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PRINCIPAL INVESTIGATOR:

Randy Strong. Ph.D.

CONTRACTING ORGANIZATION:

University of Texas Health Science Center at San Antonio
San Antonio, TX 78229

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Randy Strong. Ph.D.
Alan Frazer, Ph.D.
David Morilak, Ph.D.
email: strong@uthscsa.edu

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13. SUPPLEMENTARY NOTES

14. ABSTRACT Traumatic stress is a requirement for the development of PTSD. However, the majority of trauma-exposed persons do not develop PTSD. Therefore, examination of the typical effects of a stressor may not identify the critical components of PTSD risk or pathogenesis. One obvious explanation for individual differences in vulnerability to PTSD is that there may be genetic predisposition to susceptibility to precipitating stressors. However, to date, very few genetic polymorphisms for PTSD have been identified. An alternative mechanism that would impart lifelong vulnerability to PTSD is stable alterations in gene expression programmed by exposure to early life stressors. Therefore, the hypothesis to be addressed by this project is that early life exposure to stress or glucocorticoids programs a distinct neurochemical and behavioral phenotype during adulthood characterized by vulnerability to stressors that trigger PTSD. Moreover, we hypothesize that the susceptibility to PTSD can be reversed in adult offspring by anti-depressants which have been reported to reverse the epigenetic changes in expression of selected genes caused by stress. To address this hypothesis, the following specific aims are proposed: 1. To generate and characterize models of early life stress. 2. To determine adult predictors of vulnerability to stress: as determined by behavioral, physiological, and molecular and neurochemical measures. 3. To determine adult vulnerability to stress: Adult offspring from models developed in Specific Aim 1 are exposed to a model of traumatic stress and then a fear conditioning paradigm. Behavioral, physiological and molecular neurochemical measures are made. 4. To determine the effects of treatment with the SSRI sertraline.

15. SUBJECT TERMS
rats, prenatal stress, massed footshock, PTSD, open field test, social interaction test, fear conditioning, extinction, glucocorticoid receptors, dopamine, serotonin

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A. INTRODUCTION:

Traumatic stress is a requirement for the development of PTSD. However, the majority of trauma-exposed persons do not develop PTSD. Therefore, examination of the typical effects of a stressor may not identify the critical components of PTSD risk or pathogenesis. One obvious explanation for individual differences in vulnerability to PTSD is that there may be genetic predisposition to susceptibility to precipitating stressors. However, to date, very few genetic polymorphisms for PTSD have been identified. An alternative mechanism that would impart lifelong vulnerability to PTSD is stable alterations in gene expression programmed by exposure to early life stressors. Therefore, the hypothesis to be addressed by this project is that early life exposure to stress or glucocorticoids programs a distinct neurochemical and behavioral phenotype during adulthood characterized by vulnerability to stressors that trigger PTSD. Moreover, we hypothesize that the susceptibility to PTSD can be reversed in adult offspring by anti-depressants that have been reported to reverse the epigenetic changes in expression of selected genes caused by stress. To address this hypothesis, we proposed the following specific aims: 1. To generate and characterize animal models of early life stress. 2. To determine adult predictors of vulnerability to stress: as determined by behavioral, physiological, and molecular and neurochemical measures. 3. To determine adult vulnerability to stress: Adult offspring from animal models developed in Specific Aim 1 are exposed to a traumatic stress and then a fear conditioning paradigm. Behavioral, physiological and molecular neurochemical measures are made. 4. To determine the effects of treatments with the SSRI sertraline in trauma-exposed adults.

B. BODY:

YEAR 1:

During the initial funding period (September 1, 2008- August 31, 2009) we performed experiments to establish the methods for Model 1 (prenatal stress) to address Task 1 - Determine adult predictors of vulnerability to stress; and Task 2 - Determine adult vulnerability to stress. During this period, we concentrated on Task 2 (Steps 1 – 10), developing a model of fear conditioning and extinction and testing a model of chronic stress that sensitizes rats to fear conditioning and extinction and interferes with memory of conditioned fear extinction. We further examined neurochemical and hormonal mechanisms that may underlie resistance to conditioned fear extinction in animals exposed to maternal stress in utero as outlines in Steps 12-13 of Task 1 and Steps 11-13 of Task 2).

Experimental Design. We assessed the prenatal stress model by immobilizing timed-pregnant female rats on embryonic days (ED) 14 – 21 for one hour. Unstressed pregnant females served as a control. On post-natal day (PD) 3, the litters were culled to 8 pups. Males were weaned and pair-housed on PD 21. On PD 42, half the animals were exposed to a mild foot shock stress. We made a minor modification to the foot shock procedure in which we substituted a shock probe in a cage with bedding so that we could measure the extent to which the animals buried the probe with cage bedding after receiving a mild shock. The extent of burying behavior is associated with greater reactivity to stress. Immobility (i.e., “freezing”) in these rats is measured as well as this provides a separate measure of reactivity. We initially also exposed a group of animals to a single immobilization stress to measure the effect of this stress on their response to a battery of tests. We have not finished collecting data on this group. On day 54, we exposed the mice to a

PTSD stress model which consists of mass foot shock administered 3 times. On PD 70, half the animals were sacrificed for a number of neurochemical measures. The other half is then assessed for stress reactivity with a battery of tests on PD days 70-79. On day 73, animals are tested for hyperarousal by measuring locomotion in an open field. On day 74, the rat is exposed to another rat in the open field to measure social interaction. On day 75, state anxiety and stress reactivity are tested in the elevated plus maze. On day 76, fear conditioning is measured and on days 77 through 79, extinction of the conditioned fear is tested. Because of the logistics involved in testing a large number of animals on this battery of tests, we have performed the above with separate groups of animals and combined the data from the groups. Not all of the tests have been completed at this point, so we will present only the data for which we have enough samples for statistical analysis of the results.

Results.

Figure 1.1 shows the effect of prenatal stress on reactivity to foot shock in the shock probe defensive burying test.

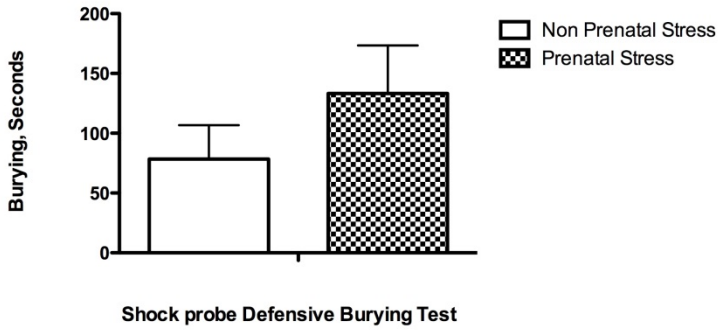

Figure 1.1 Effect of prenatal stress on the shock probe defensive burying test. The data represent the mean ± SEM for 7 – 10 animals.

Although it appears as though the offspring exposed to maternal prenatal stress show relatively greater burying activity, the difference between the two groups did not reach statistical significance.

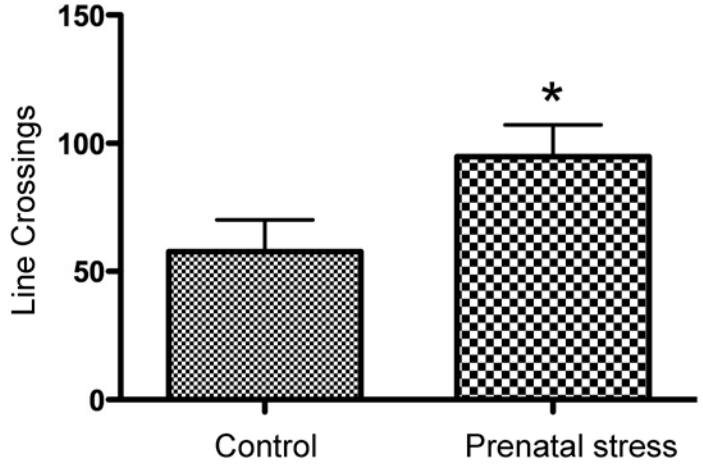

Figure 1.2. Effect of prenatal stress on adult locomotor activity. The data represent the mean ± SEM for 12-14 animals. *, p<0.05, significantly different from non prenatal stress.

The effect of prenatal stress on locomotor activity in the open field is shown in Figure 1.2. Offspring exposed to maternal prenatal stress exhibit significantly greater locomotor activity in the open field. However, there was no effect of prenatal stress on the social interaction component of the test battery.

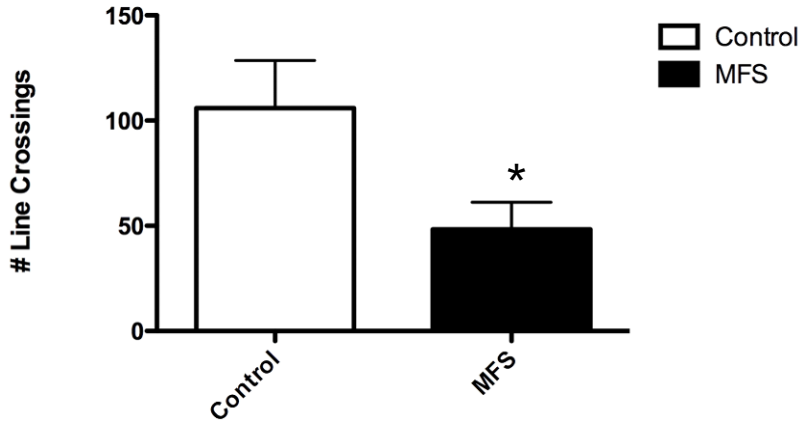

Figure 1.3. The effect of massed foot shock on locomotor activity in the open field. The data represent the mean ± SEM for 12-14 animals. *, p<0.05, significantly different from control.

The effect of massed foot shock on activity in the open field is shown in Figure 1.3. The mean number of line crossings in the open field was significantly reduced in mice after massed foot shock. However, as shown in Figure 1.4, there was no significant interaction with prenatal stress.

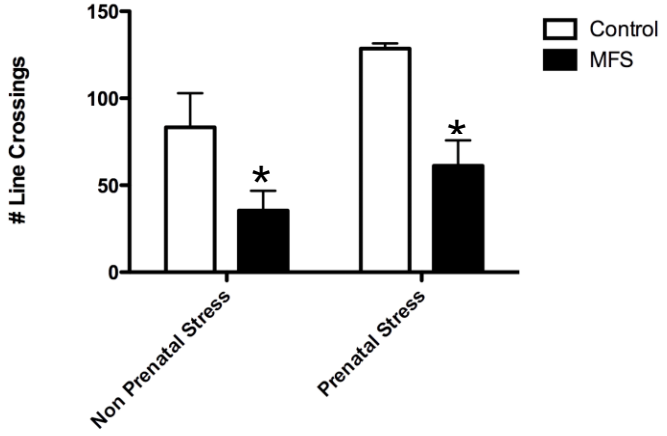

Figure 1.4. The effect of massed foot shock on locomotor activity in the open field. The data represent the mean ± SEM for 12-14 animals. *, p<0.05, significantly different from control.

Although massed foot shock was associated with a decrease in open field activity, and prenatal stress was associated with an increase in open field activity, there was no significant interaction. Thus, exposure to prenatal stress did not potentiate the effects of massed foot shock.

Figure 1.5 shows the effects of prenatal stress on the response to massed foot shock on the number of entries in the open arm of the elevated plus maze.

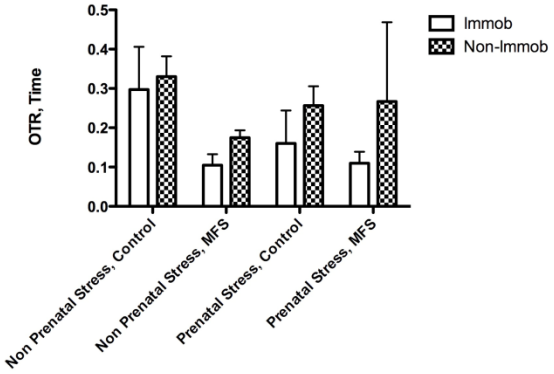

Figure 1.5. The effect of massed foot shock and prenatal stress on stress reactivity in the elevated plus maze. The data represent the mean ± SEM for 3 to 4 animals.

There was no significant interaction between prenatal stress and MFS on immobilization-induced anxiety in the elevated plus maze.

We had proposed to examine the effects of MFS on fear conditioning and extinction. However, we found a significant confounding effect of MFS on this test. Shown in Figure 1.6 are the effects of MFS on fear conditioning and extinction.

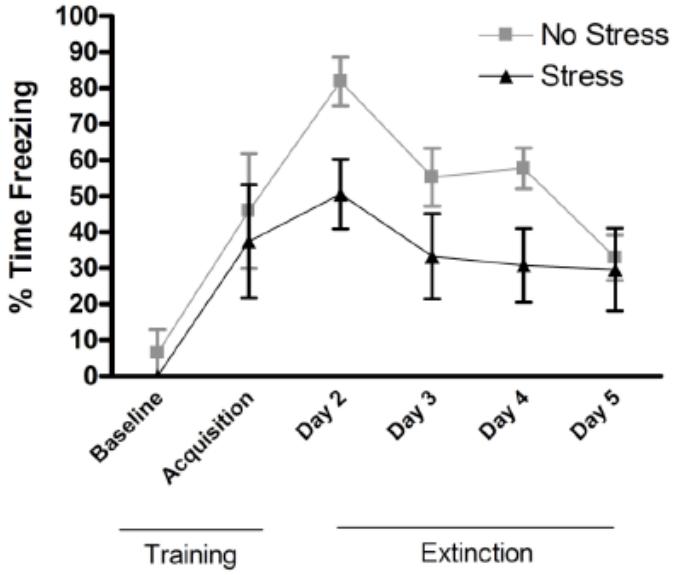

Figure 1.6. The effect of massed foot shock and prenatal stress on fear conditioning and extinction. The data represent the mean ± SEM of the percent of the time spent freezing in response to a tone that was paired with a shock.

