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14. ABSTRACT
Recent interest in the lasting effects of early-life stress has expanded to include effects on cognitive performance. An increase in circulating glucocorticoids is induced by stress exposure and glucocorticoid effects on the hippocampus likely underlie many of the cognitive consequences. Here we review studies showing that corticosterone administered to young rats at the conclusion of the stress-hyporesponsiveness period affects later performance in hippocampally-mediated trace eyeblink conditioning. The nature and even direction of these effects varies with the elevation patterns (level, duration, temporal fluctuation) achieved by different administration methods. In general, constant glucocorticoid elevations resulted in hippocampus-mediated learning deficits, whereas acute, cyclical elevations result in improved initial acquisition. Sensitivity was greater for males than for females. Further, changes in hippocampal neurogenesis paralleled some but not all effects. The findings demonstrate that specific patterns of glucocorticoid elevation produced by different drug administration procedures can have markedly different, sex-specific consequences on basic cognitive performance and underlying hippocampal physiology. Implications of these findings for glucocorticoid medications prescribed in childhood are discussed.

15. SUBJECT TERMS
Glucocorticoids, Development, Eyeblink Conditioning, Trace Conditioning, Hippocampus, Neurogenesis, Sex Differences

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Influence of postnatal glucocorticoids on hippocampal-dependent learning varies with elevation patterns and administration methods

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Abbreviations (Non-standard; In order of appearance):
HPA: Hypothalamic-Pituitary-Adrenal Axis
SHRP: Stress Hyporesponsive Period
PND: Postnatal Day
SEM: Standard Error of the Mean

Abstract

Recent interest in the lasting effects of early-life stress has expanded to include effects on cognitive performance. An increase in circulating glucocorticoids is induced by stress exposure and glucocorticoid effects on the hippocampus likely underlie many of the cognitive consequences. Here we review studies showing that corticosterone administered to young rats at the conclusion of the stress-hyporesponsiveness period affects later performance in hippocampally-mediated trace eyeblink conditioning. The nature and even direction of these effects varies with the elevation patterns (level, duration, temporal fluctuation) achieved by different administration methods. In general, constant glucocorticoid elevations resulted in hippocampus-mediated learning deficits, whereas acute, cyclical elevations result in improved initial acquisition. Sensitivity was greater for males than for females. Further, changes in hippocampal neurogenesis paralleled some but not all effects. The findings demonstrate that specific patterns of glucocorticoid elevation produced by different drug administration procedures can have markedly different, sex-specific consequences on basic cognitive performance and underlying hippocampal physiology. Implications of these findings for glucocorticoid medications prescribed in childhood are discussed.

Keywords: Glucocorticoids, Development, Eyeblink Conditioning, Trace Conditioning, Hippocampus, Neurogenesis, Sex Differences

1.0 Introduction

1.1 Stress, glucocorticoids and cognition

Exposure to stressors in childhood has been linked to a number of later psychopathological conditions, including depression (Agnew-Blais & Danese 2016; Penza et al., 2003), anxiety (Fernandes & Osório, 2015; Penza et al., 2003), schizophrenia (Fernandes & Osório, 2015; Jiang et al., 2013), and cognitive impairment (Hedges & Woon, 2011; Spies et al., 2016). Evidence for cognitive deficits derive from observations such as reductions on measures of attention, memory, and executive function in children who had experienced stress sufficient to induce PTSD (Beers & De Bellis, 2002; Moradi et al., 1999). While confounds in human studies (e.g., current PTSD status) can cloud their interpretation, well-controlled animal experiments support the conclusion of early stress disrupting cognition. Maternal separation and disturbance of normal maternal behavior, for instance, have been found to impair performance of rats in the Morris water maze and novel object recognition tests in adulthood (Aisa et al., 2007; Ivy et al., 2010). One potential mediator of such effects is an increase in circulating glucocorticoids induced by the early stress. Indeed, blockade of glucocorticoid receptors reversed the effect of maternal separation on novel object recognition (Aisa et al., 2007). In humans, there is a wealth of evidence for glucocorticoid medications negatively impacting cognitive processes (Belanoff et al., 2001). However, studies in children are sparse even though glucocorticoid therapy in childhood has been associated with reductions in cognitive ability, particularly verbal memory (Bender et al., 1988; Bender et al., 1991; Mrakotsky et al., 2013), and there is evidence that effects can persist (Hitzert et al., 2014; Lajic et al., 2008; ter Wolbeek et al., 2013). In one clear example, school age children who had been given dexamethasone for respiratory distress syndrome shortly after premature birth scored lower than a matched placebo group on several measures of cognitive ability, including verbal and performance IQ (Yeh et al., 2004).

1.2 Stress, glucocorticoids and hippocampus

The hippocampus likely underlies many of these cognitive consequences of stress- and pharmacologically-induced elevations of glucocorticoids. The hippocampus is rich with glucocorticoid receptors and plays a pivotal role in regulation of the stress response by providing negative feedback to the hypothalamic-pituitary- adrenal (HPA) system. In addition, the hippocampus is a critical structure for learning and memory including short-term memory storage, the formation of new memories, and spatial working memory (Eichenbaum et al., 2016; Mahmmoud et al., 2015; Garcia, 2001). Some glucocorticoid activation is necessary for memory formation, and acute elevations can increase hippocampal LTP to promote learning (Beylin & Shors, 2003; Blank et al., 2002). However, the abundance of glucocorticoid receptors also appears to increase the vulnerability of the hippocampus to over-activation in the presence of excess glucocorticoid levels with potentially toxic effects (You et al., 2009). Cushingoid patients who have pathologically high levels of cortisol exhibit reduced hippocampal volume and deficiencies in cognitive, particularly verbal, tasks, while treatment to lower cortisol levels results in increased hippocampal volume that is associated with improvement in verbal learning (Starkman et al., 1992; 1999; 2001; 2003). Moreover, children suffering from PTSD show significant negative correlation between cortisol levels and hippocampal volume (Carrion et al., 2007). In adult rodents, prolonged elevation of glucocorticoids in adult rodents has been shown to reduce neural volume and LTP, alter dendritic morphology, and inhibit neurogenesis (Lupien et al., 1997; Sousa et al., 1998; Tata & Anderson, 2010). In all, there is substantial evidence for stress effects on cognition mediated by the hippocampus across the lifespan in both humans and animals (for reviews see Belanoff, et al., 2001; Kosten et al., 2012; Lupien et al., 2009; Heffelfinger & Newcomer, 2001).

1.3 Glucocorticoid effects on cognitive development

Early maternal separation or even altered maternal care can also affect hippocampal structure and disrupt its intrinsic physiology in rats (Maccari et al., 2014; Dricks, 2016). Exogenous glucocorticoids administered prenatally can have lasting effects on hippocampus and behavior (Zeng et al., 2015). Early postnatal administration has been largely confined to demonstrations of massive disruption of neural development and behavioral effects when given during the first 2 weeks of life in rats (Brummelte et al., 2006; Edwards & Burnham, 2001; Ferguson et al., 2001), the time of the "stress-hyporesponsiveness period" (SHRP), when circulating levels are greatly suppressed and the brain appears to be exceptionally sensitive to glucocorticoid's catabolic effects on brain growth (Sapolsky & Meaney, 1986; Walker et al., 1991). There is some indication that hippocampal-mediated behavioral effects can be affected by glucocorticoids later in the preweaning period (Machlor et al., 2004), but this question has received scant attention. In light of the fact that the SHRP has no true equivalent in human development, and that hippocampal development continues well after the SHRP, it is remarkable that there has been so little investigation of glucocorticoid administration in the preweaning period after the SHRP on the development of hippocampal-mediated cognitive tasks. Glucocorticoid medications are widely used in our society, and altered hippocampal function may compromise development, contributing to deficits in learning and memory in a variety of neurodevelopmental disorders (e.g., ADHD, Autism, Major Depressive Disorder). Further, it is important to consider possible differential vulnerabilities in males and females to early glucocorticoid administration. Sex differences in the effects of early-life stress and of glucocorticoid administration, including effects on the hippocampus, have frequently have been observed (e.g., Bale & Epperson, 2015; Gobinath et al., 2016; Jones et al., 2014). A better understanding of how males and females differ in the response to early glucocorticoid treatment may shed light on the differential prevalence of developmental disorders in males and females (e.g., more ADHD and autism in males; more depression in females).

1.4 The eyeblink classical conditioning approach to assessing cognitive development

In our approach, rats begin testing at postnatal day (PND) 15. At this age, the SHRP has ended and there is a peak in endogenous circulating corticosterone which continues to rise into at least the fourth week of life (Walker, Perrin, Vale, & Rivier, 1986). The learning task used in our studies is eyeblink classical conditioning, which we consider to be ideal for examining the interplay between glucocorticoids and hippocampal learning in development for the following reasons. It is a simple associative learning task for which the underlying neural substrates are well known, and different versions of the eyeblink conditioning task rely on different combinations of neurological structures (Green and Woodruff- Pak, 2000; Stanton et al., 1994; Thompson and Krupa, 1994). For this reason, selective behavioral deficits might provide clues as to the specific site of action of glucocorticoid effects on cognitive development.

