstrate that oscillating corticosterone elevations produce opposite effects on this associative learning task than do sustained elevations.

Keywords

trace conditioning; eyeblink; corticosterone; hippocampus; development; learning

1. Introduction

Glucocorticoids play an important role in cognitive functions. Most often, fluctuations in glucocorticoid levels are associated with learning and memory deficits (for reviews, see

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Alderson & Novack, 2002; Lupien, Maheu, Tu, Fiocco, & Shramek, 2007; Lupien, McEwen, Gunnar, & Heim, 2009). However, glucocorticoids can also enhance learning (Roozendaal, 2002; 2003). The effects are typically described as having an inverted-U shaped relationship such that very high and very low levels of glucocorticoids impair learning, whereas moderate levels tend to facilitate learning and memory processes (Salehi, Cordero, and Sandi, 2010; Yerkes and Dodson, 1908). Glucocorticoid effects also depend on a number of other variables including task complexity, contextual and temporal factors, and the duration of the glucocorticoid exposure (reviewed in DeKloet, Oitzl, & Joels, 1999; Diamond et al., 2005; Pavlides, Watanabe, & McEwen, 2004; Salehi et al., 2010; Mateo, 2008).

The brain region most associated with learning and memory is the hippocampus. A large number of glucocorticoid receptors are found in the hippocampus, making it particularly sensitive to changes in glucocorticoid levels produced by both endogenous and exogenous sources (Bohn, 1984; Sapolsky, Meaney, & McEwen, 1985; Weinstock, 1997). It is suggested that the differential effect of glucocorticoids on hippocampal function is mediated by the ratio of occupied glucocorticoid receptor types, Type 1 (mineralocorticoid; MR) and Type II (glucocorticoid; GR). Activation of MRs, such as occurs under resting conditions, appears to *facilitate* hippocampal function, enhancing LTP and improving spatial memory in the Y-maze. Memory consolidation is impaired in the absence of glucocorticoids following adrenalectomy or administration of antagonists. In contrast, activation of GRs, such as occurs during exposure to stressors, appears to *impair* hippocampal excitability associated with learning plasticity, suppressing LTP and impairing performance on spatial memory tasks (see reviews: Conrad, 2005; deKloet et al., 1999; McEwen, 2006; Roozendaal, 2002). Prolonged glucocorticoid elevations have been found to have neurotoxic effects on the hippocampus in adult humans and in other animals, including rats (for review, see Lupien & McEwen, 1997; Lupien et al., 1998; Sousa, Madeira, & Paula-Barbosa, 1998). However, *short-term* glucocorticoid elevations have been shown to support neural plasticity and normal learning, specifically post-training memory consolidation (Keleman, Bahrendt, Born, & Inostroza, 2014; Sousa et al., 1998).

The neurotoxic effects of glucocorticoids produce devastating effects on brain development during the perinatal period. Neonatal glucocorticoid administration inhibits differentiation and growth of neurons and glia in widespread areas, but particularly those undergoing extensive postnatal differentiation, including the hippocampus (Bohn, 1984; deKloet et al., 1988; Ferguson, Paule, & Holson, 2001; Gould, 1999; Takahashi, 1997; Uno et al., 1994). A number of behavioral outcomes, including cognitive impairment, have been observed across several species. Glucocorticoid treatments for neonatal respiratory distress and asthma in children have been associated with deficits in verbal memory (Bender, Lerner, & Kollasch, 1998; Bender, Lerner, & Polland, 1991), but may also preferentially impair other cognitive tasks known to be mediated by the hippocampus (Ragnarsson et al., 2013; Yeh et al., 2004).

In studies of glucocorticoid administration three commonly used routes of administration are injection, commercially-available, timed-release, surgically-implanted biodegradable pellets, and precision-release osmotic minipumps. Glucocorticoids administered by these different methods can produce differences in temporal pattern as well as in blood levels. Pellets and

minipumps are intended to produce prolonged constant elevations, whereas injections produce relatively short-lived increases and decreases in blood levels. But even methods intended to produce constant levels might not always do so. Hermann et al. (2009) observed little consistency of circulating concentrations with different drug delivery methods, in terms of both plasma level and duration of elevation. Specifically, they reported that: (1) corticosterone pellets produced high levels at 24 hours that dropped dramatically to zero at 7 days instead of the intended 21 days. (2) osmotic mini-pumps failed to consistently alter plasma levels, and (3) injections only elevated corticosterone levels for about 4 hours. When applied to studies of learning, these results raise the possibility that performance may be affected not only by the intended dose level, but also by the inconsistency and variability in hormone administration that might follow the use of pellets or osmotic pumps. Further, the relatively short-lived increase and then decrease in circulating levels one sees following injection may also be important, in as much as Liston et al. (2013) recently reported that oscillating levels of corticosterone, such as occur during the natural circadian cycle facilitate the formation of learning –induced synapses at the peak of the cycle, whereas a prolonged constant elevation disrupted these same processes.