As shown in Figure 1.6, MFS actually decreased the response to the fear conditioning task. We hypothesize that MFS accustomed the rats to shock. Thus, when they were exposed to the milder shock in the fear conditioning and extinction test, they reacted less to the aversive stimulus.

We also made neurochemical measurements. While they are still in progress, we have included the results so far.

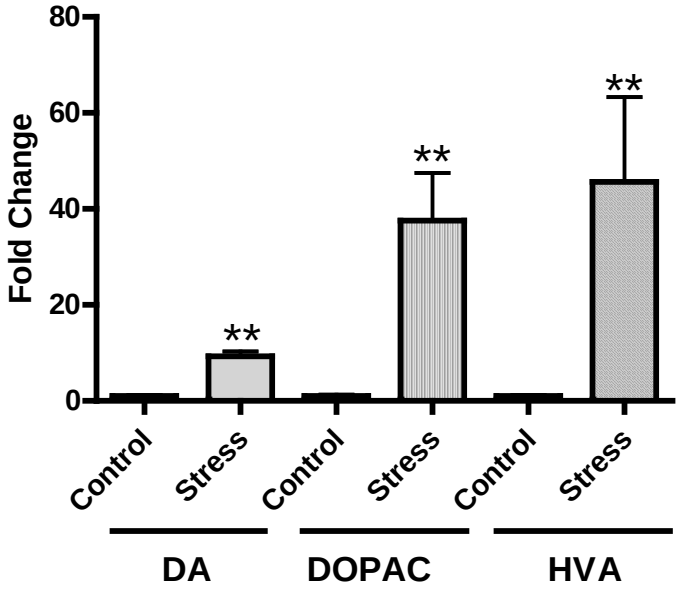

Figure 1.7: Effect of prenatal stress on neostriatal DA and metabolites. The data represent the mean ± SEM for 4-7 animals. *, p<0.05, significantly different from non prenatal stress.

Figure 1.7 shows that prenatal stress greatly increases dopamine and its metabolites in the striatum of adult rats that are the offspring of mothers who were subjected to immobilization stress during pregnancy. These data are consistent with the increased locomotor activity in these rats.

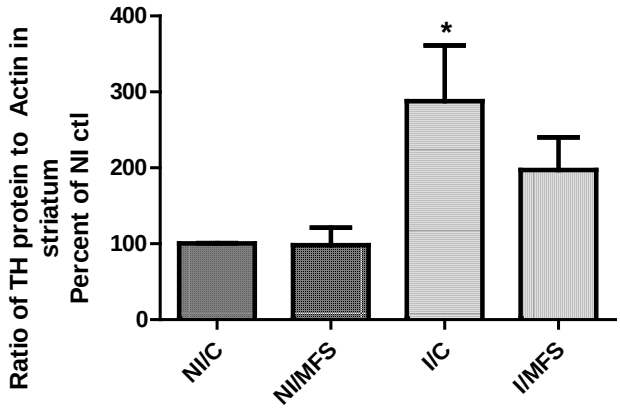

Figure 1.8: Effect of prenatal stress on neostriatal TH protein. The data represent the mean ± SEM for 4-7 animals. *, p<0.05, significantly different from rats that were not immobilized in utero.

We plan to repeat the measures in the striatum, but these data are consistent with the results of measures of tyrosine hydroxylase, the rate-limiting enzyme in the synthesis of dopamine, shown in Figure 1.8, showing a significant increase in neostriatal TH protein.

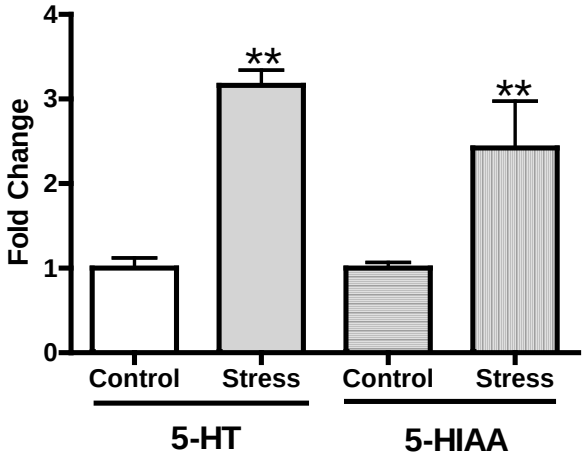

Figure 1.9: Effect of prenatal stress on neostriatal serotonin and its metabolite. The data represent the mean ± SEM for 4-7 animals. **, p<0.01, significantly different from non prenatal stress.

Furthermore, Figure 1.9 shows that serotonin and its metabolite were also significantly increased in the neostriatum.

We also measured tyrosine hydroxylase mRNA and protein in the adrenal medulla as a measure of peripheral sympathoadrenal activation (Figures 1.10 and 1.11).

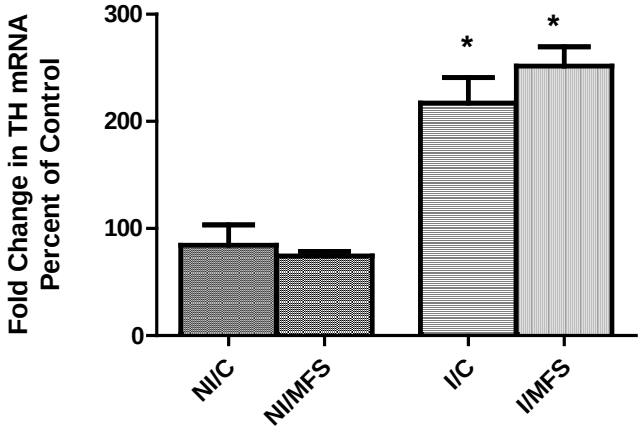

Figure 1.10: Effect of prenatal stress on adrenal TH mRNA. The data represent the mean ± SEM for 3-4 animals per group . *, p<0.01, significantly different from non prenatal stress.

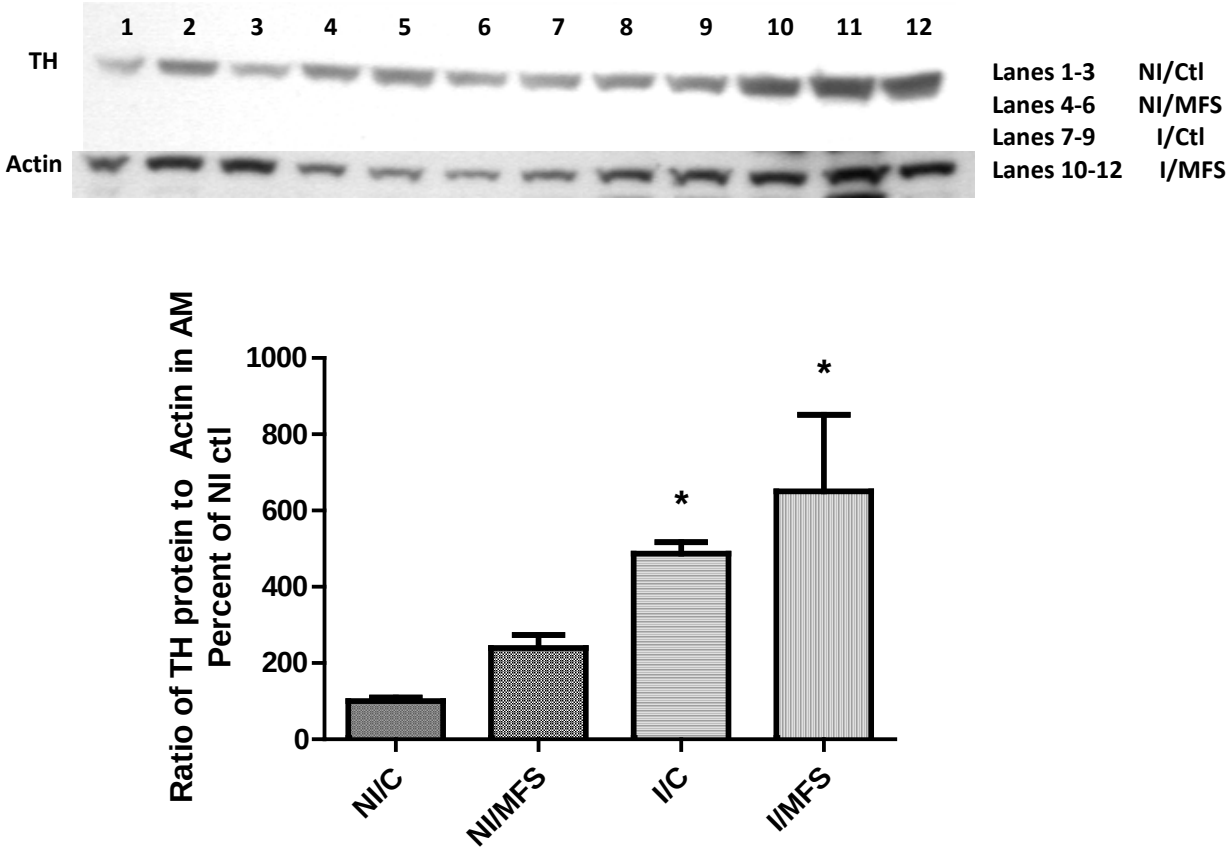

Figure 1.11: Effect of prenatal stress on adrenal TH protein. The data represent the mean ± SEM for 3 animals per group . *, p<0.01, significantly different from non prenatal stress.

The results show that prenatal stress increased both TH mRNA and TH protein in adult offspring. This is consistent with a behavioral phenotype that would be hyper-responsive to stress and is consistent with the behavioral data showing that prenatal stress increases behavioral responsiveness to mild stressors, e.g. the defensive burying shock probe test.

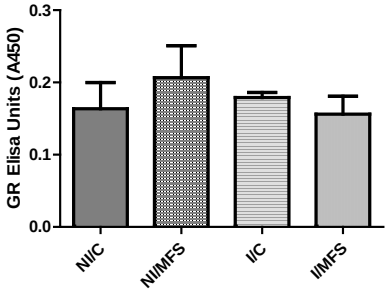

Figure 1.12: Effect of prenatal stress on glucocorticoid receptors in the hippocampus. The data represent the mean ± SEM for 3-4 animals per group .

It has been reported that stress early in life reduces the number of glucocorticoid receptors in the hippocampus in adult. The increase in these receptors causes elevation of glucocorticoids in response to stress, because of reduced feedback inhibition of ACTH production. Therefore, we also measured glucocorticoid receptors in the hippocampus,. The results are shown in Figure 1.12. There were no differences in hippocampal glucocorticoid receptors as measured by an ELISA assay for glucocorticoid receptor protein. However, as will be reported later, using Western blot techniques, we did measure changes in glucocorticoid receptors and mRNA in cortex and hippocampus.

SUMMARY: During the first year of funding, we established a model of prenatal stress that produces an adult phenotype characterized by changes in neurochemistry in the peripheral and central nervous system and increased stress reactivity in the open field. Moreover, it appeared to increase reactivity in the shock probe defensive burying test. Some of these experiments were repeated in the second year of funding as discussed below, adding additional animals to reach statistical significance. The biggest challenge that we encountered during our first year is that our PTSD-like traumatic stress, massed foot shock, appeared to have introduced a confounding variable in the fear conditioning and extinction test. Thus, in the second year of funding we developed an alternative PTSD-like stress as will be discussed below.

YEAR 2:

During the second funding period ((September 1, 2009 to August 31, 2010), we finished performing experiments to further establish the methods for Model 1 (prenatal stress) to address Task 1 - Determine adult predictors of vulnerability to stress; and Task 2 - Determine adult vulnerability to stress. During this period, we also concentrated on Task 2. (Steps 1 – 10), developing a model of fear conditioning and extinction and testing a model of chronic stress that sensitizes rats to fear conditioning and extinction and interferes with memory of conditioned fear extinction. We further examined neurochemical and hormonal mechanisms that may underlie resistance to conditioned fear extinction in animals exposed to maternal stress *in utero* as outlined in Steps 12-13 of Task 1 and Steps 11-13 of Task 2).

Experimental Design. Tasks 1 and 2; Steps 1 – 3: We assessed the effects of prenatal stress by immobilizing timed-pregnant female rats for 1 hr/day on embryonic days (ED) 14 – 21. Unstressed pregnant females served as controls. On post-natal day (PD) 3, the litters were culled to 8 pups. Males were weaned and pair-housed on PD 21. Steps-4 and 5: On PD 42, half the animals were tested on the shock-probe defensive burying test. In this procedure, the rats received a single brief and mild footshock in a cage filled with excess bedding, allowing us to measure the extent to which the animals buried the probe after receiving the shock. The extent of burying behavior reflects an active response to stress, and immobility (i.e., "freezing") reflects a passive response. Together, these measures indicate the degree of stress reactivity. On day 54, rats were exposed to the PTSD stress model (Step 10). Other rats served as unstressed controls. On PD 70, half of the animals in each condition were sacrificed for neurochemical and hormonal measures (Task 1 Steps 12-14), and the other half assessed for behavioral stress reactivity on a battery of tests on PD days 70-79. On day 73, animals were tested for hyperarousal by measuring locomotion in an open field as part of the Social Interaction Test (Task 1 Step 7 and Task 2 Step 6). On day 74, the rats were tested for social interaction (Task 1 Step 7 and Task 2 Step 6)). On day 75, state anxiety and stress reactivity were tested on the elevated plus maze (Task 1 Step 8 and Task 2 Step 7). On day 76, fear conditioning was measured and on days 77 through

79, extinction training and memory of extinction training was tested (Task 1 Steps 10 and 11; Task 2 Steps 9-10). Because of the logistics involved in testing such a large number of animals on this battery of tests, we performed the above in several cohorts of rats and combined the data from the groups. We reported results on Task 1, Steps 1-9 and Task 2, Steps 1-8 in the year 1 annual report. We had technical difficulties with Tasks 1, Steps 10 and 11 and Task 2 Steps 9 and 10. We solved the problem, which was reported in the year 1 annual report and is now reported here (Task 1 Steps 10 and 11 and Task 2 Steps 9 and 10. We found that the only measures that were informative (i.e. showed significant differences) were in Task 1 Steps 10 through 14 and Task 2 Steps 9 through 13. These Tasks and Steps were repeated reliably with the same results and the results are shown here.