We studied two forms of eyeblink classical conditioning: delay and trace (see Figure 1). In delay eyeblink classical conditioning, an auditory conditioned stimulus (CS) overlaps the presentation of a periocular shock, which serves as the unconditioned stimulus (US). Delay eyeblink classical conditioning is mediated chiefly by circuits in the cerebellum and brainstem. In contrast, trace eyeblink classical conditioning engages the hippocampus and prefrontal cortex, as well as the basic associative circuits underlying delay eyeblink classical conditioning, and is our primary procedure for assessing hippocampal-mediated glucocorticoid effects on learning. During trace eyeblink classical conditioning the CS terminates before the onset of the US, so that the subject must retain a ‘‘memory trace’’ of the CS to form the association with the subsequently presented shock. Acquisition of the CS-US pairing (i.e., learning) is measured by the percentage and amplitude of conditioned responses (CRs), responses which occur after the CS but before the US and therefore indicate anticipation of the US. The frequency and strength of the responses typically increase across training trials. When the timing of conditioned responses is such that the peak is within the 200 ms immediately preceding the shock US the CRs are labelled as “adaptive” or well-timed responses, although any response within the CS-

US window can be counted in a “total” CR measure. All of the data presented in the following figures will be of adaptive conditioned responses.

Based on the known susceptibility of the hippocampus to glucocorticoid effects, we hypothesized that glucocorticoid administration—specifically corticosterone, the primary endogenous glucocorticoid in the rat—would impair trace eyeblink classical conditioning, but have no or less effect on delay eyeblink classical conditioning. Developmentally, the ability to acquire trace eyeblink conditioning emerges postnatally and becomes robust by about 28 days of age in the rat, corresponding to the prolonged maturation of hippocampal and cortical circuits during the first several weeks (Ivkovich et al., 2000b; Travaglia et al., 2016). In humans with *hyper*cortisolism, trace eyeblink conditioning deficits were observed (Grillon et al., 2004) while metyrapone- induced acute, mild *hypo*cortisolism facilitated trace but not delay eyeblink conditioning (Nees et al., 2008), confirming that the trace eyeblink conditioning task may be sensitive to glucocorticoid effects mediated by hippocampus.

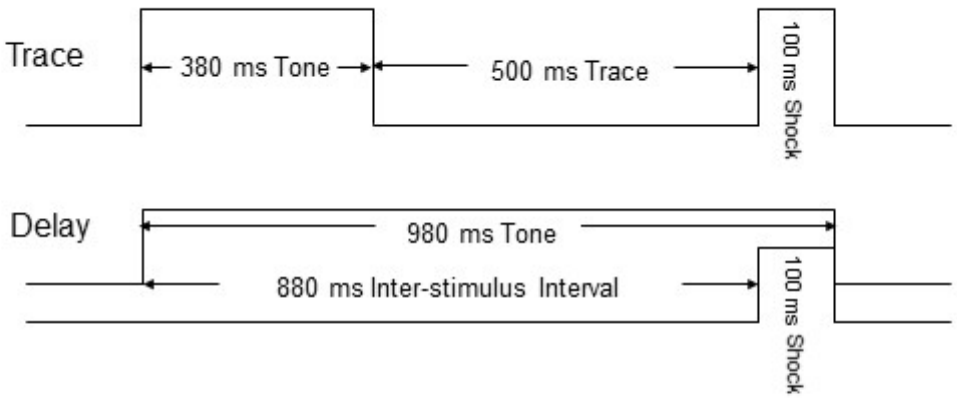


Figure 1. Schematic diagram of Trace versus Delay classical conditioning as used in the studies described here. A tone conditioned stimulus is paired with a periorbital shock unconditioned stimulus separated in time for trace conditioning, but overlapping in delay conditioning. Trace conditioning engages forebrain structures, including the hippocampus, in addition to the brainstem structures necessary for simpler delay conditioning.

2.0 Corticosterone effects on eyeblink trace conditioning

2.1 Corticoserone pellets- chronic high dose

In an initial study (Claflin et al., 2005), corticosterone was administered by way of 21-day timed-release subcutaneous pellets in order to examine the effects of chronic elevation of corticosterone beginning immediately post SHRP on later learning. Either a 35 mg corticosterone pellet or an equivalent-size inert placebo pellet was surgically implanted at the nape of the neck on PND 15. Two weeks later, on PND 28, acquisition of delay and trace eyeblink conditioning was compared between treatment groups and between males and females. Although initial acquisition of delay conditioning was slower for corticosterone-treated animals, they easily reached asymptotic performance during the 4th of 6 training sessions, with no sex differences (Figure 2, left panel). In contrast, for hippocampal-dependent trace eyeblink conditioning (Figure 2, right panel) there was a clear impairment in learning, as measured by the percentage of adaptive conditioned responses, for corticosterone-treated male rat pups only. These males showed no significant improvement across trials, whereas control males, as well as corticosterone-treated and control females, exhibited gradual learning that reached asymptote in the 4th or 5th sessions of training. Conditioned-response amplitude measures followed the same patterns.

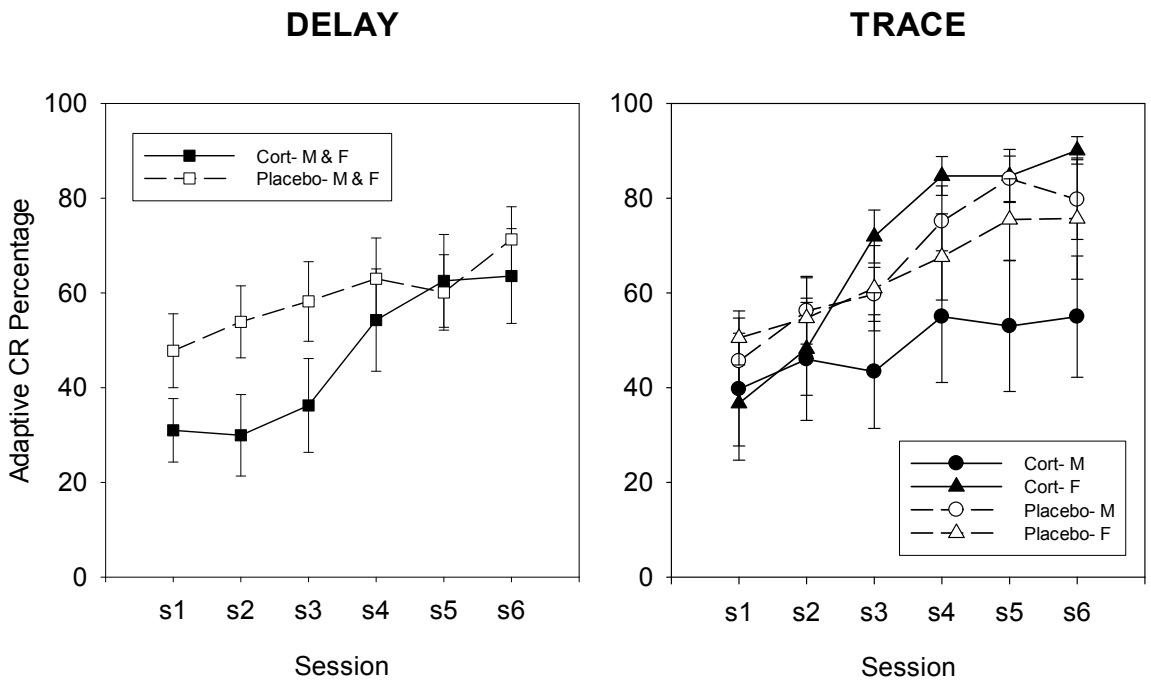


Figure 2. Percentage conditioned responses for delay (left) and trace (right) conditioning on PND 28-29, two weeks after corticosterone or placebo pellets were implanted on PND 15. Effects of corticosterone were not significant for delay conditioning but a significant impairment in acquisition of trace was observed for males only.

However, the physical appearance of both males and females was also affected by corticosterone pellet treatment. Their coats were scruffy and weight gain retarded (59 +/- 2 g for treated males and females vs. 84 +/- 2 g for controls on PND 27). Assessment of circulating corticosterone concentrations revealed that hormone treatment had produced a much greater and briefer elevation than intended: pharmacological levels of about 80 µg/dl were detected 3 days after implant on PND 18, but had declined to the levels of animals treated with placebo pellets (~ 20 µg/dl) by 6 days after implant. Inspection of the pellets (designed for 21-day release) indicated a deteriorating pellet mass that appeared to have become encapsulated by scar tissue, potentially preventing further regular release of the hormone. In all, the results of this experiment demonstrated a clear sex-specific vulnerability of males in a hippocampal-mediated learning task. Furthermore, since the corticosterone elevations were not maintained

throughout the study, the deficit we observed was due to a lasting effect produced by earlier hormone administration. Finally, our results, like those of others (Hermann et al., 2009), serve as a caveat for the importance of verifying actual blood levels following hormone pellet administration.

2.2 Corticosterone by mini-pumps- steady moderate elevation

If three days of pharmacologically elevated corticosterone could produce a sex-specific, later impairment in a hippocampal-mediated task, the next obvious question seemed to be whether a more physiologically relevant elevation of the same duration would have a similar outcome. To achieve a more-controlled delivery, we turned to osmotic mini-pumps. On PND 15, pups were implanted subcutaneously with pumps containing corticosterone or vehicle that were designed for a 3-day period of delivery. Trace eyeblink conditioning was again evaluated beginning on PND 28 (Claflin et al., 2014).