In a previous study we used corticosterone pellets to assess the effects of elevated hormone levels on hippocampal-mediated associative learning during the preweaning period. The neural substrates of trace classical eyeblink conditioning have been well established and are known to be hippocampus-mediated (Ivkovich & Stanton, 2001; Moyer Deyo & Disterhoft, 1990; Weiss, Boumeester, Power, & Disterhoft, 1999). We found that the pellets, implanted subcutaneously on postnatal day 15, produced an initial marked elevation in circulating corticosterone reaching a pharmacological level of 80µg/dl on Day 3 post-implant which returned to control levels by Day 6. Associative learning, as measured by an increase in conditioned responding using trace eyeblink conditioning procedures was assessed on postnatal day 28. Learning was impaired, but only in males, suggesting that the effects of corticosterone on learning lasted beyond the drug's effective period and that sex differences in vulnerability to corticosterone were present at this stage in development (Claflin et al., 2005). In order to determine the effects of corticosterone within a more relevant *physiological* range, we then implanted subcutaneous minipumps designed for a slow and constant rate of release over 3 days. The result was corticosterone levels in the moderate physiological range of 12 µg/dl at 24 hr. Once again learning was impaired, but in this case males and females showed comparable deficits (Claflin et al., 2014)

The present study examined whether corticosterone elevations at the same approximate levels would produce the same pattern of deficits if corticosterone was administered by injection. Rats were injected twice daily with doses producing peak plasma levels that approximated those of our earlier studies with pellets and minipumps. Injections continued over 3 days to mimic the length of exposure in the previous studies. Effects on trace eyeblink conditioning were then assessed 10 days later.

2. Material and methods

2.1. 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 until weaning 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. Animals were randomly assigned to one of three dose groups (high, low and control) and groups were roughly balanced for sex.

Starting on PND 15, two subcutaneous injections were given daily for 3 days, one at 0900 and one at 1700 hrs. 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 on PND 28-29. After surgery, and during behavioral training, animals were housed individually.

The final data are derived from 57 Long-Evans rat pups, 29 female and 28 male sampled from 15 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 20 pups (12 f, 8 m), the low dose group consisted of 20 pups (9 f, 11 m) and the control group consisted of 17 pups (8 f, 9 m).

2.2 Procedures

2.2.1 Injections—Corticosterone (*Sigma* C2505) was dissolved in sesame oil (*Sigma* S-3547), vortexed and placed in a warm water bath 24 hours prior to injections and maintained at 37°C until injections were completed. In order to establish doses that produced blood levels of corticosterone that at least roughly approximated the pharmacological levels obtained in our previous study with mini-pumps (Claflin et al., 2014), a pilot study was conducted in which Day 15 pups were injected at 0730-0800 hours with one of three doses of corticosterone (5, 10, and 20 mg/kg body weight) and had blood samples collected at either 1, 2, 4, 8, 16, or 24 hr. Based on the results (Fig. 1), we chose the low and high doses (5 and 20 mg/kg) for inclusion in the main study. The low dose peaked in the physiological range (44.2 ug/dl), whereas the high dose produced elevations that peaked in the pharmacological range (103 ug/dl). Therefore, in the present study, pups received either 5 or 20 mg/kg body weight of corticosterone or were injected with the sesame oil vehicle.

The decision to inject on PND 15 was made both for consistency with our previous studies and to avoid the stress-hyporesponsive period (SHRP) in the rat which occurs between Days 4 and 14. During the SHRP, endogenous stress hormones are greatly reduced and minimally responsive to increases in stressful stimuli (Sapolsky & Meaney, 1986; Walker, Scribner, Cascio, & Dallman, 1991). The SHRP is followed by a peak of endogenous corticosterone around PND 15, and a continual rise into at least the fourth week of life, aiding in development (Walker, Perrin, Vale & Rivier, 1986). Because the rat is especially sensitive to

the damaging effects of glucocorticoids during the SHRP, exogenous glucocorticoids at this time would likely lead to widespread undifferentiated neural damage. We chose to study the effects of glucocorticoids at a time after the SHRP when the physiological responses are returning to normal, selective effects on neural development in different brain regions would seem more likely and the hippocampus is undergoing substantial developmental changes.