Results.

Behavioral measures: In year 1 we reported on the effect of prenatal stress on reactivity to foot shock in the shock probe defensive burying test- Task 1 , Steps 1- 5. Although in our first cohorts there appeared to be significant effects, the effects were lost when we added in the results of subsequent cohorts. Subsequent to applying the PTSD stress model we were using at the time, we saw no significant effects on social withdrawal (Task 1, Step 6 and Task 2, Step 7) as measured in the social interaction test or in locomotor activity measured in the open field. Because we see no significant differences on these tests, we do not plan to continue measuring them in subsequent cohorts using the maternal stress model.

Our most significant accomplishments in the second year were in developing an effective and valid stress model that induces behavioral changes most relevant to PTSD and that is also sensitive to prenatal stress, and in establishing a method to testing one of those key behaviors, fear conditioning and extinction (Task 1 Steps 9 and 10 and Task 2 Steps 9 and 10). In particular, we were able to overcome a confounding interaction in which the original model of PTSD-like traumatic stress interfered with fear conditioning.

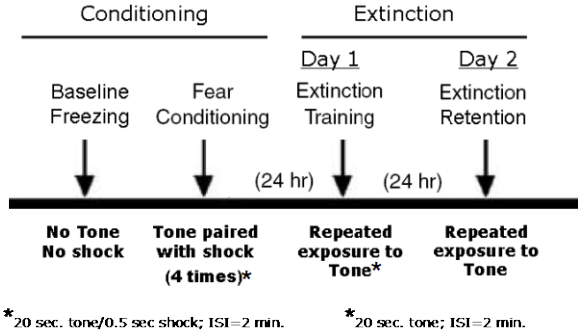

Figure 2.1. Procedure for measuring fear conditioning and extinction.

Figure 2.1 shows the procedures for measuring fear conditioning and extinction (Tasks 1 and 2, Steps 9 and 10. Freezing was first measured in the absence of conditioned and unconditioned stimuli to establish a baseline for each animal and then each animal was exposed to a series of four shocks paired with a tone. Each tone lasted for 20 seconds and ended with a brief shock during the last 0.5 seconds of the tone. The tone-shock pairing was repeated 4 times with an inter-stimulus interval of 2 minutes. The effects of cold stress and prolonged stress (CAPS) and prenatal stress (PNS) on acquisition of conditioned fear is shown in Figure 2.2.

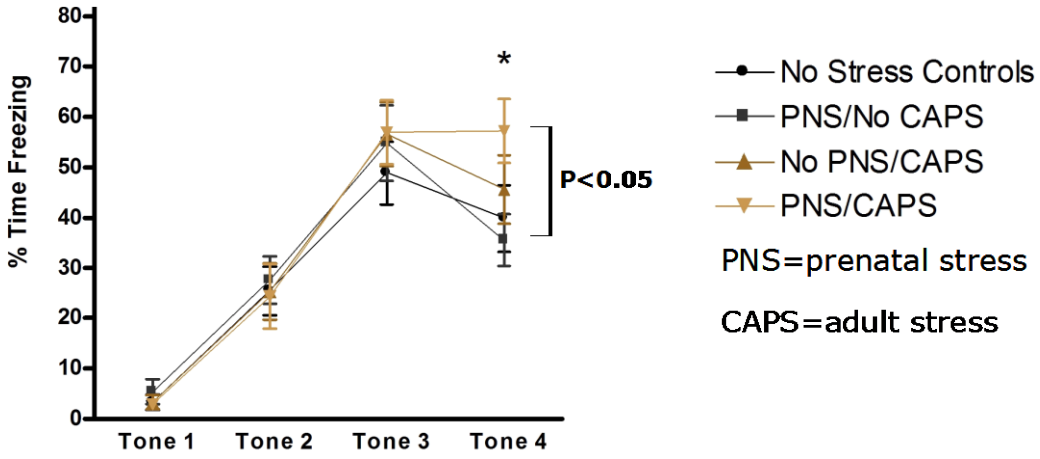

Figure 2.2. The effect of prenatal stress and adult stress on acquisition of conditioned fear The data represent the mean ± SEM for 23 to 25 subjects. Data were analyzed by a 3-way ANOVA followed by post-hoc tests of significance of differences between individual means.

Using these procedures, we found that prenatal stress plus adult stress sensitized subjects to the acquisition of conditioned fear (Tasks 1 and 2; Step 9). There was a significant difference in the mean percent of time freezing between the groups exposed to prenatal stress plus the adult stress (PNS/CAPS group) and the group exposed to prenatal stress but no adult stress (PNS/No CAPS) group.

Figure 2.3. The effect of prenatal stress and adult stress on acquisition of conditioned fear extinction The data represent the mean ± SEM for 23 to 25 subjects. Data were analyzed by a 3-way ANOVA.

Figure 2.3 shows the effects of prenatal stress and adult stress on conditioned fear extinction training. (Tasks 1 and 2 Step 10). There was no effect of prenatal stress on this measure. However, there was a significant effect of adult stress (CAPS) on acquisition of extinction training and a significant interaction between tone number and CAPS. This result is interpreted as showing that prior exposure to chronic stress during adulthood hinders subsequent acquisition of the extinction of conditioned fear.

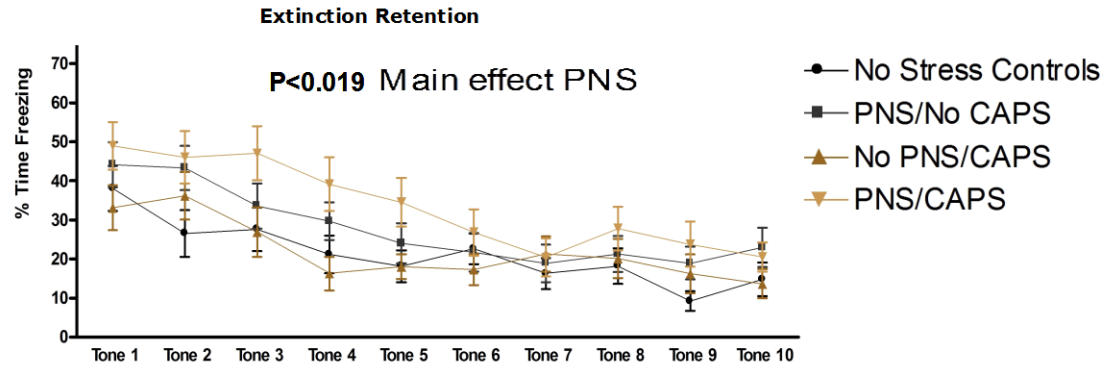

Figure 2.4. The effect of prenatal stress and adult stress on retention of conditioned fear extinction The data represent the mean ± SEM for 23 to 25 subjects. Data were analyzed by a 3-way ANOVA.

As shown in **Figure 2.4**, there was a significant main effect of prenatal stress on the retention of the memory of conditioned fear extinction (Tasks 1 and 2; Step 10). This is more clearly shown in **Figure 2.5** in which only the groups that received adult stress (CAPS) are shown. Animals exposed to prenatal stress and adult stress (PNS/CAPS) show greater deficits in retention of conditioned fear extinction as compared to the animals not exposed to prenatal stress (No PNS/CAPS).

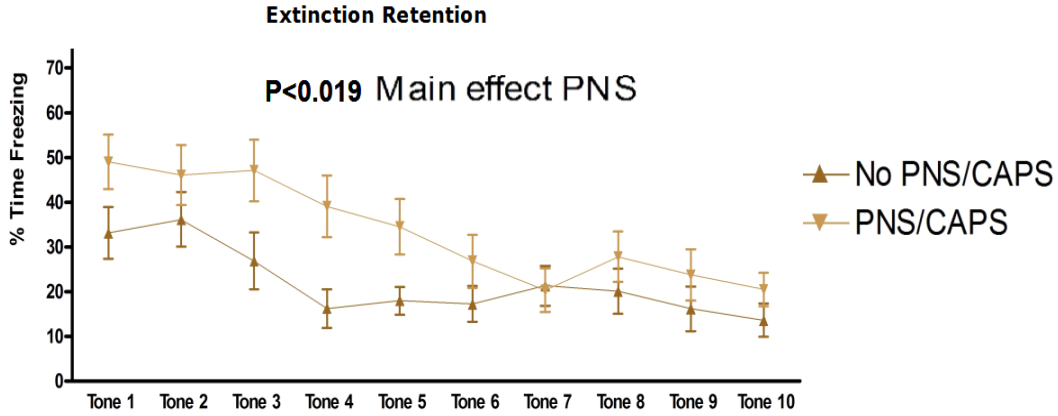

Figure 2.5. The effect of prenatal stress and adult stress on acquisition of conditioned fear extinction The data represent the mean ± SEM for 23 to 25 subjects. Data were analyzed by 3-way ANOVA.

Thus, these data reveal that prenatal stress plus traumatic adult stress impairs memory of conditioned fear extinction. Taken together, **these results provide a rationale for investigating the factors invoked during prenatal stress that may predispose for individual differences in adult susceptibility to PTSD, and perhaps also for differences in treatment response**.

Neurochemical and hormonal measures (Task 1 Steps 12, 13 and 14; Task 2 Steps 11, 12 and 13): The noradrenergic system plays a key role in central modulation of stress responses and memory for fear extinction. Noradrenergic signaling in prefrontal cortex strengthens memory for fear extinction (Mueller et al., 2008; Milad and Quirk 2002). Furthermore, work from our

laboratory showed that the WKY genetic rat model of impaired stress adaptation (and hence, a commonly used rat model of PTSD) exhibits impaired synthesis of tyrosine hydroxylase (TH) mRNA in the locus ceruleus in response to stress (Sands et al., 2000). Consistent with that finding, induction of FOS expression by acute immobilization stress is reduced in locus ceruleus of WKY rats (Ma and Morilak 2004). Tyrosine hydroxylase (TH) is the rate-limiting enzymatic step in the synthesis of norepinephrine. Therefore, alterations in TH gene expression directly affect noradrenergic neurotransmission. The locus ceruleus is the source of noradrenergic innervation of the prefrontal cortex. Therefore, we measured mRNA for TH in the brainstem region containing the locus ceruleus as a first step in determining whether changes in noradrenergic function play a role in the effects of PNS and CAPS on memory for conditioned fear extinction.

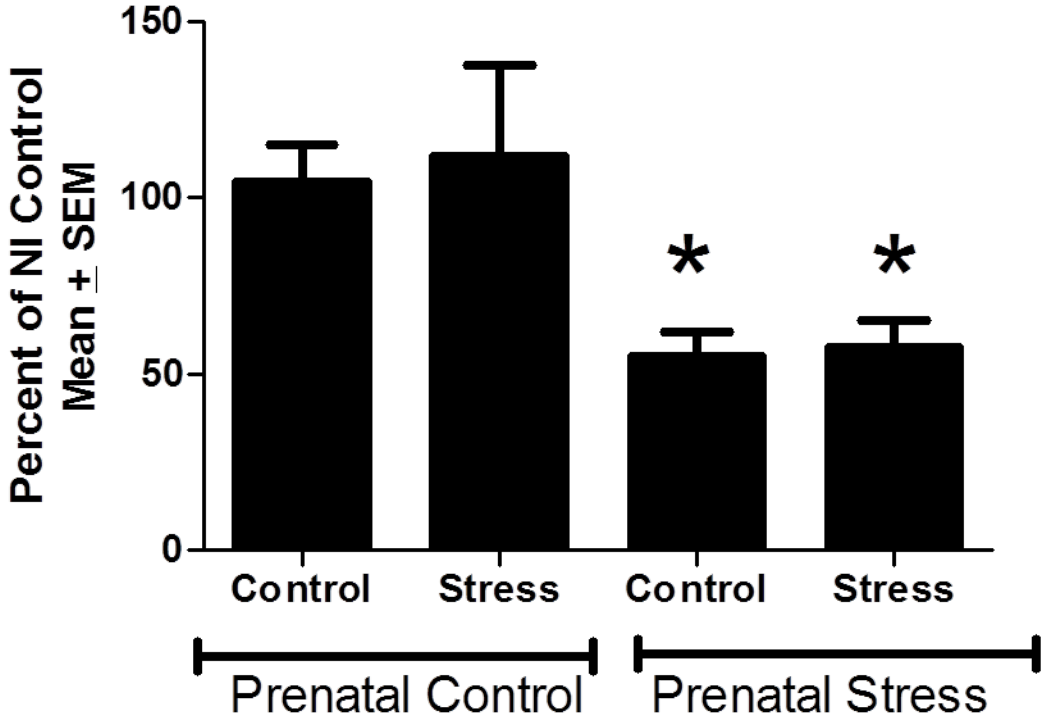

Figure 2.6: Effect of prenatal stress on TH mRNA in brain stem. The data represent the mean ± SEM for 17-18 animals. *, $p<0.05$, significantly different from non-prenatal stress controls. NI=no prenatal immobilization.