Unlike the previous experiment, there was no effect of corticosterone on the appearance or body weights of pups. Furthermore, the pumps were successful at achieving an elevation of plasma levels 24 hours following implantation (12 μg/dl vs 6 μg/dl for controls) that might best be considered to be in the low to moderate physiological stress range. During trace conditioning almost 2 weeks later, percentage of adaptive CRs was numerically lower for corticosterone-treated animals relative to controls, but not statistically different (Figure 3, left panel). However, the difference as measured by percentage of "total" CRs did reach significance (see Figure 4) and resembled the data for amplitude of the adaptive conditioned responses which were also found to be significantly different (Figure 3, right panel). Both males and females were impaired in this study, but visual inspection of the data separated by sex suggest a tendency again for males to exhibit greater impairment than females (Figure 4). In summary, a short-term, modest elevation of corticosterone at the conclusion of the SHRP was sufficient to disrupt acquisition of trace eyeblink conditioning at 4 weeks of age. And while there was some suggestion of greater

vulnerability in males, animals of both sexes exhibited deficits following corticosterone treatment.

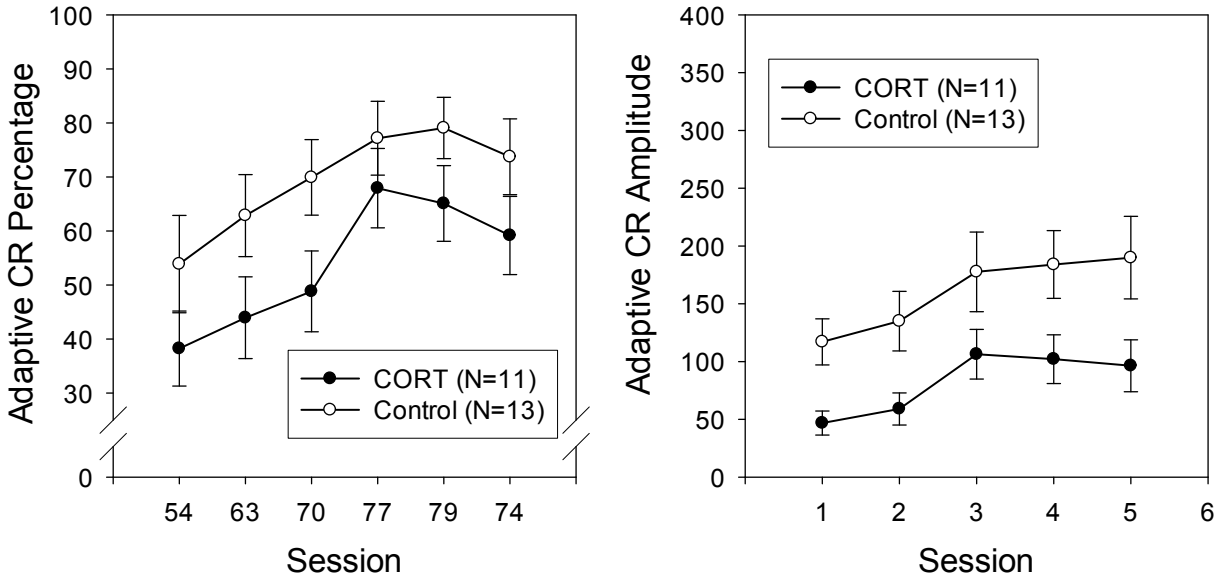


Figure 3. Percentage and amplitude of adaptive conditioned responses for trace conditioning on PND 28-29, two weeks after corticosterone or vehicle mini-pumps were implanted on PND 15. Impairment in CR amplitude was statistically significant.

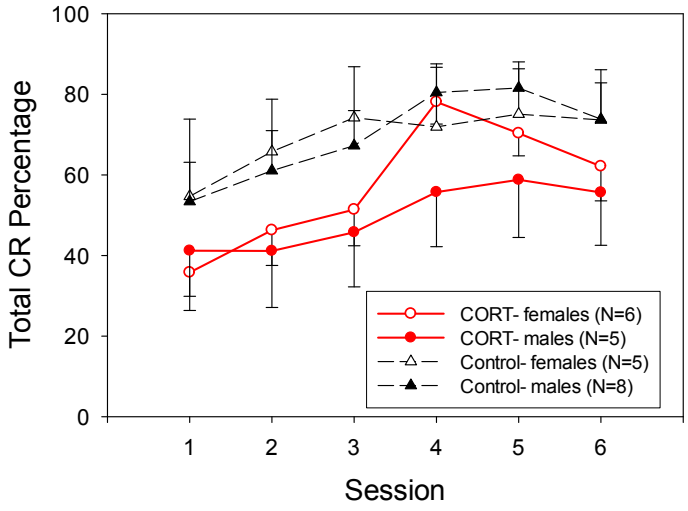


Figure 4. Percentage of total CRs plotted separately for males and females. Impairment following corticosterone delivery was significant regardless of sex, though males administered corticosterone exhibited numerically lower percentage of responses than did corticosterone-treated females.

2.3 Corticosterone by injection – fluctuating elevation

Though osmotic mini-pumps successfully achieved circulating corticosterone levels in the normal range, they are not designed to mimic normal temporal variation in hormone levels. For one, they obscure natural circadian and ultradian cycles. Further, because the output is consistent, mini-pumps eliminate the repeated elevation and descent of circulating levels that characterize acute stress exposures or intake of steroid medications. One drug delivery method that better approximates this pattern of temporal fluctuation is injection. Accordingly, pups were injected with corticosterone in our next study. Initial testing revealed that doses of 5 and 20 mg/kg produced peak plasma elevations within an hour of the injection in the physiological range for the low dose (44.2 ug/dl) and in the pharmacological range for the high dose (103 ug/dl), both of which declined precipitously, returning to control levels by 4 hours post-injection (Wentworth-Eidsaune et al., 2016). For this reason, subjects were treated twice daily, at 0900 and 1700, for 3 days—PND 15, 16, and 17. Once again, corticosterone treatment affected trace eyeblink performance and, as in our initial study, the effect was limited to males. However, rather than impairing performance, corticosterone—particularly the higher, pharmacological dose—*facilitated* acquisition (Figure 5). Moreover, it was only the initial acquisition of the response that was enhanced since effects were limited to the first of six sessions, each of which consisted of 90 paired CS-US trials. In other words, fluctuating glucocorticoid levels in the period immediately following the end of the SHRP enhanced the males' initial phase of acquisition of trace eyeblink conditioning 2 weeks after drug treatment was initiated.

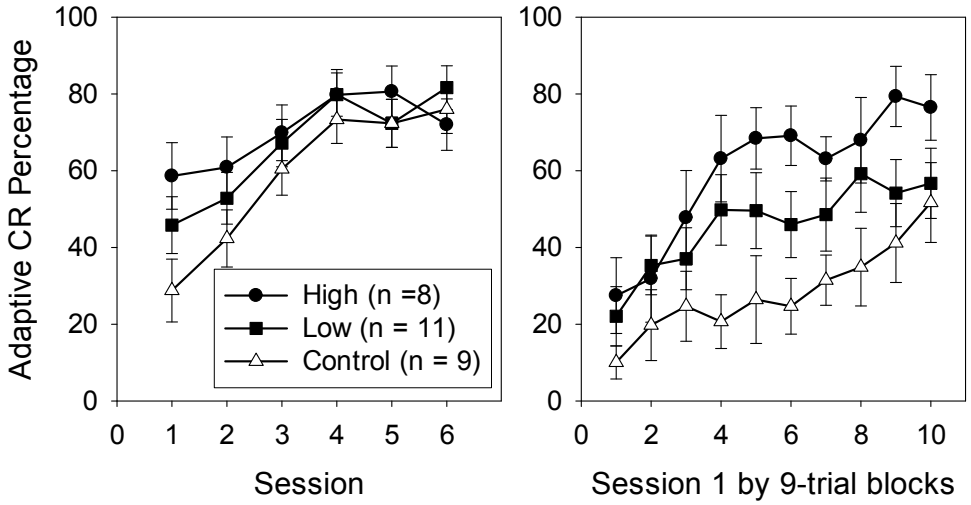


Figure 5. Percentage of adaptive conditioned responses for males only during trace conditioning on PND 28-29. Facilitation in CR percentage was statistically significant in the first session (left). Right panel shows Session 1 data only, across blocks of 9 trials. Facilitation of initial acquisition during Session 1 was statistically significant the high dose corticosterone-treated males relative to the control males.

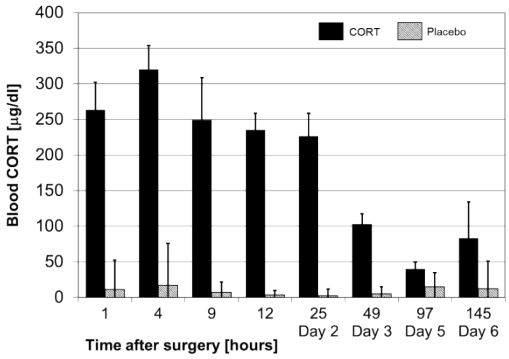
Overall, we found that three different methods of administering corticosterone during the post-SHRP portion of the preweaning period all had lasting effects on hippocampal-mediated trace eyeblink conditioning. Yet, the nature of the outcomes observed varied with the pattern of elevation (level, duration, temporal fluctuation) that each administration procedure achieved. Nonetheless, our assessment of blood levels was based on limited samples so as to constrain the conclusions that can be drawn for these results. In the first experiment, we know that pellets dramatically increased corticosterone concentrations at 24 hours and that the increase had disappeared by 3 days, but we do not know how quickly the elevations subsided. Similarly, in the second study, physiological levels were observed 24 hours following implantation of the mini-pumps, but the duration of this elevation is unclear. And in the third experiment, we cannot comment on how repeated injection at different times of the day or on consecutive days may have affected the pattern of elevation. Therefore, we conducted a systematic study of plasma

corticosterone levels following implantation of pellets or mini-pumps, or administration of repeated injections, at multiple time points.