2.2.2 Blood collection—For the pilot study, animals within each dose and sex group were randomly assigned to have blood samples collected at 1, 2, 4, 8, 16 or 24 hours after the injection. Animals were anesthetized with CO₂ prior to blood sampling via decapitation. Blood was collected within less than 4 minutes of disturbance in order to minimize any effect of the procedure on corticosterone levels in the samples obtained (Coover et al., 1979). Blood was centrifuged to separate plasma, which was then frozen until assay at a later date. Assays were run in duplicate with a radioimmunoassay kit (Siemens, ¹²⁵I Rat Corticosterone). Intra- and Inter-assay variability was calculated to be less than 5%.

2.2.3. Eyeblink Electrode Surgery—On PND 26, two days before conditioning procedures took place, 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.

Each animal was anesthetized with a ketamine and xylazine cocktail (75 mg/kg ketamine, and 5 mg/kg xylazine) administered intraperitoneally. A midline incision was made on the scalp and two triangular-shaped, two-pronged wire hooks were inserted between bregma and lambda landmarks to serve as anchors for the electrodes and dental acrylic. The two EMG electrode wires were threaded through the upper eyelid muscle of the left 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 (3 mg/kg) 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.

2.2.4. Eyeblink conditioning apparatus—Animals were allowed to move freely in a Plexiglas test chamber (28 × 24 × 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 kHz 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 secured to the animals' heads were connected to 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.

2.2.5. Trace Eyeblick Conditioning procedures—Six eyeblick conditioning sessions were administered over PND 28 and 29 as described below. This age is one at which trace conditioning is robust but it precedes adolescence which begins around PND 32. We now appreciate that adolescence is another developmental period during which unique and complex changes take place. The procedures for eyeblick 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.

2.2.6. Behavioral recordings—EMG signals from the eyelid 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).

2.2.7. Measures of learning—Learning was measured by an increase in the percentage of conditioned responses (CRs) across the 6 conditioning sessions. “adaptive” CRs were eyeblicks that occurred within the 200 ms just prior to the US onset, or 680 ms after tone onset. The “total” CR measure took into account all responses occurring after an initial 80 ms startle period (see *Control Measures*, below). 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 best 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.

2.2.8. Control measures—Differences in sensitivity to the CS or US can influence acquisition of eyeblick conditioning, and were therefore compared to the pattern of behavioral differences observed during acquisition. The startle response (SR) was used to measure sensitivity to the tone, and the unconditioned response (UR) was the standard measure of sensitivity to the US. SRs were responses occurring during the first 80 ms after the CS, indicating a reflexive/orienting response to the sound. URs were measured 140 ms after the US, indicating a reflex 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.

2.3. Data Analysis

The design of this study was 3 (Dose) $\times$ 2 (Sex) $\times$ 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.

3. Results

Eyeblink conditioning was affected by corticosterone treatments, but only in males, and only during Session 1 of acquisition. Significant differences were found for CR percentage between the high dose and control treatments. The high dose males produced significantly more CRs than the control males. There were no significant treatment 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.

3.1. CR Percentage

3.1.1. 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 $\times$ Sex $\times$ Session was also observed, $F(5.2, 133.5) = 2.3$, $p = 0.048$. To follow up, separate ANOVAs (Dose $\times$ Session) were performed for males and females.

There were no significant differences found for the females. Females, therefore, were not analyzed further (see "Supplemental Materials" for illustration female data). However, a significant Dose $\times$ 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 ($58.6 \pm 8.0\%$ s.e.m.) than the control-treated males ($28.8 \pm 7.2\%$ 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 $45.8 \pm 7.6\%$ s.e.m. CRs. It is important to note that all the facilitation occurred in Session 1 (see Figure 3B). Analysis of Session 1 alone [3 (Dose) $\times$ 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 $\times$ Block interaction. Although the interaction was not significant, inspection of Figure 3 indicates that divergence among the groups became apparent after the first several blocks of the first session. High dose males started at 27.5% and increased to 76.5% over the 10 blocks in Session 1.

3.1.2. Total CRs—A similar pattern of effects was observed for the total number of CRs. There were no main effects for dose or sex. There was a significant main effect of session [$F(2.8, 142.6) = 28.1; p = 0.001$] and a significant three-way interaction of 3 (Dose) $\times$ 2 (Sex) $\times$ 6 (Session), $F(5.6, 142.6) = 2.5, p = 0.026$, so separate ANOVAs (Dose $\times$ Session) were performed for males and females.