Figure 2.6 shows that prenatal stress reduced adult TH gene expression in the brain stem region containing the locus ceruleus. It is not surprising that there wasn't an effect of adult stress on mRNA levels of TH, because the rats were sacrificed 16 days after the adult stress treatment. Previous studies have shown that acute stress produces a transient increase in mRNA for TH followed by a more stable increase in TH protein. Since TH expression controls the capacity for noradrenergic neurotransmission in the prefrontal cortex, these results suggest a possible mechanism underlying resistance to extinction of conditioned fear that we observed in rats exposed to prenatal stress. Specifically, a reduction in the capacity for NE synthesis would impair noradrenergic modulation of the cognitive processes underlying memory for extinction. Circulating corticosteroids and brain glucocorticoid receptors are also reported to play a role in

responses to stress and in memory of fear extinction. Corticosterone exposure has been reported to regulate fear extinction (Gourley et al., 2009). Glucocorticoids and norepinephrine have been reported to act on the medial prefrontal cortex to modulate PTSD symptoms (Bremner et al., 2008) in humans. Furthermore, disruption of the glucocorticoid negative feedback system is induced in animals by chronic stress and involves down regulation of glucocorticoid receptors in the prefrontal cortex (Mizoguchi et al., 2003). Therefore, we measured plasma corticosterone levels and levels of glucocorticoid receptor protein in the prefrontal cortex and hippocampus. Figure 2.7 shows the effects of adult and prenatal stress on plasma corticosterone levels (Task 1 Step 12; Task 2, Step 11). Prenatal stress significantly increased basal levels of plasma corticosterone. Since the animals were exposed to adult stress 16 days before blood was taken for these measures, it is not surprising that there was no effect of adult stress on this measure. Thus, in studies planned for this year, we will measure both basal and acute stress-induced levels of corticosterone and ACTH.

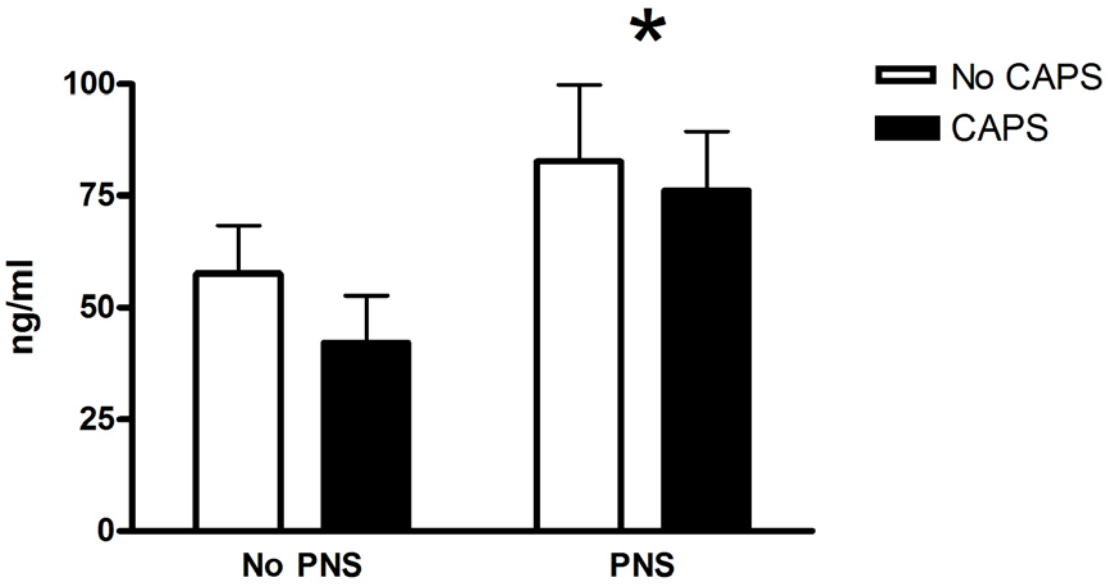

Figure 2.7: Effect of prenatal stress and adult stress on plasma corticosterone. The data represent the mean ± SEM for 5 animals per group. *, $p<0.05$, significantly different from non-prenatal stress groups.

We then measured glucocorticoid receptor (GR) protein in the prefrontal cortex (Task 1 Step 14 and Task 2 Step 13). As shown in Figure 2.8, adult stress (CAPS) had a significant effect on GR protein that was evident 16 days after CAPS ended. Moreover, rats exposed to prenatal stress showed a significant reduction in mean GR protein in the absence of exposure to CAPS. There was no further reduction in GR protein in rats exposed to both stresses. There was no effect of either stressor in the hippocampus. These results suggest that the chronically elevated corticosterone levels caused by prenatal stress alone, which are comparable to those induced following the period of chronic adult stress, may explain the reduction in GR protein in the prefrontal cortex.

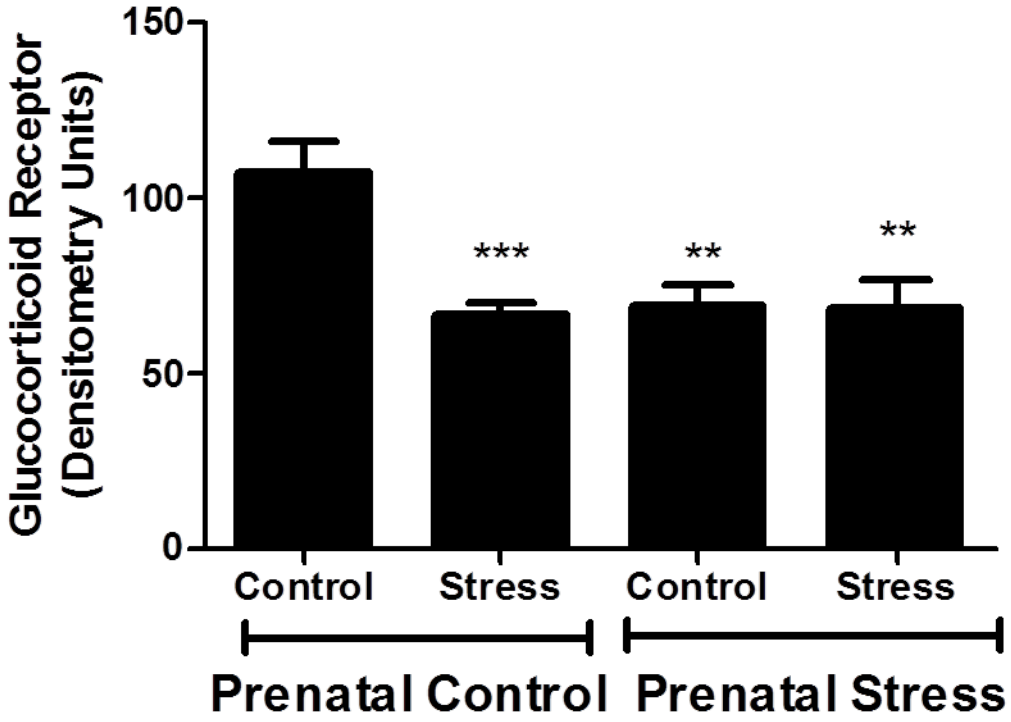

Figure 2.8. Effect of prenatal stress and adult stress on glucocorticoid receptors in the prefrontal cortex. The data represent the mean ± SEM for 18 to 19 animals per group . **, $p<0.01$, significantly different from non-prenatal stress. *, $p<0.001$, significantly different from non-stressed control group.

SUMMARY OF SECOND YEAR FINDINGS: In the first year, we reported that our model of PTSD-precipitating traumatic stress appeared to be deficient in several ways. It didn't show an interaction with prenatal stress in the open field or in the immobilization-induced effects on exploratory activity in the elevated plus maze. It also appeared to have introduced a confounding variable in the fear conditioning and extinction test. Thus, our goal for the second year was to develop an alternative stress model that would trigger PTSD-relevant behavioral phenotypes. We developed a new PTSD-like stress (CAPS) which has face validity as a PTSD-precipitating stressor. Moreover, we developed an improved measure of fear conditioning and memory of conditioned fear extinction. We found that prenatal stress programs adult sensitization to conditioned fear and resistance to conditioned fear extinction. We also discovered that prenatal stress programs a unique neurochemical and hormonal phenotype that suggests a mechanism by which it sensitizes to acquisition of conditioned fear and impairs memory of conditioned fear extinction. Thus, we observed that prenatal stress reduces TH expression in noradrenergic neurons in the LC region of the brainstem, increases basal corticosterone levels and reduces GR protein in prefrontal cortex. The chronically elevated corticosterone may explain the reduction in GR protein in the prefrontal cortex. Thus, for the following year we focused additionally on noradrenergic-corticosterone interactions in studies of the role of early life stress in increased vulnerability and reduced resilience stressors that precipitate PTSD.

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YEAR 3:

During year 3 (September 1, 2010 to August 31, 2011), we completed the experiments on Model 1 (prenatal stress) to address Task 1 - Determine adult predictors of vulnerability to stress; and Task 2 - Determine adult vulnerability to stress. The results of these studies were published in the journal Neuroscience and appeared in the online version on June 22, 2011.

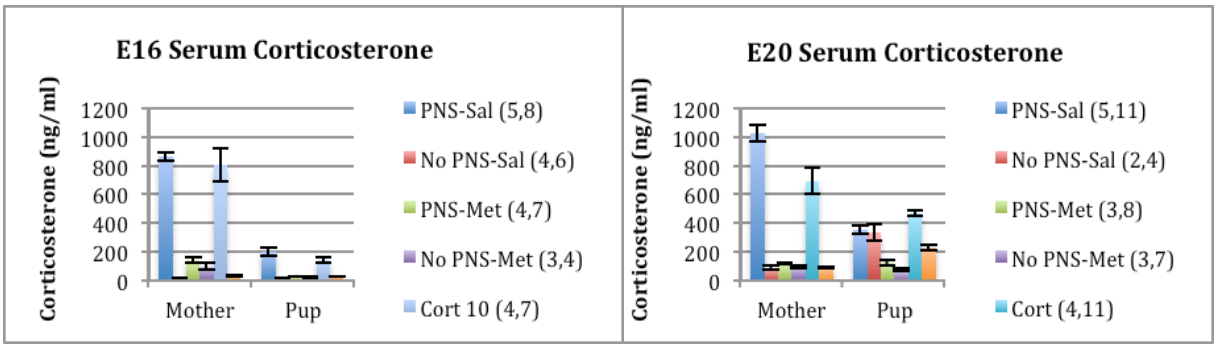
This report covers additional progress from studies begun in the previous year and completed in year 3 as well as the initiation of experiments in Year 3 to begin testing of the second of four models of early life trauma, i.e. testing the effects of pharmacological manipulation of corticosteroid function during pregnancy to determine the role of corticosterone during maternal stress on behavioral and neurochemical phenotypes in adult offspring (Model 3). We tested Model 3 before Model 2, because it more logically follows from the studies employing Model 1 (the effects of maternal restraint stress on adult predictors and adult vulnerability to traumatic stress). We made a minor modification to the approach in which, instead of testing dexamethasone (a synthetic glucocorticoid) in utero, we administered the natural glucocorticoid agonist corticosterone. We also began another model in which we hoped to measure the effect of the corticosteroid antagonist metyrapone administered during prenatal stress to determine the role of glucocorticoids in the effects of prenatal stress on subsequent adult changes in neurochemistry and behavior in the offspring. For Model 3**,** we decided against using the synthetic glucocorticoid receptor agonist dexamethasone to determine if it would mimic the effects of stress, because it is a pure glucocorticoid receptor agonist whereas the endogenous glucocorticoid (corticosterone) is an agonist at both glucocorticoid and mineralocorticoid receptors. Therefore, the natural glucocorticoid more closely mimics the effects of stress.

Moreover, by using the antagonist metyrapone and the agonist corticosterone, we hoped to be able to determine unambiguously whether corticosterone is involved in the effects of maternal stress. In order to design an intervention, we needed to know whether the naturally occurring corticosterone is involved in the effects of maternal stress, as opposed to other stress-responsive hormones such as catecholamines.

Results:

Initial studies (Model 3, Task 1, Steps 1-4) were done to establish a range of effective doses of CORT and metyrapone which could then be tested in the pregnant females. These doses were initially established using DMSO as a vehicle for both drugs. However due to several concerns, including potential toxicity of DMSO, pup loss, stress caused by the strong odor emitted by DMSO-treated animals, and later observations of behavioral inconsistencies, the vehicles were changed to saline (for metyrapone) and sesame oil (for CORT), and the doses of metyrapone and CORT were re-established. For CORT, groups of time-pregnant SD rats were injected once daily with a range of doses (5-30 mg/kg, s.c) starting at embryonic day E14. A positive control PNS-treated group was injected with vehicle and immobilized 1hr/day. Dams were sacrificed by decapitation on day E16 or E20, and maternal trunk blood was collected. Fetuses were then rapidly removed (<5 min) and trunk blood collected with heparinized capillary tubes. Serum CORT was measured by RIA.

To establish a dose of metyrapone that blocks the stress-induced elevation in endogenous CORT by PNS exposure, pregnant rats were treated with metyrapone (50-100 mg/kg s.c.) 1 hr. prior to immobilization each day. A second PNS group was injected with vehicle 1 hr. before immobilization. Results for CORT (10 mg/kg in sesame oil) and Metyrapone (100mg/kg in saline) are shown in **Figure 3.1**. At both E16 and E20, PNS and CORT (10 mg/kg) induced comparable CORT increases in both maternal and fetal serum. Further, PNS-induced CORT release was effectively blocked in both mothers and fetuses by pretreatment with metyrapone. These data indicate that over the course of daily PNS, rat fetuses are exposed to a level of CORT that can be mimicked by 10 mg/kg exogenous CORT given daily, and blocked with 100 mg/kg metyrapone pretreatment.