3.0 Corticosterone elevation patterns over time

Specific procedures and doses were as described for the previous experiments. Implantation or first injection occurred at 0900 on Day 15, with blood samples collected 1, 4, 9, and 12 hours later. Further blood collection was spaced according to the expected reliable duration of effect for a particular treatment method (see Figure 6). For injection, only the high dose of 20 mg/kg was used here, and sampling occurred 1 hour and 4 hours after each injection. Samples from pups undergoing control procedures for each method were collected for comparison. In all, 149 pups from 17 litters were each sampled twice—initially via cardiac withdrawal and the second time following decapitation, at a minimum interval of 12 hours. Data for each time point for each method are based on between 6 and 9 samples. Blood was collected within 4 minutes of home cage disturbance in order to minimize any effect of the procedure on corticosterone levels in the samples obtained (Coover, Heybach, Lenz, & Miller, 1979). Plasma samples were run in duplicate with radioimmunoassay procedures.

A



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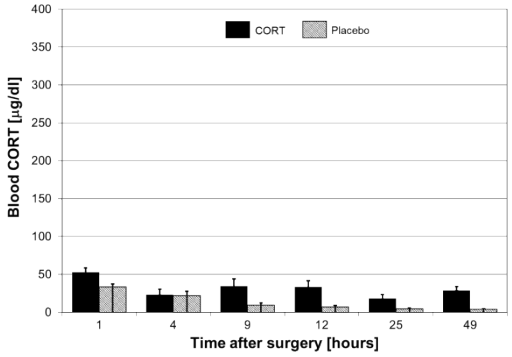

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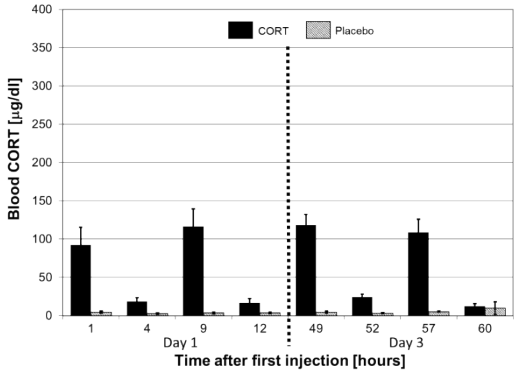

Figure 6. Circulating corticosterone levels over time (hours and days) for the three administration methods, pellets (A), mini-pumps (B), and injection (C).

Once again, pellets yielded the largest elevations. Though designed for release over 21 days, plasma corticosterone concentrations of pellet-implanted pups rose to pharmacological levels and then fell sharply on day 2 and were no different than controls by day 5. Mini-pumps yielded a relatively steady elevation in the physiological stress range into the 3rd day. Injections yielded the same oscillating peaks and troughs whenever administered, with significant elevations persisting for less than 4 hours. It is clear, therefore, that the three administration methods result in very different levels, temporal fluctuations, and durations of circulating glucocorticoid elevation, which apparently have distinct individual consequences for underlying brain development.

4.0 Corticosterone effects on neurogenesis

While there are a number of mechanisms by which glucocorticoids may be affecting brain development to produce the different behavioral outcomes we have reported, one of the first to come to mind is by altering hippocampal neurogenesis. To assess this possibility, we examined potential differences in neurogenesis in the dorsal dentate gyrus following corticosterone administration by the same three methods. Neurogenesis in this region of the hippocampus has most frequently been observed in adults to correlate with changes in cognitive performance (Gould et al., 1999; Leuner et al., 2006). As described above, corticosterone administration began on PND 15. Daily bromodeoxyuridine (BrdU) injections (i.p., 50mg/kg) began one day later and were administered once a day for 3 days, on PND 16-18, to label newly dividing cells during this critical time window when corticosterone levels were known to be elevated. We examined brain samples of 4-7 pups, approximately balanced for sex, in each corticosterone and corresponding control group. An additional 4 brain samples served as baseline non-manipulated controls. Brains were harvested and preserved on PND 28 to correspond with the day of behavioral testing in our other studies. Coronal sections (80-μm)

were collected, yielding 7-9 sections per animal, for immuno-histochemical staining with Anti-BrdU, anti-NeuN, anti-GFAP, and Sox10 as the primary antibodies followed by secondary antibodies FITC, Cy3, and DyLight649 to allow for confocal imaging (Figure 7). Stereological techniques were used to obtain counts of the total number of newly generated neurons (both mature and immature), their density, and the volume of the dorsal dentate gyrus. Values were estimated using a rare event version of the optical fractionator (Gundersen et al., 1988) and Cavalieri methods (Gundersen et al., 1988b) with Stereo Investigator software.

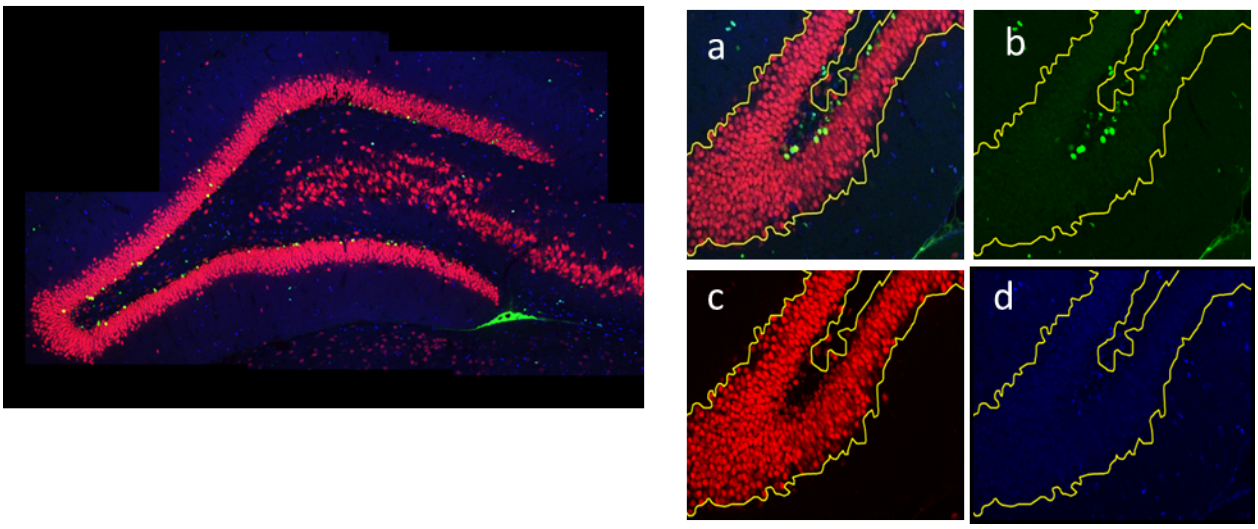

Figure 7. Confocal images of dorsal dentate gyrus showing all fluorescent co-labelling (left). Immunohistochemical staining (right) allowed us to identify newly generated neurons: a) combined fluorochromes, b) BrdU labeled cells (any dividing cells), c) NeuN labeled mature neurons, and d) GFAP/Sox-10 labeled glia. The yellow perimeter tracing was used for calculating the cross-sectional area/volume of the dorsal dentate gyrus.

Among the different administration method groups, statistically significant differences were observed for the pellet groups only and the direction of the effects was sex dependent (Figure 8). There was a significant *decrease* in neurogenesis for corticosterone-treated male

pups relative to placebo control males but a significant *increase* in neurogenesis for the female corticosterone-treated group relative to placebo females. These data are consistent with the significant behavioral deficit in learning we observed in corticosterone-pellet-treated males. Interestingly, examination of control groups indicated that for both placebo pellet controls and untreated controls there was a significantly higher rate of neurogenesis for males than females. Although not significant for the other control groups, this post-SHRP increased rate of neurogenesis for males is consistent with a growth spurt period in hippocampus (Bayer, 1980; Bayer & Altman, 1974; Travaglia et al., 2016) and makes the decrease in neurogenesis for corticosterone-pellet-males even more significant. It is possible that the increased rate of neurogenesis during this developmental window produces a vulnerability to glucocorticoid perturbation specifically in males. The volume of the dentate and cell density of new neurons did not differ between the corticosterone-treated and control animals, regardless of sex or drug administration method.