Again, there were no significant treatment differences for females, but a significant Dose $\times$ Session interaction for males, $F(5.1, 64.7) = 3.1, p = 0.013$ (see Figure 4A). Although post-hoc multiple comparisons revealed no significant dose differences within any single session, visual inspection of Figure 4 once again reveals different learning curves across the sessions with both corticosterone groups producing more CRs overall and early in training, as if asymptote has been reached in Session 1 (see Figure 4B).

3.2. CR Amplitude (CRA)- Adaptive and Total CR

There was a significant main effect of session for both 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 or interactive effects of dose or sex in these measures.

3.3. 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 (more finely tuned) 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 two-way or three-way interactions.

3.4. 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. The mean percentage of startle responses for the high, low, and control groups during the first conditioning session were 22.4 ± 10 , 36.8 ± 9 , and 29.7 ± 12 , respectively, and remained steady across all 6 sessions. This pattern in SR percentage did not parallel group differences in learning. UR amplitude was very similar across groups averaging 9.7 ± 10 EMG units.

4. Discussion

In the pilot experiment, we determined that plasma concentrations of corticosterone produced by the high dose remained elevated for at least 4 hours. Our earlier studies showed

that a sustained high dose produced impairment of the acquisition of trace eyeblink conditioning in male rats and led us to expect the possibility that males would be more sensitive to changes in corticosterone elevations. In fact, our data demonstrated a *facilitation* of learning in males. Whereas the present study demonstrated no effects of corticosterone for females, there was significant *improvement* in conditioning seen for males, but only for those treated with the high dose of corticosterone, and only during Session 1. The frequency of startle responding somewhat exceeded those one would like to observe in demonstration of associative learning effects. However, in our experience moderate variability in startle measures is not uncommon in studies of young rats. Moreover, our data analysis confirms that the startle response was consistent across training and did not mimic the group differences or changes in CR patterns across acquisition sessions. Our study is not alone in finding contrasting effects of corticosterone in developmental studies depending on means of delivery. Catalani et al. (2011) reported improved learning and reduced fearfulness in offspring of mothers administered CORT in the drinking water during lactation, whereas Brummelte et al., (2012) found that CORT injections during gestation and postpartum increased behavioral measures of anxiety and depression. Nonetheless, our current findings pose an interesting conundrum given previous corticosterone administration studies from this lab showing impairment, not facilitation, of learning for males given sustained doses of corticosterone via pellets (~ 80 µg/dl; Claflin et al., 2005) and osmotic mini-pumps (~ 12 µg/dl; Claflin et al., 2014).

If we consider the inverted U-shaped function of corticosterone's effects on cognition, the acute nature of the dosing in this present study (rapid elevations and declines twice a day for 3 days) may have produced behavioral effects such as one might expect to see with a sustained elevation intermediate to those produced by our earlier procedures and represented at the peak of the inverted U. That is, short-lived oscillating elevations in the pharmacological range may have effects greater than a sustained physiological elevation and less than a sustained pharmacological elevation, thereby facilitating learning.

It is important to remember, however, that the corticosterone was administered 10 days prior to conditioning, and so the effects lasted beyond the drug administration period. Administration of exogenous corticosterone just after the SHRP was shown to decrease baseline corticosterone levels at PND 30 and produce a prolonged response to a restraint challenge in adulthood (longer duration of secretion of corticosterone; Ordyan, Pivina, Rakitskaya, & Shalyapina, 2002). It is possible, therefore, that the early corticosterone injections modified later corticosterone levels and responses which in turn affected performance on the associative learning task. Another possibility is suggested by studies showing that increasing levels of corticosterone at the end of the SHRP (~ Day 15) engage the amygdala to promote the development of avoidance conditioning in developing rats (e.g., Moriceua, Roth, & Sullivan, 2010). It is known that, in addition to the hippocampus, the amygdala plays a modulatory role in eyeblink conditioning (Burhans and Schreurs, 2008, Ng & Freeman, 2014). It may be that the relatively brief, but large, elevations of corticosterone resulting from our injection procedure enhanced this effect on the amygdala, which then facilitated later learning to avoid the aversive blink-eliciting shock.

Perhaps in order to get a sustained, long-term damaging effect, corticosterone 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 corticosterone 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. It is striking that corticosterone exposure for 1-7 days after the SHRP (osmotic mini-pump vs. pellets) is sufficient to produce measurable declines in trace eyeblink conditioning for male rats (Claflin et al., 2005; 2014).