<u>Figure 3.1:</u> Serum CORT following treatment with PNS, metyrapone, or CORT Treatment was started at embryonic day E14 and measured at day E16 (left) or E20 (right). Data are expressed at the mean ± SEM for the number of animals in parentheses (mothers, pups).

Some pups in each treatment group were tested on behavioral and molecular biology tests as adults (Model 3, Tasks 1 and 2, Steps 4 and 5).

Behavioral Testing: Due to the very long time between prenatal treatment and adult testing, we initiated pilot studies investigating the potential role of prenatal CORT on the adult behavioral effects of PNS even as we were still establishing the optimal doses for prenatal treatment. From E14 to parturition, an initial 2 groups of pregnant rats were either given daily injections of metyrapone prior to immobilization stress, or given injections of exogenous CORT (5 mg/kg) in lieu of immobilization stress, as described above. The offspring of these rats and their vehicle-treated/non-stressed controls were allowed to mature to late adolescence, at which point they were subject to CAPS stress, then tested in the fear conditioning and extinction paradigm Model 3, Task 2, Steps 5 and 11). However, in these groups we did not replicate the previous effect of PNS-CAPS-induced deficits in fear extinction. We believe that this was due to the use of DMSO as the prenatal vehicle, as described above, and we repeated this experiment with oil and saline as respective vehicles as will be discussed later.

We also examined changes in coping style as a result of prenatal stress and/or CAPS (Model 3, Task 1, Step 6; Task 2, Steps 5 and 6) substituting shock probe defensive burying for cognitive deficits (Model 3, Task 2, Step 6). Timed-pregnant female rats were singly housed throughout pregnancy. Half the pregnant females were immobilized daily for 1 hr, from day 14 of pregnancy until birth. Unstressed control pregnant females were left undisturbed during this same time period. On postnatal day (PD) 5, litters were culled to 8 pups each, maximizing the number of males retained, and weaned on PD 21. Upon weaning, male pups were pair-housed with a littermate until PD 51-53, at which time they were singly housed. A subset of the rats that received prenatal stress and a subset of those that did not were then subjected to adult CAPS stress or served as unstressed controls.. The combination of PNS and adult CAPS treatment resulted in 4 treatment groups: No PNS/No CAPS (i.e., unstressed controls; n=20), PNS/No CAPS (n=7), No PNS/CAPS (n=10), and PNS/CAPS (n=13). One day after CAPS (or the comparable control time, at PD 66-68), the rats were tested in the shock probe defensive burying test. They were placed into a modified cage containing 5 cm of bedding, with a shock probe protruding 6 cm into one end of the cage. The probe was set to deliver 2 mA of current when the probe was touched. After the rat made contact with the probe and received a shock, the current was shut off and the 15 min test began. Behavior was recorded using a CCD camera mounted above the cage and stored to video files for offline scoring and analysis. The dependent measures were the amount of time spent immobile and the amount of time spent engaged in actively burying the probe.

The results are shown in **Figure 3.2.** CAPS-treated rats displayed a significant reduction in the amount of time spent burying ($F(1,44)=4.323$, $p<0.05$). Likewise, these rats displayed an increase in immobility ($F(1,44)=20.65$, $p<0.0001$). CAPS-treated rats displayed a significant decrease in the burying ratio, calculated as (time spent burying)/(time spent burying + time spent immobile)* 100 ($F(1,44)=11.02$, $p<0.01$), reflecting a change in coping strategy, from a predominantly active strategy, as seen in the non-CAPS groups, to a predominantly passive strategy. This further validates the utility of the CAPS treatment as a model of traumatic stress, and indicates that an important component of CAPS-induced behavioral pathology may be reflected in a shift from an adaptive active coping strategy to maladaptive passive coping. Further, in replication of the results in our initial publication, there was again no effect of PNS

alone on burying behavior or on immobility, nor was there an interaction between PNS and CAPS.

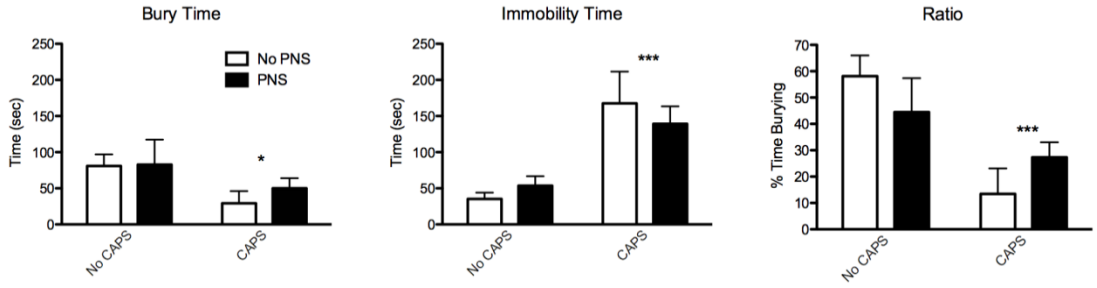


Figure 3.2. Effects of prenatal stress and adult stress on passive and active coping in the shock probe defensive burying test. Data are the mean ± SEM for 7 to 20 subjects.
***P<0.05, significantly different from No CAPS.**
***P,0.001, significantly different from No CAPS.**

Molecular biological analysis: Concurrent with the behavioral experiments described above, we investigated the role of prenatal CORT exposure on adult expression of TH mRNA (qPCR) and GR (ELISA). (Model 3, Tasks 1 and 2, Steps 14 and 15). Littermates of the rats used for behavioral experiments were allowed to grow to adulthood. We have shown that the effects of PNS on TH and GR expression are independent of adult stress, so these animals were not subject to CAPS stress (Task 2, Step 5). As shown in **Figure 3.3**, prenatal stress (PNS) was associated with decreased glucocorticoid receptor protein in the medial pre-frontal cortex. This replicated the finding that PNS decreases GR protein in the mPFC in our recently published studies. Interestingly, Metyrapone also had an effect, decreasing mPFC GR levels by itself. Similar to the behavioral results, we believe these molecular studies may also have been confounded by the use of DMSO as a vehicle. Therefore, a second study was initiated as will be discussed.

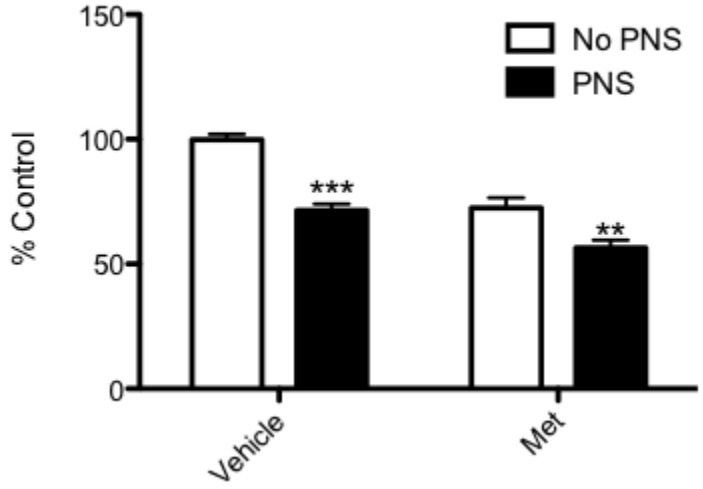


Figure 3.3. Effects of prenatal stress and metyrapone on glucocorticoid receptor protein in the medial prefrontal cortex. Data are expressed as the mean ± SEM.
**, P<0.01, significantly different from No PNS.
***, P,0.001, significantly different from No PNS.

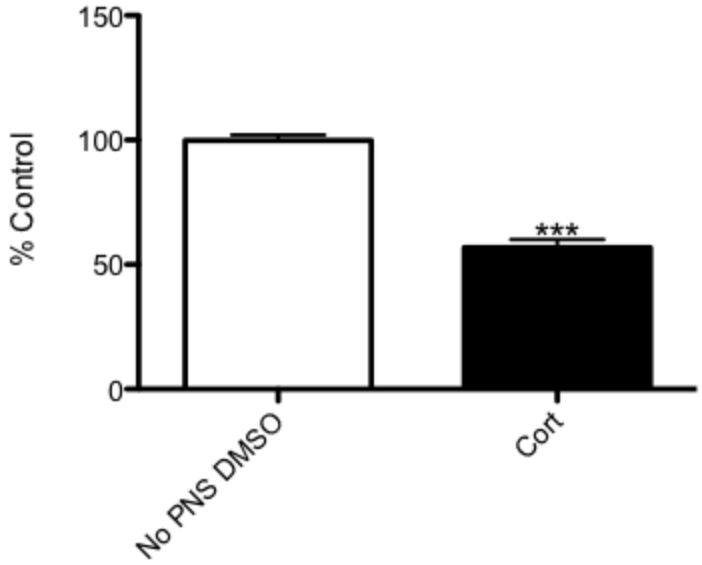


Figure 3.4. Effects of prenatal corticosterone on glucocorticoid receptor protein in the adult medial prefrontal cortex. Data are expressed as the mean ± SEM.
***, P<0.001, significantly different from vehicle control .

Figure 3.4 shows the effects of prenatal treatment with corticosterone on glucocorticoid receptors in the mPFC of adult rats. Like PNS, corticosterone administered during the last week of pregnancy caused a significant decrease in glucocorticoid receptor expression in the adult offspring. These data support the idea that the effects of PNS on glucocorticoid receptors in adulthood are mediated by corticosterone.

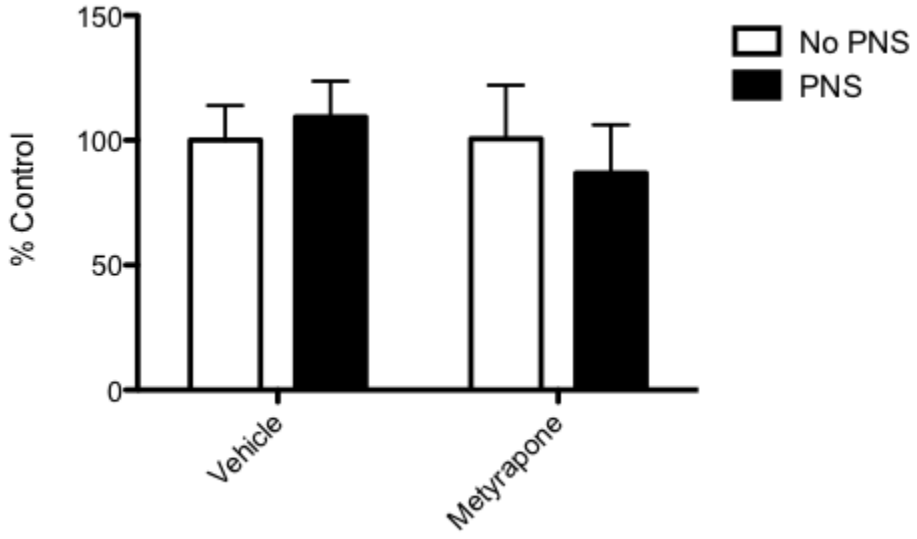


Figure 3.5. Effects of prenatal stress and metyrapone on TH mRNA in the brainstem region containing the locus ceruleus. Data are expressed as the mean ± SEM.
**, P<0.01, significantly different from No PNS.
***, P,0.001, significantly different from No PNS.

Figure 3.5 shows the effects of prenatal stress and metyrapone on TH mRNA in the brainstem region containing the locus ceruleus. In contrast to our previously published results, exposure to the DMSO vehicle and prenatal stress did not result in a reduction in TH mRNA. Moreover, metyrapone did not affect TH mRNA. As mentioned previously, we believe that this study may also have been confounded by the use of DMSO as a vehicle. Therefore, we repeated the study.

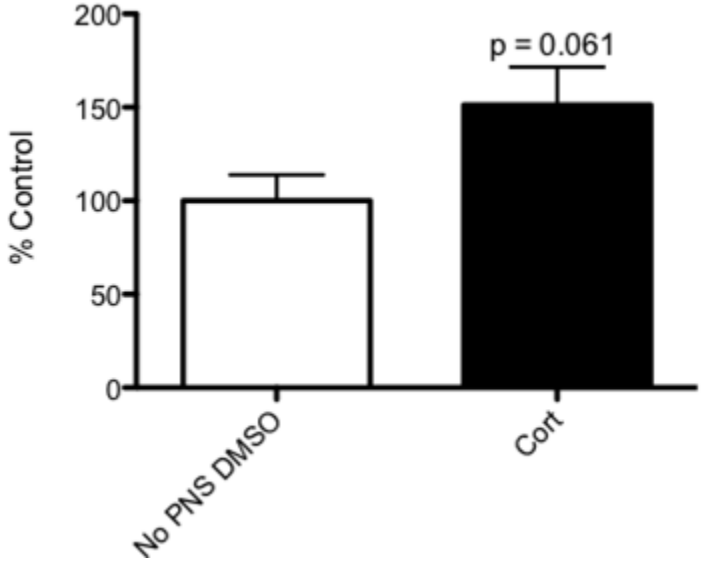


Figure 3.6. Effects of prenatal corticosterone treatment on TH mRNA in the brainstem region containing the locus ceruleus. Data are expressed as the mean ± SEM.

We also observed that prenatal corticosterone treatment caused a marginally significant increase in TH mRNA (**Figure 3.6**). This is in contrast to the effects we observed in our published study (Green et al., 2011) on the effects of prenatal stress, which showed that maternal stress was associated with significant decreases in TH mRNA in the adult brainstem. Thus, corticosterone may not mediate this effect of prenatal stress. As mentioned above, we repeated these studies with a bigger N as discussed later.