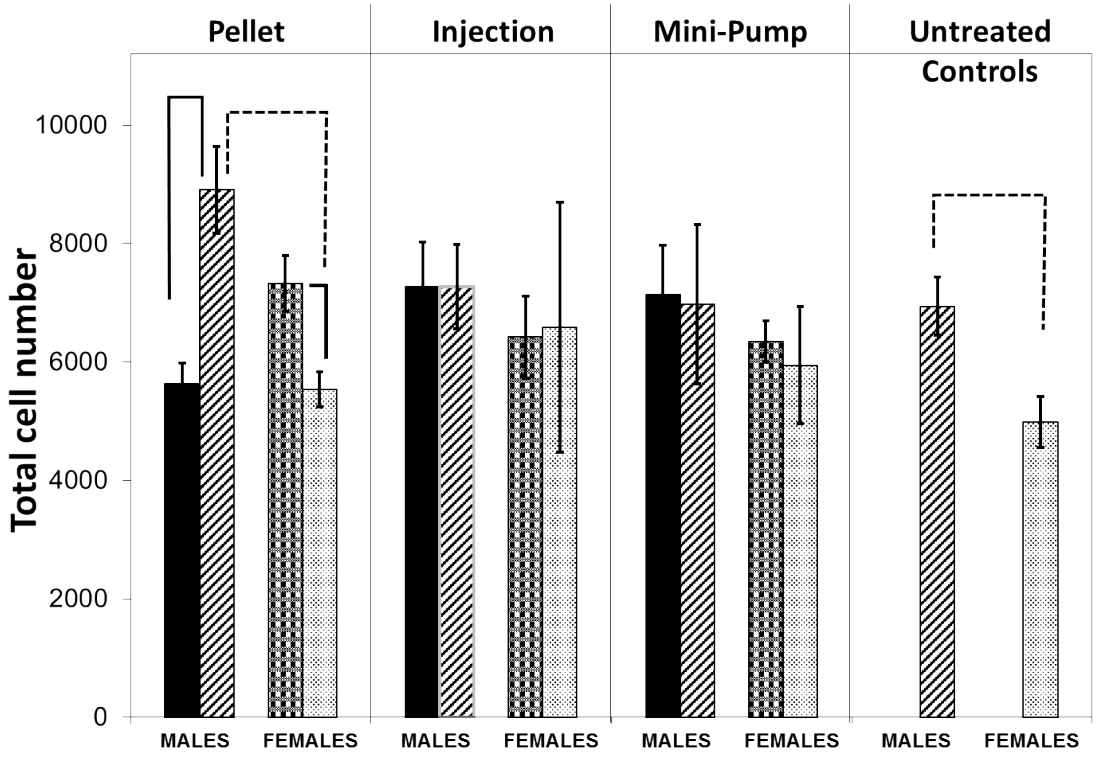

Figure 8. Total number of newly generated neurons (includes both mature and immature neurons) on PND 28, two weeks after corticosterone treatments began by pellet, injection, and mini-pump methods. Statistically significant effects of corticosterone treatment were found within the pellet groups only. Untreated control data are provided for comparison.

5.0 General Discussion

It is clear that the pattern of circulating glucocorticoids (level, duration, temporal fluctuation) is affected by the drug administration method and that even seemingly modest differences (i.e., output of mini-pump vs injection) can determine the direction of a behavioral effect. Our work has focused on exploring the consequences of raising glucocorticoid levels starting on PND 15, immediately after the SHRP, and the potential effect on the developing hippocampus, as assessed 2 weeks later, on PND 28. Although none of the drug administration methods we evaluated affected hippocampal volume, neurogenesis in the dentate was altered by the pharmacologically high levels of corticosterone delivered by pellets but not by the more modest levels produced by mini-pumps and injections. Moreover, the effect was sex specific.

Although across all control groups, males generally demonstrated more neurogenesis in the PND 16-28 period assessed, there was a drastic decrease in newly-generated-neurons for corticosterone-pellet-treated males relative to placebo controls. Our results are consistent with those of others who have found that postnatal exposure to glucocorticoids through nursing or early postnatal injections reduced neurogenesis in males and in specific regions of hippocampus, like the dentate gyrus (Brummelte et al., 2006 and Gould, 1991, respectively). So, although pharmacological doses of corticosterone may impair neural development in males, this reduced neurogenesis argument cannot explain the learning deficits observed with lower levels of corticosterone delivered by mini-pump or the facilitated learning observed with injections.

One prevailing view for some time has been 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). Rising glucocorticoid concentrations first saturate the high-affinity MR receptor sites, but with continuing elevation, glucocorticoids increasingly occupy GR receptors, which are found in abundance in the hippocampus. Initial activation of MRs appears to facilitate hippocampal function, enhancing LTP and improving spatial memory in the Y-maze (Conrad et al.1996, 1997; deKloet et al., 1999). So long as the exposure is short-term and at low-moderate levels, glucocorticoid elevations appear to support neural plasticity and specifically post-training memory consolidation. For example, intra-hippocampal infusion of corticosterone improves memory consolidation in awake but not sleeping rats (Kelemen et al., 2014). Memory consolidation is impaired in the absence of glucocorticoids following adrenalectomy or administration of antagonists (e.g., Marin et al., 2011; Nees et al., 2008). In contrast, prolonged exposure to stressors activates GRs, which appears to impair hippocampal excitability associated with learning plasticity, altering NMDA receptor expression (Lee et al., 2003), suppressing LTP, and

impairing performance on declarative and spatial memory tasks and memory retrieval (see reviews: Conrad et al., 2005; DeKloet et al., 1999; McEwen et al., 2006, 2007; Roosendaal, 2002). One suggestion is that chronic, prolonged elevation produces genomic level changes that specifically affect hippocampal functions during later-stage memory processes (Roosendaal, 2002; Schwabe et al., 2012). In addition, prolonged glucocorticoid elevation and GR stimulation can have neurotoxic effects on the hippocampus in adult humans and in other animals, including rats (for review, see Lupien et al., 1997, 1998; Sousa et al., 1998).

We cannot rule out the possibility that GR activation in the non-hippocampal basic eyeblink conditioning circuits may produce the effects we observed in trace eyeblink conditioning. Certainly Wilber et al., (2007, 2010, 2011) demonstrated that early postnatal maternal separation and corticosterone administration during the SHRP resulted in impaired *delay* eyeblink conditioning for males in adulthood, but not females, and that the deficits corresponded with enhanced GR expression in the interpositus nucleus of the cerebellum, an area critical for this form of associative learning. However, our results obtained by manipulating corticosterone *after* the SHRP are consistent with adult human studies suggesting that hippocampus-mediated trace, but not delay conditioning, is more sensitive to these later variations in glucocorticoid levels. When cortisol levels are endogenously high (Cushing's syndrome) trace eyeblink conditioning is impaired and correlates with hippocampal-declarative memory deficits (Grillon et al., 2004). On the other hand, when cortisol production was inhibited by metyrapone in healthy volunteers, facilitation of trace eyeblink conditioning was observed while delay conditioning remained unaffected (Nees et al., 2008). It is possible that the later, but not earlier, postnatal glucocorticoid effects on cognition are mediated by hippocampus, especially since the immediate post-SHRP period of PND 15-18 has been reported as a time when there is a noticeable spurt in hippocampal cell maturation, in particular in the dentate gyrus (Bayer & Altman, 1974). It has been argued that brain areas are more vulnerable

precisely during such developmental spurts than they are either before or after reaching an adult state (Rice & Barone, 2000)

Beyond the potential glucocorticoid effects on hippocampal structure and function are the effects exogenous glucocorticoids can have on endogenous activity via feedback systems in the HPA Axis. It is possible that the chronic, though low level, corticosterone supplementation provided by minipumps impacts the natural physiological variations in corticosterone levels associated with rhythmic ultradian and circadian cycles of the hormone and regulatory feedback effects on the HPA axis. Minipumps may obscure natural circadian rhythms of corticosterone by producing a constant output that raises physiological levels above normal circulating levels for an extended period. In contrast, injections produce rapid fluctuations superimposed on the circadian cycle, thereby adding in additional periods of elevation into the natural cycle, which are likely to better mimic real-life repeated acute stressor effects. In one study in which early-life stress exposure *enhanced* later fear conditioning in adulthood, similar to our injection effects, the diurnal corticosterone cycle was disrupted so as to produce a second daily peak (Poulos et al., 2014). Some researchers are proposing that disruptions of the natural diurnal corticosterone cycle may prove to be useful in modelling PTSD symptoms in rodents (Hall et al., 2015; Poulos et al., 2014).

Peaks and troughs in the circadian cycle may be particularly important to normal cognitive development. Liston et al. (2013) determined that peaks in circadian glucocorticoid levels promoted dendritic spine growth in cortical tissue of mice whereas troughs in the circadian cycle were periods of stabilization for the new spines. An injection of glucocorticoids during the trough period enhanced spine growth in mice to similar levels as those seen during normal peak circadian periods. A similar beneficial effect on memory function was observed in human young adults administered hydrocortisone during the evening trough period (Lupien et al., 2002). It is increasingly clear that endogenous and exogenous glucocorticoids will interact to

alter the natural cyclical patterns of elevation in ways that may produce either facilitative or deleterious effects on cognition.

Although excessive glucocorticoid elevation over a prolonged period is generally detrimental, and likely to result in hippocampal damage, acute elevations such as those produced by injections or by administration at specific times of day, may provide beneficial effects by way of a different mechanism. Facilitative effects on cognition have been demonstrated following cortisol administration in humans, specifically enhanced memory for emotional material (Abercrombie et al., 2003; Buchanan & Lovallo, 2001) and improvement in working memory (Stauble, et al. 2013) . Inhibitory avoidance, contextual-fear conditioning, and water maze spatial learning in rodents (reviewed in Roozendaal, 2002) were similarly improved. Acute administration of glucocorticoids also appears to follow the hormetic, or U-shaped, dose-response function, with beneficial effects at lower doses (Lupien et al., 2005). It is proposed that the enhanced learning observed following acute stress or glucocorticoid exposure is the result of better memory encoding and consolidation processes, the early phases of memory formation, which are modulated by amygdalar-noraderenergic interactions with the learning circuits (Roozendaal, 2002). Given the delay between glucocorticoid treatment and assessment of learning in our paradigm, our findings may reflect a lasting increase in amygdalar-noradrenergic activity or excitability that promotes faster acquisition of trace eyeblink conditioning at a later time. According to this model, there would also be no negative effect on hippocampal function, so the basic neural substrates for performing the task would remain intact.