The hippocampus and the amygdala, while related to stress and HPA regulation, are not the only structures involved in eyeblink conditioning that may also be affected by glucocorticoids. The foundational circuitry for forming basic associations in eyeblink conditioning has been localized to the cerebellum and brainstem (for review, see Thompson, 2013). Administration of glucocorticoids during the SHRP period (PND 2-14) in rats impaired hippocampus-independent delay eyeblink conditioning in adulthood and reduced expression of the glucocorticoid receptors in the interpositus nucleus of the cerebellum (Wilber, Lin, Wellman, 2011). In addition, trace eyeblink conditioning, while engaging higher order brain regions such as the hippocampus during acquisition also involves the prefrontal cortex in later retrieval and execution of the learned response (Hattori, Yoon, Disterhoft, and Weiss, 2014). Prolonged stress and dysregulation of the HPA axis have been shown to impair cognitive abilities and affect prefrontal function by potentially several different pathways (dopamine, GABA, glutamate; e.g., see Mizoguchi, Ishige, Takeda, Aburada, & Tabira, 2004). Thus, while we believe the hippocampus and amygdala most likely underlie the effects we observed, other brain regions may also have contributed to the effects. Regardless of the explanation for the current findings, the data point to the need for better monitoring of plasma levels of corticosterone over time in studies such as these. Our findings, together with those of Hermann et al (2009), which specifically compared glucocorticoid delivery techniques, emphasize the need to carefully choose the technique that best fits a particular study's objectives, and then to monitor the circulating levels that result, a procedure that is not routinely adhered to in the literature. This may be particularly important in developmental studies given the moving baseline created by SHRP, the initial post-SHRP peak, and the gradual rise in glucocorticoid levels for several weeks after that aids in normal developmental processes.

Another point of interest for developmental scientists is the unique sensitivity of infant male rats to even small changes in corticosterone levels. 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. The sex-specific differences are likely due to adrenal and reproductive hormones and differences in glucocorticoid receptor expression throughout development (Takahashi, 1997; Wood et al., 2001; Matthews, 2001; Owen & Matthews, 2003). Twenty-four hours of maternal separation during the first week of life significantly increased neurogenesis in the already GC receptor rich hippocampus of male rats, but significantly decreased neurogenesis in female rats (Oomen et al., 2009). Other data suggest that adolescent female rats may be more resistant to acute stressors in a Y-maze or open field test (Conrad et al., 2003; Spivey

et al., 2009). It is also possible that there is a shift in the sex differences and vulnerability to acute stressors across development. Acute stress has been shown to facilitate performance of adult males in the Morris water maze (Sandi et al., 1997). While acute stress may facilitate performance for young male rats, an opposite effect may be observed for adults (Wolf et al., 2001). Indeed, adult female rats that experienced acute stress displayed impaired learning during trace eyeblink conditioning (Hodes & Shors, 2005; Wood et al., 2001; Wood & Shors, 1998).

The findings reported here highlight the importance of understanding the nature of changes in glucocorticoid levels and exposure durations that might be beneficial versus detrimental to cognitive function across the life span. In addition, this line of research may give us better insight into stress-related neurodevelopmental disorders that have a higher prevalence in males versus females, such as attention deficit hyperactivity disorder (ADHD) and schizophrenia (Cuffe, Moore, & McKeon, 2005; Iacono & Beiser, 1992). Further studies are needed to examine critical periods in the developmental timeline during which stress or elevated glucocorticoids may produce lasting detrimental neurophysiological effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

MR	Mineralocorticoid receptor
SR	Startle Response
GR	Glucocorticoid receptor
SEM	Standard Error of the Mean
LTP	Long Term Potentiation
HPA	Hypothalamic-Pituitary-Adrenal Axis
PND	Postnatal Day
SHRP	Stress Hyporesponsive Period
EMG	Electromyography
CPR	Conditioned Response Percentage
CRA	Conditioned Response Amplitude
CL	Response Onset Latency
CML	Latency to Maximum Peak

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HIGHLIGHTS

- Oscillating corticosterone elevations facilitate trace eyeblink conditioning
- Corticosterone's effects appear to be specific to male rats
- Direction of corticosterone effect on conditioning varies with delivery method

Figure 1.

Plasma levels of corticosterone at 1, 2, 4, 8, 16, and 24 hours (x-axis) after injection. Values represent mean plasma concentrations ($\mu\text{g/dl} \pm \text{SEM}$) for 4 dose groups, 0, 5, 10, or 20 mg/kg. Data from 4-7 subjects were used in each data point.

Figure 2. Trace eyeblink conditioning paradigm.

Figure 2.
Trace eyeblink conditioning paradigm.

Figure 3.

Mean ($\pm$ 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.

Figure 4.

Mean ($\pm$ 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.