SUMMARY OF YEAR 3 FINDINGS

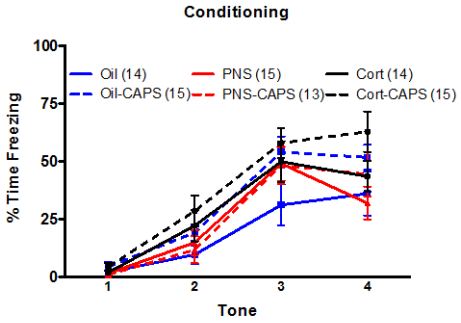
During this year we began testing the second of four models of early life trauma, i.e. testing the effects of pharmacological manipulation of corticosteroid function during pregnancy to determine the role of corticosterone during maternal stress on behavioral and neurochemical phenotypes in adult offspring (Model 3). We established a dose of corticosterone that mimics the levels of corticosterone in mothers and pups exposed to prenatal stress. We established a dose of metyrapone that blocks the increase in corticosterone induced by prenatal stress. We found that adult traumatic stress, independent of prenatal stress, causes changes in active and passive coping in the shock probe defensive burying test. We found that prenatal corticosterone treatment programs a neurochemical phenotype similar to prenatal stress characterized by reduced GR protein in prefrontal cortex. We also found that, unlike prenatal stress, prenatal corticosterone had no effect on TH mRNA, suggesting that prenatal stress produces its effects on TH mRNA through a mechanism independent of corticosterone. In the next year, we further examined these mechanisms and compared the two models.

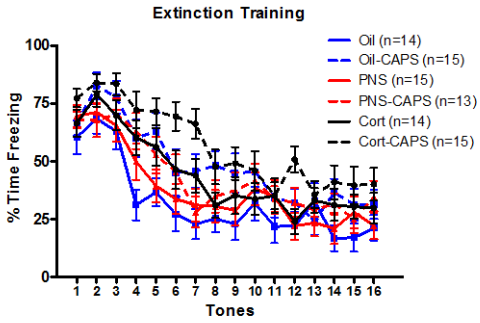
YEAR 4:

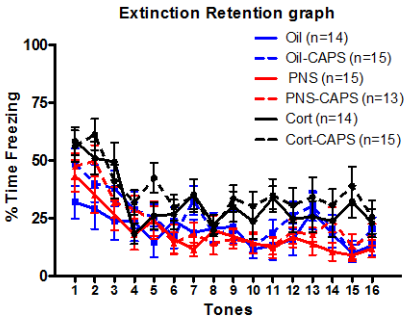
At the end of the previous year (August 31, 2011) we initiated experiments to begin testing the second model of early life trauma, i.e. testing the effects of pharmacological manipulation of corticosteroid function during pregnancy to determine the role of corticosterone during maternal stress on behavioral and neurochemical phenotypes in adult offspring (Model 3). As discussed in the year 3 report, we made a minor modification to the approach in which, instead of testing dexamethasone (a synthetic glucocorticoid) in utero, we administer the natural glucocorticoid agonist corticosterone. During year 4 (September 1, 2011 to August 31, 2012), We also began another model in which we measured the effect of the corticosteroid antagonist metyrapone administered during prenatal stress to determine the role of glucocorticoids in the effects of prenatal stress on subsequent adult changes in neurochemistry and behavior in the offspring. As discussed previously, we decided against using the synthetic glucocorticoid receptor agonist dexamethasone to determine if it would mimic the effects of stress, because it is a pure glucocorticoid receptor agonist whereas the endogenous glucocorticoid (corticosterone) is an agonist at both glucocorticoid and mineralocorticoid receptors. Therefore, the natural glucocorticoid more closely mimics the effects of stress. Moreover, by using the antagonist metyrapone and the agonist corticosterone, we hoped to be able to determine unambiguously whether corticosterone is involved in the effects of maternal stress. In order to design an intervention, we needed to know whether the naturally occurring corticosterone is involved in the effects of maternal stress, as opposed to other stress-responsive hormones such as catecholamines.

Results: Some pups in each treatment group were tested on behavioral and molecular biology tests as adults (Model 3, Tasks 1 and 2, Steps 4 and 5).

Behavioral Testing:







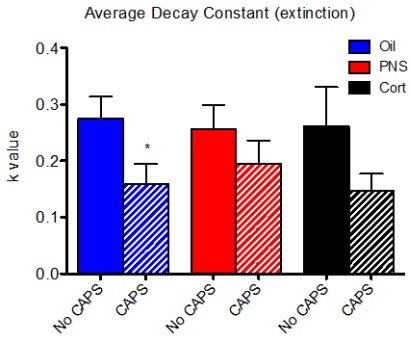


Figure 4.1. The effects of prenatal stress (PNS) or prenatal cort (CORT) on fear conditioning and extinction.

Fear Conditioning: As shown in **Figure 4.1,** there was a main effect of CAPS, resulting in an overall increase in freezing in animals stressed as adults, regardless of prenatal treatment.

Fear Extinction: Also shown in **Figure 4.1**, similar to our published data (Green et al., 2011), we found a significant main effect of adult CAPS exposure, and a CAPS by tone interaction on fear extinction. There was a main effect of prenatal treatment that was driven by prenatal CORT (significantly different than Oil on tones 4, 6-7), indicating that both prenatal CORT and CAPS increase freezing and delay extinction. There was no CAPS by CORT interaction, indicating that that the CAPS and CORT effects were additive. Likewise, when the rate constant was analyzed for each individual animal, we found a significant main effect of CAPS treatment with a significant post-hoc difference in Oil.

Fear Retention: There was a main effect of prenatal treatment and prenatal treatment by tone interaction. CORT increases overall freezing and delays extinction, presumably because baseline-freezing levels are higher. Overall, the data indicate that, in terms of behavior, prenatal corticosterone exposure alone elicits a distinct behavioral profile from prenatal stress, even though the dose of CORT was titrated to mimic the fetal exposure from maternal stress. This may suggest a compensatory mechanism, activated by maternal stress, which buffers prenatal corticosterone exposure in some manner.

Neurochemical Testing:

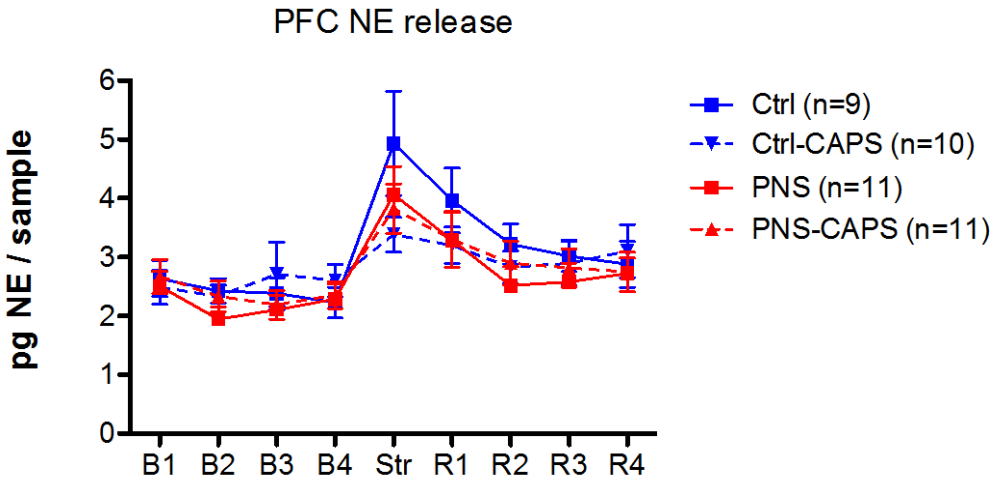


Figure 4.2. Microdialysis in PNS vs Saline animals with and with out CAPS.

We have repeatedly measured reductions in TH mRNA in the pontine region that contains the locus ceruleus. However, the functional significance of the increase in TH mRNA had not been tested. We hypothesized that the decrease in TH mRNA would be reflected in a reduction in norepinephrine (NE) release in the prefrontal cortex, suggesting a mechanism

whereby prenatal stress impaired stress-responsiveness. We therefore measured NE release by microdialysis in the prefrontal cortex of rats exposed to adult stress (CAPS) and/or prenatal stress. We found a CAPS by sample interaction on NE released in the mPFC. CAPS treatment blunted the immobilization induced increase in NE in saline treated animals only. CAPS had no effect in the PNS treated animals, which were non-significantly lower than saline animals. This CAPS effect in control animals may have been due to a habituation, as the immobilization stress used to evoke NE release was also used 5 days prior as part of their CAPS treatment. When the difference from baseline to the first stress sample was analyzed, we found again found a main effect of CAPS.

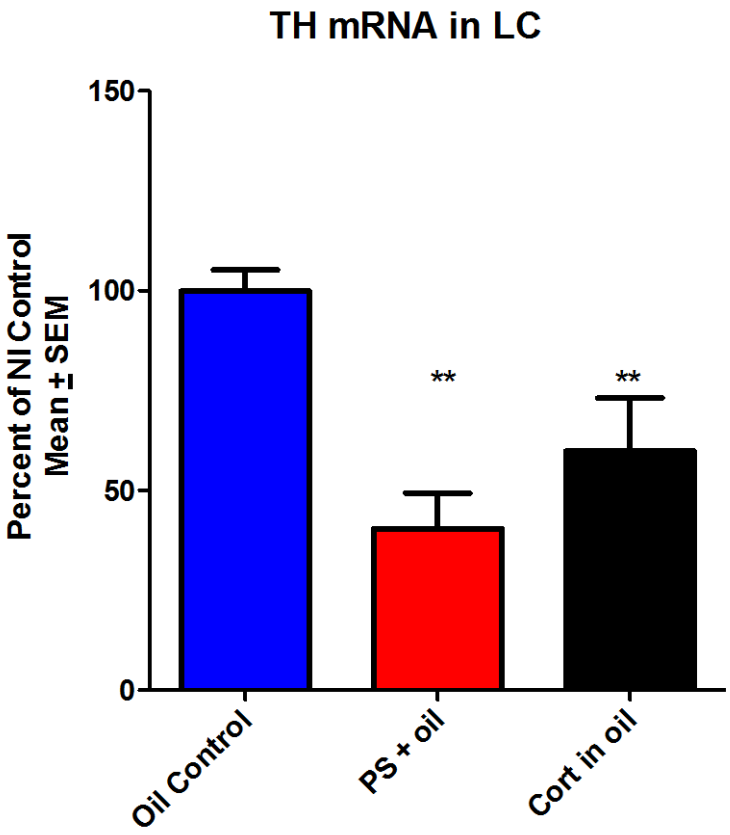


Figure 4.3. Comparison of the effects of prenatal corticosterone (CORT) and prenatal stress (PS) on TH mRNA in the pontine region of the brain containing the locus ceruleus.

** $p < 0.01$ Significantly different from oil control.

As discussed above, we have repeatedly measured by RT-PCR a reduction in TH mRNA in the region of the pons containing the locus ceruleus in adult rats exposed to prenatal stress. Therefore, to determine if this effect could be due to prenatal exposure to corticosterone, we compared the effects of prenatal CORT and prenatal stress on TH mRNA in the pontine region containing the locus ceruleus. As shown in **Figure 4.3**, there was a significant reduction in TH mRNA in rats exposed to prenatal stress as we have reported previously. Similarly, prenatal CORT also reduced TH mRNA. These results are consistent with the conclusion that prenatal stress may produce an effect on TH mRNA by increasing CORT. However, the significance of

this is not clear since the effect on NE release in the PFC was not significantly altered by PNS, although there was a trend in that direction.

We also measured TH mRNA in the locus ceruleus (LC) by in situ hybridization to see if the reductions in TH mRNA measured by RT-PCR were localized to LC neurons. The results are shown in **Figure 4.4**.

We found no effect of PNS or CAPS on LC TH signal. However, these data came from animals that had been previously subjected to surgery for microdialysis cannulation, with and without the actual microdialysis, therefore the data may not be directly relatable to the qPCR.

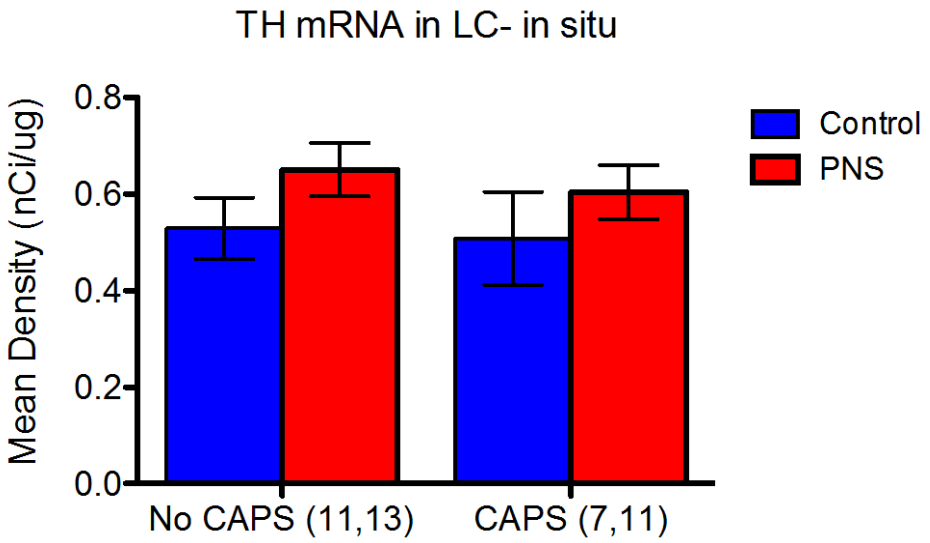


Figure 4.4. Effect of adult stress and prenatal stress on LC TH mRNA by ub situ hybridization. Data are expressed as the mean ± SEM of the number of animals in parentheses.