6.0 Summary and conclusion

Our results offer caveats not just for the delivery of glucocorticoids in the laboratory, but also for their administration in a clinical setting. Results confirm that hippocampally-mediated

cognitive function appears to be especially vulnerable to early glucocorticoid administration, but that the observed effects may be in different directions. Early glucocorticoid manipulation may influence hippocampal development during a critical period of vulnerability for males moreso than for females. Our data show fairly consistent evidence for male sensitivity to glucocorticoid effects, both positive and negative, at PND 15, a time that corresponds with a significant growth spurt in the hippocampus in the rat. Glucocorticoids may alter the trajectory of brain development to affect cognitive development in a sex-specific manner (Howard et al., 2006, Kosten, 2012). Because the effects of glucocorticoids on cognition may vary greatly with factors beyond just dose (e.g., timing, developmental period, sex), adverse effects may be difficult to detect without thorough testing. Systematic study of these various parameters using animal models for cognitive assessment may be able to provide clues to increase our understanding of the development, sex specificity, and underlying mechanisms contributing to certain learning disorders.

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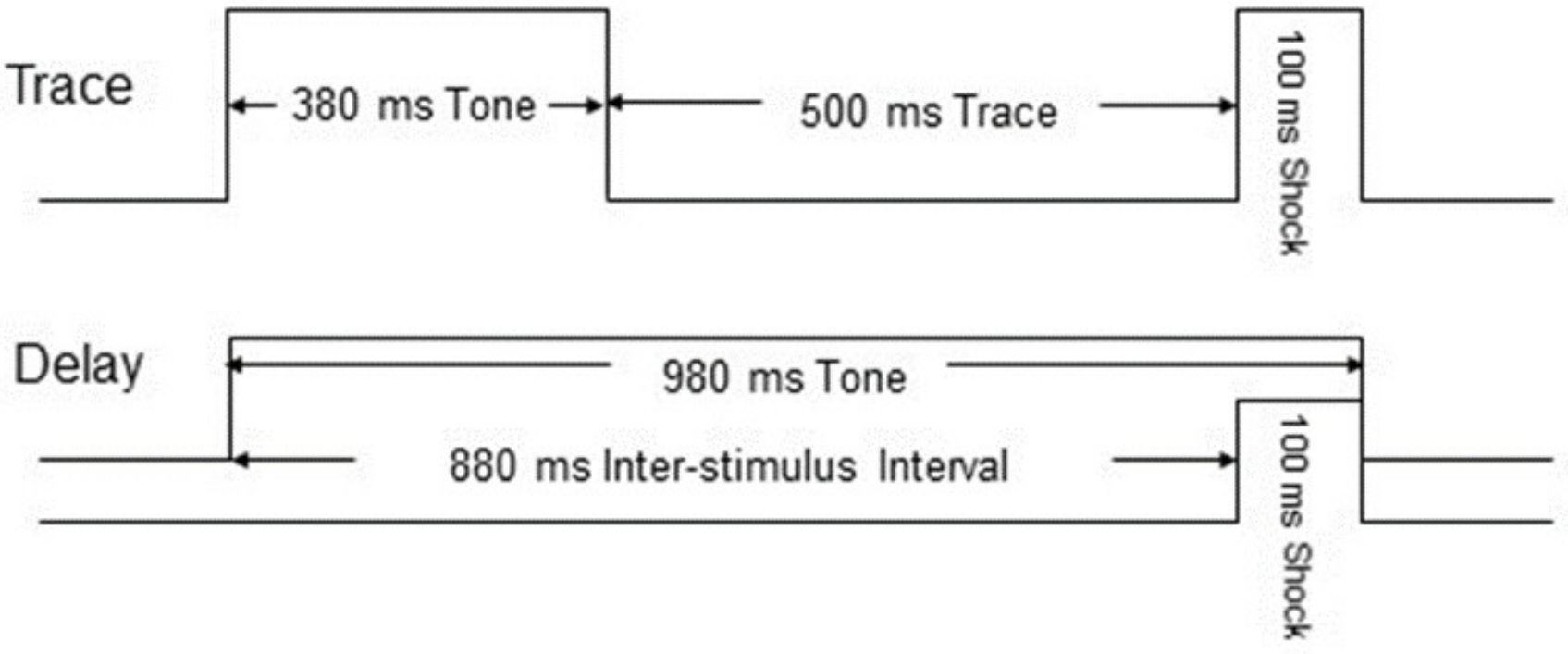