We have consistently found that prenatal stress (PNS) reduces glucocorticoid receptors in the medial prefrontal cortex and the hippocampus in adulthood. We therefore determined whether this might be mediated by prenatal increases in corticosterone (CORT).

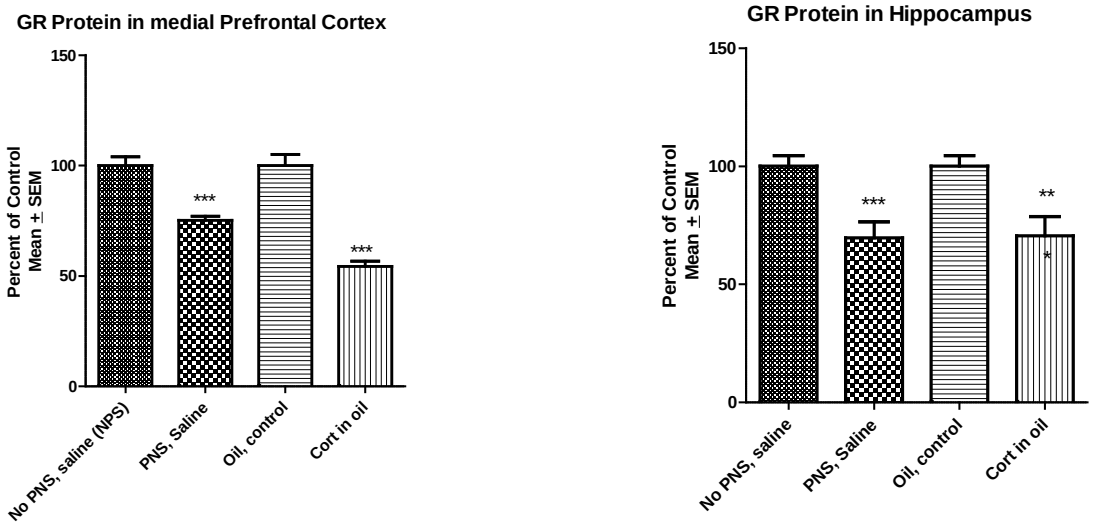


Figure 4.5. Effect of adult stress and prenatal stress on glucocorticoid receptor protein in the medial prefrontal cortex and hippocampus. Data are expressed as the mean ± SEM. **, significantly different from no PNS or Oil controls.

As shown in **Figure 4.5**, prenatal corticosterone at a level produced by maternal stress reduced glucocorticoid receptor protein in the medial prefrontal cortex and the hippocampus, indicating that elevated corticosterone during prenatal stress is sufficient to explain the alterations in GR protein in adults exposed to prenatal stress.

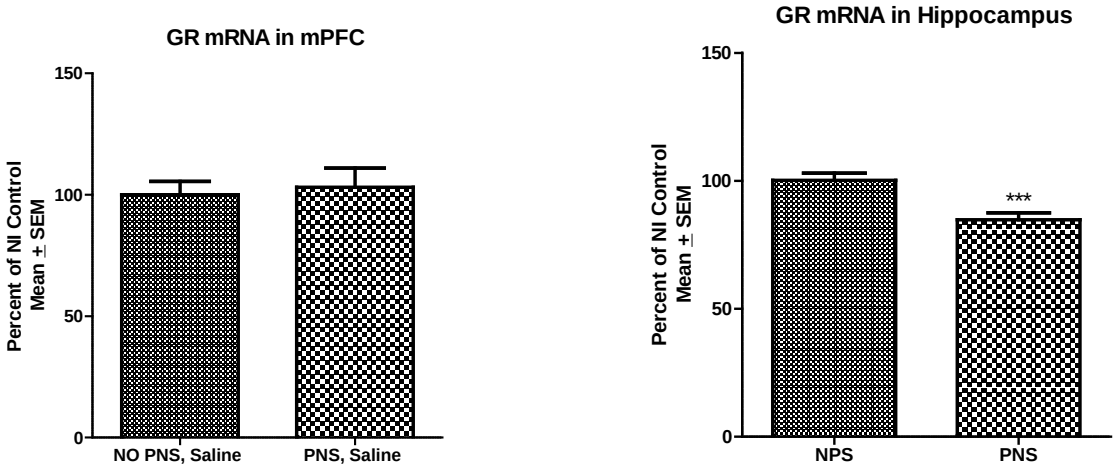


Figure 4.6. Effect of adult stress and prenatal stress on glucocorticoid receptor mRNA in the medial prefrontal cortex and hippocampus. Data are expressed as the mean ± SEM. **, significantly different from no PNS controls.

We began to determine whether the increases in GR protein in the mPFC and Hippocampus resulted from reduced mRNA. The results are shown in **Figure 4.6**. As can be seen the results

were mixed. There was no effect of PNS on GR in the mPFC, but there was a small but significant decrease in GR mRNA in the hippocampus in adult rats exposed to maternal stress.

SUMMARY OF YEAR 4 RESULTS: During Year 4 (September 1, 2011 to September 28, 2012), we continued testing the effects of pharmacological manipulation of corticosteroid function during pregnancy to determine the role of corticosterone during maternal stress on behavioral and neurochemical phenotypes in adult offspring (Model 3). We found that prenatal corticosterone treatment programs a neurochemical phenotype similar to prenatal stress characterized by reduced GR protein in prefrontal cortex and hippocampus and reduced TH mRNA in the locus ceruleus. We also found that prenatal corticosterone also influenced responses to fear conditioning and extinction, independent of adult stress.

YEAR 5 AND EXTENSION WITHOUT FUNDS:

Following from results reported in the progress report from the previous years, in years 5 and 6 (September 1, 2012 to August 31, 2014) we completed a series of experiments designed to determine if prenatal CORT exposure was sufficient to recreate the previously described effects of PNS on fear extinction learning and stress neurobiology that we reported previously. Specifically, we finalized results from Model 3, Tasks 1 and 2, Steps 4 and 5 in which we exposed rats to corticosterone (CORT) in utero to mimic the levels of CORT observed in prenatal stress. The behavioral assays were substantially completed at the end of year 4 and we produced preliminary results on neurochemical and molecular biological assays on the brain tissues from those rats. We completed the neurochemical/molecular biological assays by adding more samples. The completed work was published on August 6, 2013 (Bingham et al, 2013). Please see the attached reprints. We began testing of Model 4, i.e. treatment of prenatally stressed dams with the CORT synthesis blocker, Metyrapone. Please note that this was discontinued because of adverse effects on maternal and fetal survival, i.e. the metyrapone treatment was lethal.

***Behavioral Results*:** To summarize the behavioral experiments, pregnant female rats were delivered and divided into 3 prenatal treatment groups (vehicle controls, PNS, or CORT) and a subset of their male offspring were subject to control or CAPS stress from p46-p60. These animals (n=15/group) were then tested in the fear conditioning and extinction paradigm following the end of CAPS stress. We found that both prenatal CORT and adult CAPS stress independently delayed extinction learning while prenatal CORT treatment impairs the retention of extinction learning. We worked with Jim Mintz, PhD (Director of the STRONG STAR Data Management and Biostatistics Core) to refine our novel analysis of the extinction behavior using single exponential decay fit. We developed a statistical analysis that better allows us to compare the different phases of fear extinction behavior between groups. Analysis of the decay constants derived from the regression lines indicate that both prenatal CORT and adult CAPS stress reduced the rate of extinction learning. By contrast, analysis of the plateau value indicates no effect of either prenatal CORT or CAPS on the final level of freezing behavior displayed at the end of the extinction learning session. With respect to the regression analysis of extinction retention curves, Analysis of the decay constants indicates that all groups showed equivalent rates of extinction re-learning. However, analysis of the plateau term indicated that rats exposed to prenatal CORT treatment were unable to re-extinguish to the same final level of freezing behavior as controls. In sum, these data demonstrate that prenatal CORT exposure, similar to

prenatal stress, impairs the extinction of learned fear in an additive and distinct manner from adult exposure to CAPS stress.

Neurochemical results: With respect to the neurobiological consequences of PNS or prenatal CORT, sibling male offspring from the animals described above were allowed to grow to adulthood undisturbed, at which point they were sacrificed and the medial prefrontal cortex (mPFC), hippocampus, hypothalamus, and a section of the rostral pons containing the locus coeruleus (LC) were dissected. Both PNS and prenatal CORT treatment decreased glucocorticoid receptor protein levels in the mPFC, hippocampus, and hypothalamus when compared to control offspring. Both treatments also decreased tyrosine hydroxylase levels in the LC. Prenatal CORT also resulted in a small, but significant decrease in hippocampal BDNF expression. These behavioral and neurobiological results were published online August 6, 2013 in *Psychoneuroendocrinology* (Bingham, Sheela Rani et al. 2013). See attached reprint.

Preparation for SSRI testing: During year 5 and the year of extension without funds, we also began work on Specific Aim 4, i.e. Task 3, to determine the efficacy of SSRI treatment in the PTSD model that we developed. These studies were not completed during year 5 of the award because of the delay in developing a suitable adult stress model, because of the failure of the massed foot shock model in year 1. We applied for and we were approved for an extension without funds to complete the work. First, in preparation for the experiments implementing chronic drug treatment using minipumps to test the efficacy of SSRIs in alleviating the CAPS-induced extinction deficit, several experiments had to first be conducted to establish suitable procedures and conditions. In all of these experiment, the animals were fear conditioned prior to beginning the CAPS stress procedure to allow us to directly investigate the effects of stress on extinction processes without the conflict of stress effects on fear acquisition. Also, because SSRIs must be administered chronically, our initial intent was to implement a "reversal" paradigm (beginning drug treatment after the end of CAPS stress) rather than a "prophylactic" paradigm, ie beginning drug treatment before CAPS and continuing throughout the stress procedure. We first had to establish how long after CAPS treatment the extinction deficit persists, as this would determine the minimum time during which we could administer chronic drug treatment after CAPS prior to behavioral testing. Rats were fear conditioned and exposed to 15 days CAPS treatment as usual, then extinction learning was tested at varying time points after the end of CAPS (3 days, 5 days, 10 days, 14 days). Subsets of a single control group were tested at the same time points after fear conditioning, with no CAPS treatment. As in our previous experiments, CAPS induced an increase in freezing and a delay in the extinction of conditioned fear over 16 tones that was evident at 3 days and 5 days post-CAPS, but had disappeared by 10 days. This is not sufficient time to implant minipumps and initiate chronic steady-state drug treatment after the end of the CAPS stress and prior to behavioral testing.

Thus, we considered an alternative approach. Rather than begin drug treatment after the end of CAPS (precluded by the time course results above), or begin drug treatment prior to beginning the stress treatment (which has minimum translational relevance to real life treatment of PTSD), we instead attempted a compromise, by prolonging the CAPS treatment, essentially doubling it by taking the animals through two full rounds (2 x 15 days). Our previous results indicate that the first round will be sufficient to induce the extinction deficit, thus modeling the induction of PTSD after traumatic stress. After the first round, we would include a 3-day gap to allow for surgical implantation of minipumps to initiate chronic drug treatment prior to beginning a second round of CAPS treatment, which should be sufficient to sustain the extinction deficit in the

absence of any drug treatment. Thus, this compromise approach would allow us to implement a "reversal of deficit" treatment strategy rather than a prophylactic strategy, while still allowing sufficient time for effective chronic drug treatment before behavioral testing, with stress continuing throughout the period of drug treatment. To use this strategy, we first had to demonstrate that the 2 X CAPS treatment would produce a comparable deficit in extinction learning as the regular CAPS treatment, or that conditioned fear would even still be evident nearly 5 weeks after conditioning. Six groups of rats (approximately 12-14 rats per group) were fear conditioned as usual, and CAPS treatment was then initiated in four of the groups. The first two CAPS groups were tested for extinction of conditioned fear at 3 or 5 days after the end of this single round of CAPS treatment, along with one of the control groups. The remaining groups were given a 3 day break after the end of CAPS (to accommodate minipump surgeries in future studies). On the third day, a single tone-shock "reminder" was administered to reactivate the original conditioning. A second round of CAPS was then initiated, and these groups were also tested 3 or 5 days after the end of the second CAPS treatment, along with the second control group. Both control groups showed comparable conditioned fear and comparable extinction. All of the CAPS-treated groups showed a comparable deficit of extinction. The effects were more robust 3 days post-CAPS compared to 5-days, regardless of the duration of the CAPS treatment, but the significant effect seen at 5 days after the second round of CAPS treatment (Figure 5.1: approx. 20% freezing vs 40% freezing on tones 4-7) allows us a potential window within which to implement additional adjunct treatments in future studies. Interestingly, we found that the repeated CAPS stress procedure also increased freezing during extinction learning (Figure 5.1, panel C). The impairment in retention of extinction learning produced by repeated CAPS was a new effect of CAPS that we think more closely models the deficits in fear extinction that occur in PTSD patients. Thus, these results cofirm that it would be possible for us to institute this prolonged stress treatment to first establish the CAPS-induced extinction deficit, then begin chronic drug treatment of sufficient duration to have a therapeutic effect, while continuing the stress treatment for a second round.