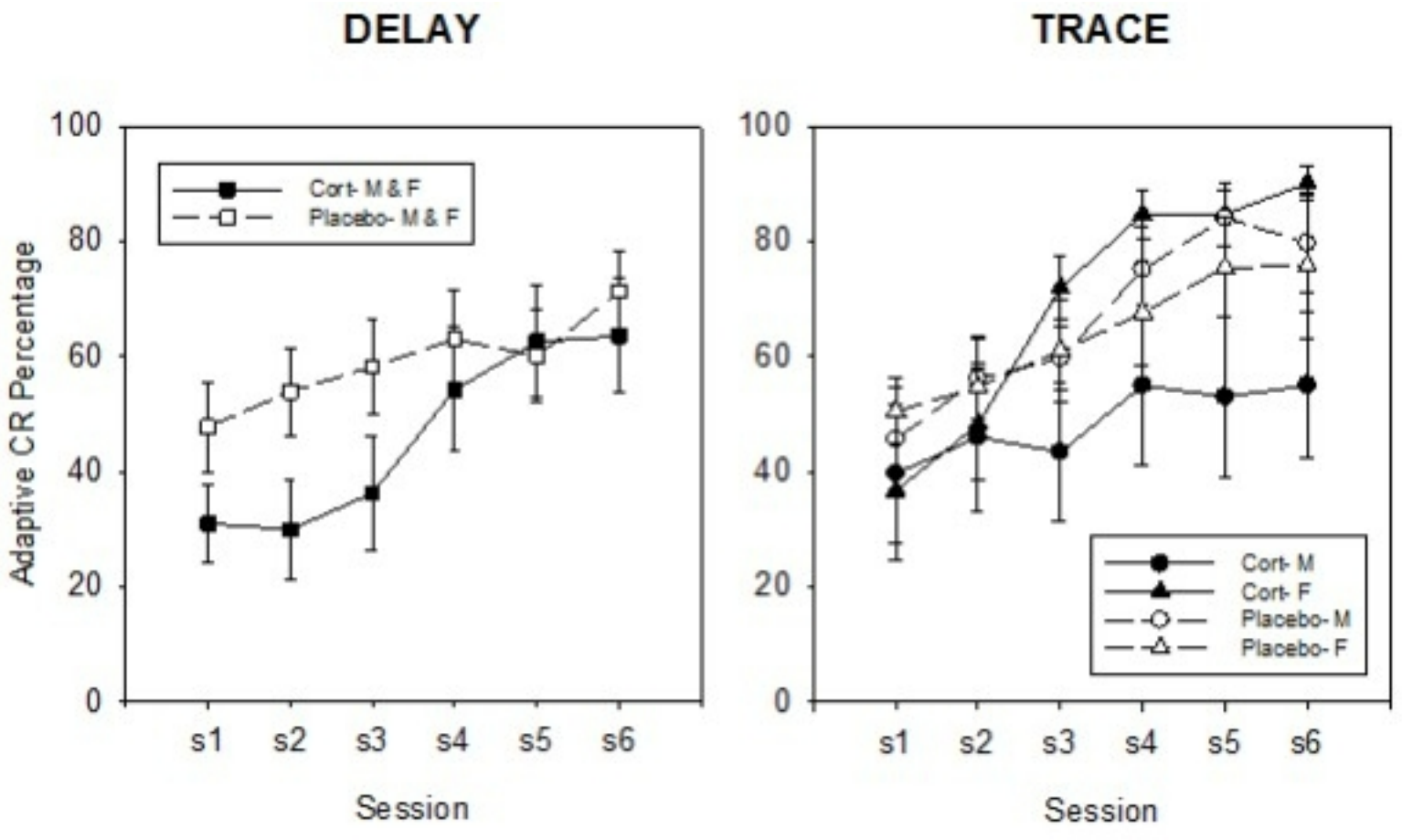

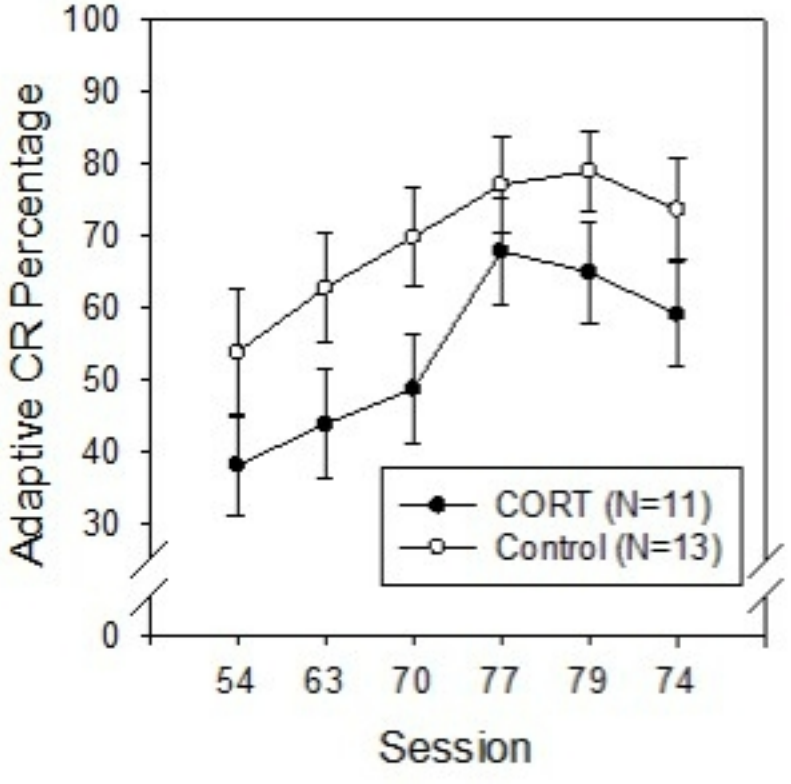

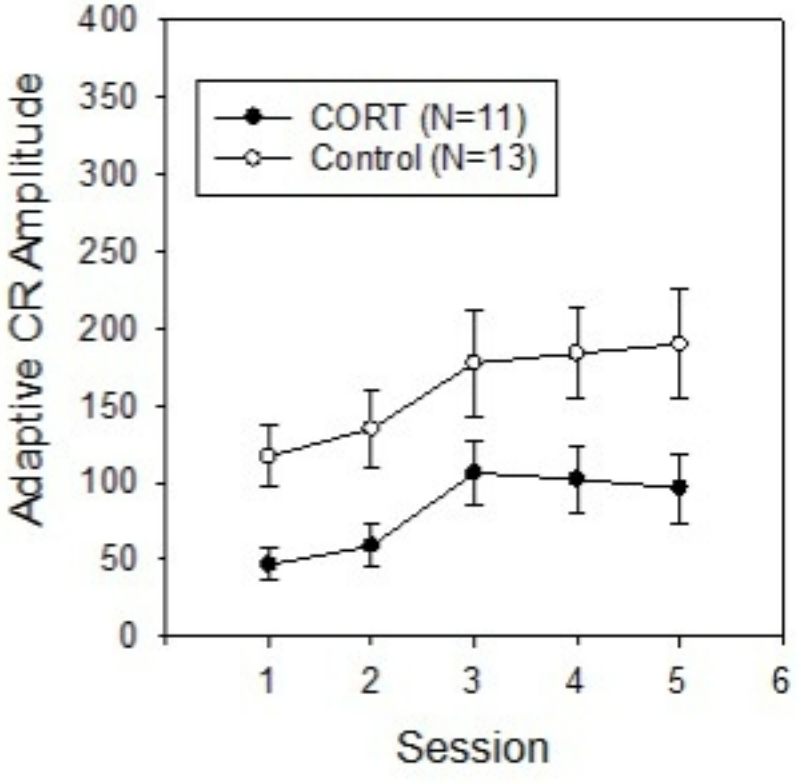

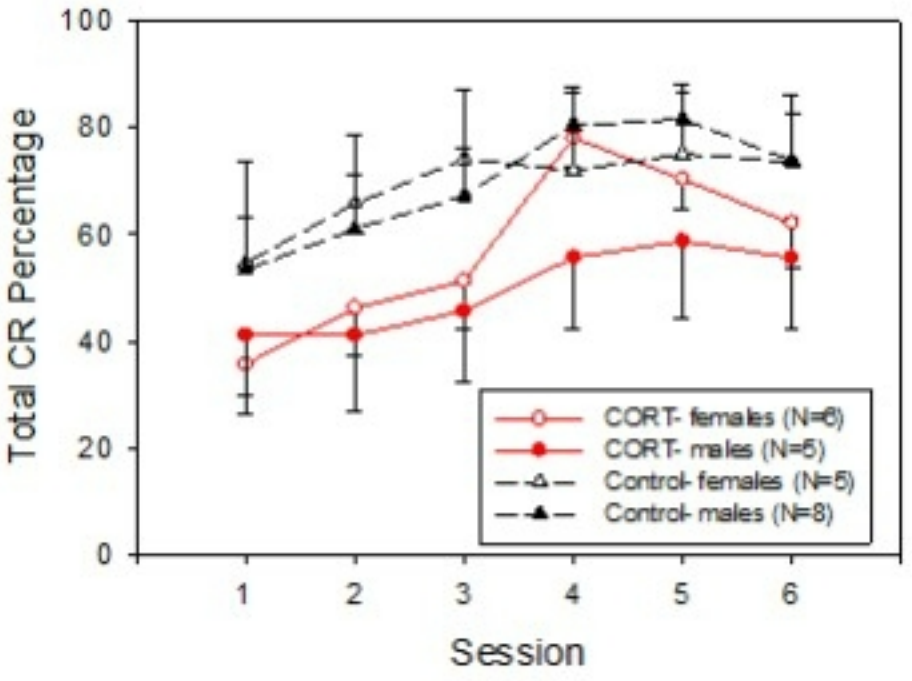

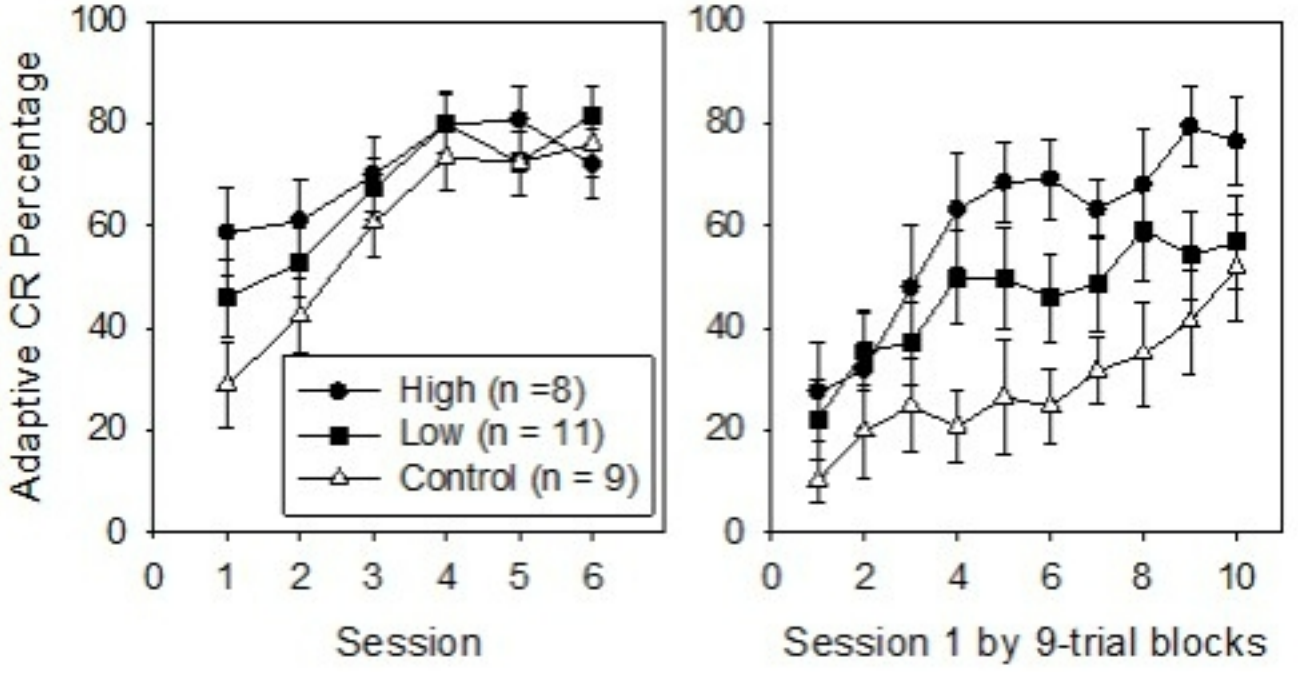

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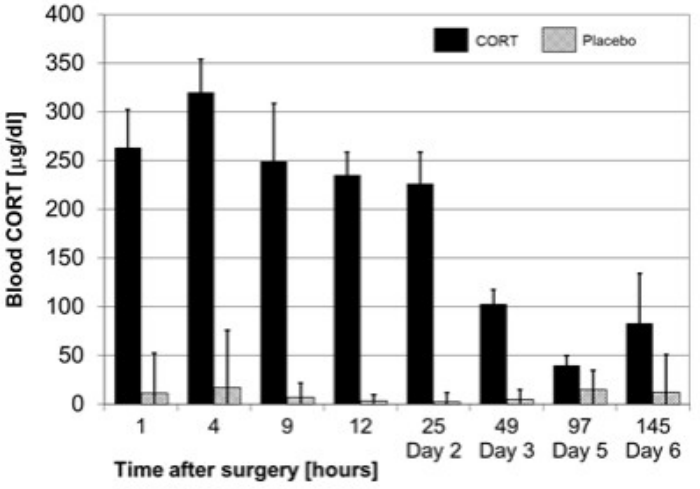

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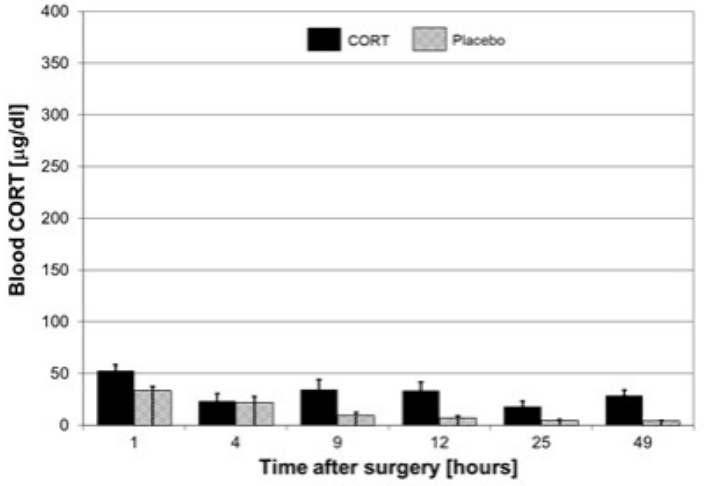

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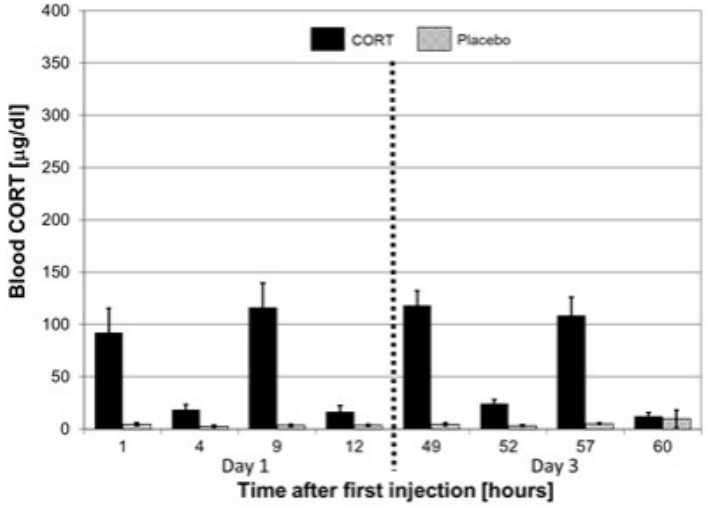

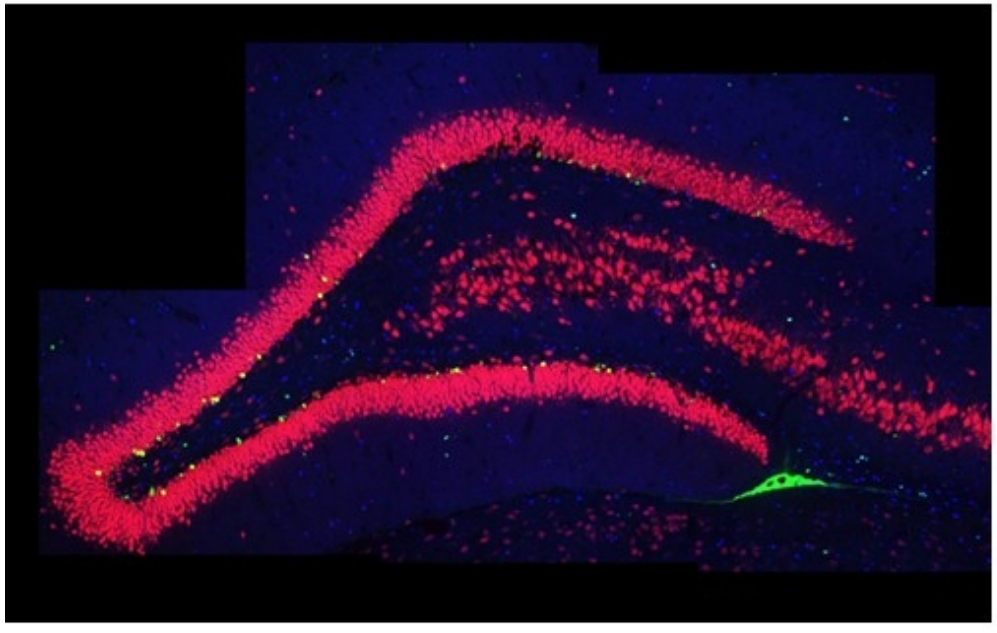

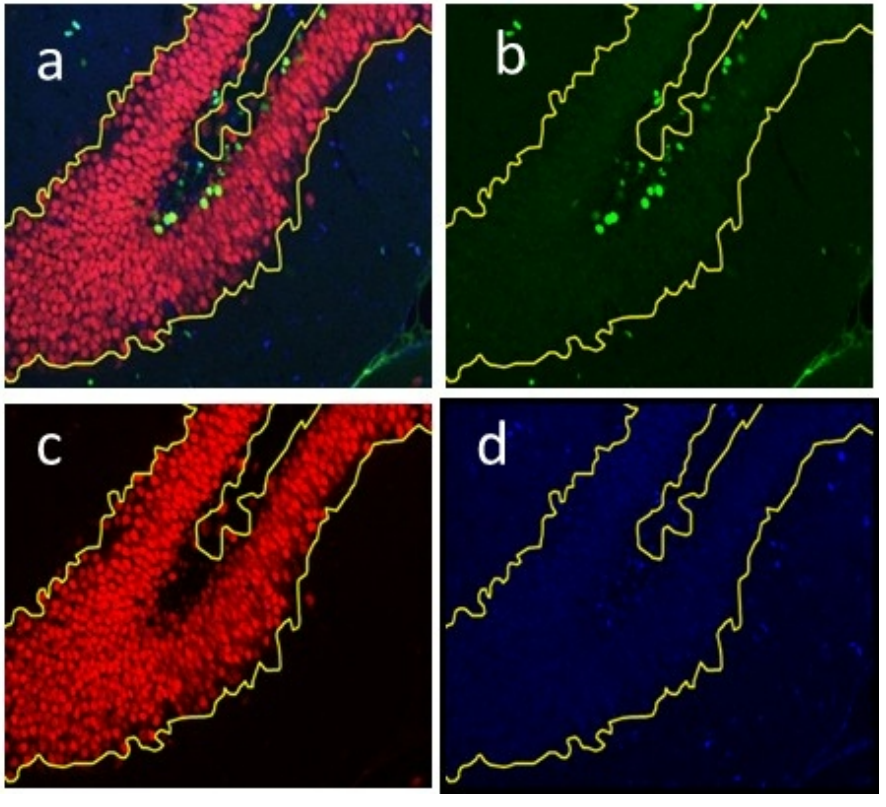

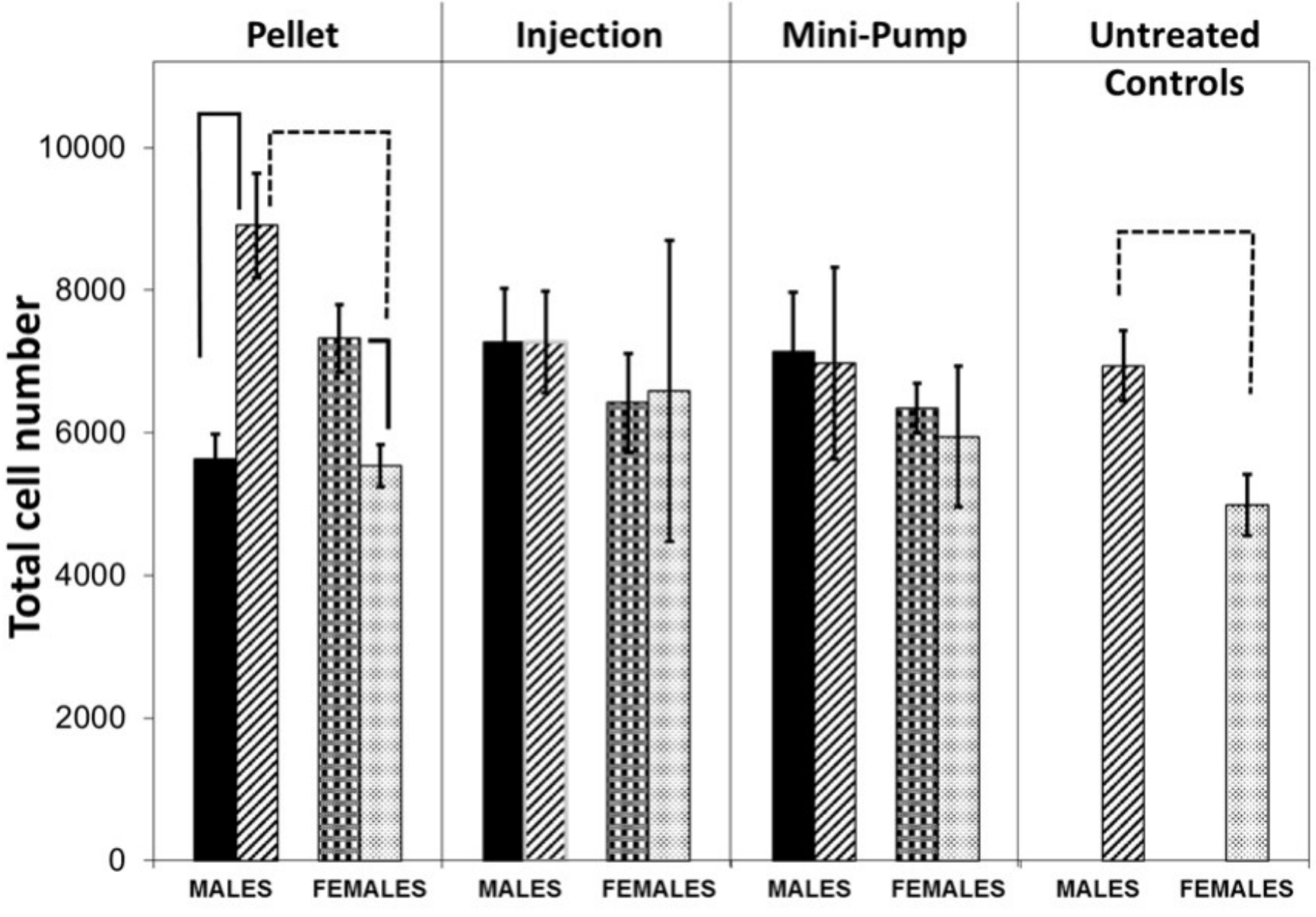

Highlights:

- Preweaning glucocorticoids affect later eyeblink trace conditioning
- Varying glucocorticoid elevation patterns differentially affect later cognition
- Greater sensitivity of males to glucocorticoid effects on hippocampal tasks