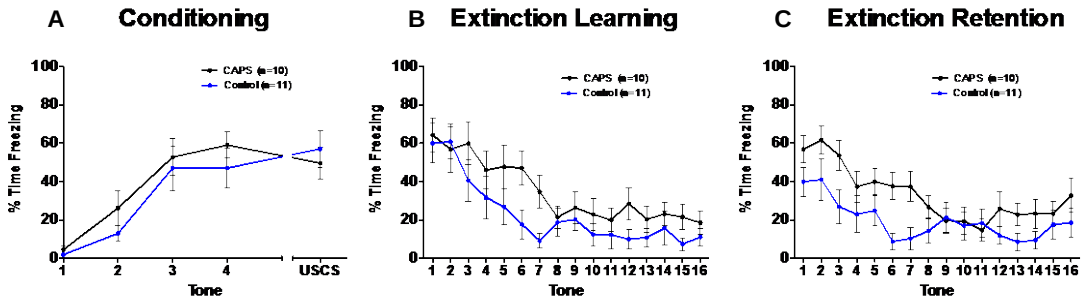


Figure 5.1 . Effect of repeated CAPS stress on fear extinction learning and retention tested 5 days after the end of the second round of CAPS treatment. A) All animals were conditioned previous to any manipulation and were divided into groups so that there were no differences in acquisition of freezing behavior. In addition, there were no significant differences in freezing during the "reminder" tone-shock pairing administered between sessions of CAPS stress. B) Repeated CAPS stress increased freezing during extinction learning. C) Repeated CAPS stress increased freezing during the extinction test as well as a trend to a tone by stress interaction.

At this point, however, the funds in our award were expended, and these experiments were ended prior to completing SSRI testing. However, we established methods during the period without funds that are necessary for testing SSRI in our model. We had intended to continue this line of study in our continuation project as part of the renewed STRONGSTAR Consortium to Alleviate PTSD (CAP); however, because of the lengthy process securing CAP leadership, peer review, and CAP Government Steering Committee approvals, we decided to pursue other funding through a VA merit award.

REPORTABLE OUTCOMES:

ABSTRACTS

Strong R, Joshi A, Rodriguez GA, Martinez PA, Fernandez E, Frazer A, Morilak DA (2009) Mechanisms of vulnerability to PTSD: The role of early life stressors. Congressionally Directed Medical Research Programs Military Health Research Forum, Kansas City, MO, Aug 31-Sept 3, 2009.

Green MK, Joshi A, Frazer A, Strong R, Morilak DA (2010) Prenatal stress increases stress-reactivity and impairs fear extinction after adult stress: A model of PTSD. Soc Neurosci Abstr 36 Online Program no. 809.11

Green MK, Joshi A, Shah A, Frazer A, Strong R, Morilak DA (2011) Effects of prenatal stress and combined chronic plus acute adult stress on anxiety-like behavior and evoked HPA axis activity in rats. Soc Neurosci Abstr 37, Online Program no. 190.06.

Bingham B, Kaddapakam S, Strong R, Morilak DA (2011) Potential role of corticosterone in the long-lasting effects of prenatal stress. Soc Neurosci Abstr 37, Online Program no. 190.09.

Strong R, Morilak D, Frazer A (2010) Mechanisms of Vulnerability to PTSD: The Role of Early Life Stressors. Oral presentation at the National Trauma Institute Annual Symposium, San Antonio TX, August 31, 2010

JOURNAL ARTICLES

Green MK, Rani CS, Joshi A, Soto-Piña AE, Martinez PA, Frazer A, Strong R, Morilak DA. Prenatal stress induces long term stress vulnerability, compromising stress response systems in the brain and impairing extinction of conditioned fear after adult stress. Neuroscience. 29; 192:438-451, 2011.

Roth, M.K., Bingham, B., Shah, A., Joshi, A., Frazer, A., Strong, R., Morilak, D.A. Effects of chronic plus acute prolonged stress on measures of coping style, anxiety, and evoked HPA-axis reactivity. Neuropharm. 63:1118-26, 2012.

Bingham, B.C., Rani, S., Frazer, A., Strong, R., & Morilak, D.A.; for the STRONG STAR Consortium. (2013). Exogenous prenatal corticosterone exposure mimics the effects of prenatal stress on adult brain stress response systems and fear extinction behavior. *Psychoneuroendocrinology, 38*(11), 2746-2757. doi:10.1016/j.psyneuen.2013.07.003

APPENDIX A

REPRINTS

Neuroscience 192 (2011) 438–451

PRENATAL STRESS INDUCES LONG TERM STRESS VULNERABILITY, COMPROMISING STRESS RESPONSE SYSTEMS IN THE BRAIN AND IMPAIRING EXTINCTION OF CONDITIONED FEAR AFTER ADULT STRESS

M. K. GREEN, C. S. S. RANI, A. JOSHI, A. E. SOTO-PIÑA, P. A. MARTINEZ, A. FRAZER, R. STRONG AND D. A. MORILAK*

Department of Pharmacology and Center for Biomedical Neuroscience, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78229, USA

Abstract—Stress is a risk factor for the development of affective disorders, including depression, post-traumatic stress disorder, and other anxiety disorders. However, not all individuals who experience either chronic stress or traumatic acute stress develop such disorders. Thus, other factors must confer a vulnerability to stress, and exposure to early-life stress may be one such factor. In this study we examined prenatal stress (PNS) as a potential vulnerability factor that may produce stable changes in central stress response systems and susceptibility to develop fear- and anxiety-like behaviors after adult stress exposure. Pregnant Sprague–Dawley rats were immobilized for 1 h daily during the last week of pregnancy. Controls were unstressed. The male offspring were then studied as adults. As adults, PNS or control rats were first tested for shock-probe defensive burying behavior, then half from each group were exposed to a combined chronic plus acute prolonged stress (CAPS) treatment, consisting of chronic intermittent cold stress (4 °C, 6 h/d, 14 days) followed on day 15 by a single session of sequential acute stressors (social defeat, immobilization, cold swim). After CAPS or control treatment, different groups were tested for open field exploration, social interaction, or cued fear conditioning and extinction. Rats were sacrificed at least 5 days after behavioral testing for measurement of tyrosine hydroxylase (TH) and glucocorticoid receptor (GR) expression in specific brain regions, and plasma adrenocorticotropic hormone (ACTH) and corticosterone. Shock-probe burying, open field exploration and social interaction were unaffected by any treatment. However, PNS elevated basal corticosterone, decreased GR protein levels in hippocampus and prefrontal cortex, and decreased TH mRNA expression in noradrenergic neurons in the dorsal pons. Further, rats exposed to PNS plus CAPS showed attenuated extinction of cue-conditioned fear. These results suggest that PNS induces vulnerability to subsequent adult stress, resulting in an enhanced fear-like behavioral profile, and dysregulation of brain noradrenergic and hypothalamic–pituitary–adrenal axis (HPA) activity.

*Corresponding author. Tel: +1-210-567-4174; fax: +1-210-567-4303.
E-mail address: morilak@uthscsa.edu (D. A. Morilak).

Abbreviations: ACTH, adrenocorticotropic hormone; CAPS, chronic plus acute prolonged stress; CORT, corticosterone; GR, glucocorticoid receptor; HPA axis, hypothalamic–pituitary–adrenal axis; HR, high responder rats; ITI, inter-trial interval; LC, locus coeruleus; NE, norepinephrine; PD, postnatal day; PNS, prenatal stress; PTSD, posttraumatic stress disorder; TH, tyrosine hydroxylase; WKY, Wistar-Kyoto rats.

Key words: prenatal stress, traumatic stress, vulnerability, fear extinction, tyrosine hydroxylase, HPA axis.

Affective disorders, such as depression, post-traumatic stress disorder (PTSD) and other anxiety disorders, have long been considered to be stress-related/stress-initiated disorders. Responses to acute stressors are thought to be adaptive in the short term by increasing, for example, access to energy stores, increasing cardiovascular tone, and enhancing behavioral response capabilities. However, when these systems are repeatedly activated, as with chronic stress, dysregulation of a number of hormonal and neurotransmitter systems may occur (McEwen, 2003). Chronic stress is a risk factor, and possibly a causal factor, in the development of depression and anxiety disorders (Kendler et al., 1999; Koenen et al., 2002, 2007; Gilmer et al., 2005; Jordanova et al., 2007). Indeed, a number of physiological and anatomical alterations associated with chronic stress are hallmarks of depression and anxiety disorders (Board et al., 1956; Nemeroff et al., 1984, 1991; Gold et al., 1986; Holsboer et al., 1986; Weisse, 1992; Heuser et al., 1998; Arborelius et al., 1999; Manji et al., 2001; McEwen, 2003). In addition to cumulative or chronic stress, severe acute stress is also associated with mood and anxiety disorders, most prominently with PTSD. (Jordanova et al., 2007; American Psychiatric Association, 2000). However, not all individuals exposed to chronic or acute-traumatic stress in adulthood develop depression or anxiety disorders, suggesting that some other factor or factors, either genetic, epigenetic, or experience-based, contribute to susceptibility to develop affective disorders. Therefore, to fully understand the mechanisms underlying these disorders, it is insufficient to simply examine the response to stressors. Rather, the factors involved in predisposing for failure to recover from the normal response to stress must be identified (Yehuda and LeDoux, 2007).

Early life stress is one potential factor. For example, early life stress is a risk factor for PTSD, specifically, a history of trauma, childhood abuse/neglect, low education and IQ, low socio-economic status, or loss of a parent in childhood (Bremner et al., 1993; Breslau et al., 1999; Widom, 1999; Koenen et al., 2002, 2007). In rodents, prenatal stress (PNS) produces several behavioral and physiological changes that may be indicative of later stress vulnerability (e.g., Weinstock et al., 1992; Valle et al., 1997; Lemaire et al., 2000).

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Two of the most prominent systems involved in stress adaptation, the brain noradrenergic system and the hypothalamic–pituitary–adrenal (HPA) axis, have also been implicated in stress-related pathology. Norepinephrine (NE) is released in response to stress (Morilak et al., 2005; Aston-Jones et al., 1999), chronic stress alters noradrenergic signaling (Buffalari and Grace, 2009; Kitayama et al., 2008; Ma and Morilak, 2005), and noradrenergic dysregulation is reported in numerous affective disorders, including depression and PTSD (Ressler and Nemeroff, 1999; Strawn and Geracioti, 2008). Likewise, the HPA axis is activated in response to acute stress, resulting in release of adrenocorticotropic hormone (ACTH) and corticosterone (CORT) and this response is altered after chronic stress (Dallman, 1993; Ma and Morilak, 2005). Furthermore, HPA axis dysregulation is a consistent component of several affective disorders, including depression, panic disorders, obsessive-compulsive disorder, and PTSD (Nemeroff et al., 1984; Gold et al., 1986; Souetre et al., 1988; Abelson et al., 2007; Kluge et al., 2007; Mason et al., 1986; Pitman and Orr, 1990).

It is being increasingly recognized that changes in executive function and cognitive capability are also prominent features of mood and anxiety disorders (Beck, 1976; Beck et al., 1987; Mathews and MacKintosh, 1998; Coles and Heimberg, 2002). Moreover, in the context of stress, both the brain noradrenergic system and the HPA axis are involved in regulation and dysregulation of cognitive processes such as learning and memory (de Quervain et al., 2009), including specifically conditioned fear and extinction learning (McIntyre et al., 2002; Mueller et al., 2008; Gourley et al., 2009). Impaired cognition, maladaptive fear responses, and impaired extinction of learned fear are primary symptoms of a number of affective disorders, with these fear-related symptoms being most relevant to anxiety disorders such as panic disorder, phobias, obsessive-compulsive disorder, and PTSD (Sutker et al., 1995; Fossati et al., 1999; Koenen et al., 2001; Moritz et al., 2002; Kangaratnam and Asbjørnsen, 2007; Blechert et al., 2007; Wessa and Flor, 2007). Therefore, it is possible that the mechanisms by which vulnerability factors such as prenatal stress may induce long-lasting susceptibility to develop psychopathology upon adult stress exposure could include dysregulation of the HPA axis and/or brain noradrenergic system, resulting specifically in maladaptive responses to fear-provoking stimuli and an impaired ability to extinguish fear responses in non-stressful conditions.

Thus, the purpose of the present study was to examine neurobiological correlates of adult stress vulnerability induced by PNS exposure. We measured the effects of PNS followed by a combined chronic plus acute prolonged stress (CAPS) treatment as adults, on tyrosine hydroxylase (TH) expression in the locus coeruleus (LC) and adrenal medulla, HPA status, and glucocorticoid receptor (GR) protein levels in the prefrontal cortex (PFC) and hippocampus. In the same rats, we also tested the vulnerability of PNS-exposed adult rats to develop fear or anxiety-like behaviors following exposure to CAPS treatment, on measures of acute stress reactivity, social interaction, fear conditioning, and extinction. We hypothesized that PNS exposure would produce stable, long-term changes in central and peripheral stress response systems, and a vulnerability to subsequent adult stress exposure such that the behavioral impact of adult stress would be greater. Portions of this work have been presented in abstract form (Green et al., 2010).

EXPERIMENTAL PROCEDURES

Animals

Timed-pregnant (6 days pregnant upon arrival) female Sprague–Dawley rats (Harlan, Indianapolis, IN, USA) were singly housed throughout pregnancy. On postnatal day (PD) 5, litters were culled to eight pups each, maximizing the number of males retained (typically, three to six per litter), and weaned on PD 21. Upon weaning, male pups were pair-housed with a littermate until PD 41–45, depending on the experiment, at which time they were singly housed prior to starting the adult stress or unstressed control treatments. The rats were housed in Plexiglas cages ($25\times45\times15$ cm^3) on a 12/12 h light-dark cycle (lights on at 7:00 h) with food and water available *ad libitum*. In total, 141 adult male offspring (from 63 litters—33 stressed and 30 unstressed) were used in these experiments. In addition, for the social defeat procedure, six adult male Long–Evans rats (Harlan), weighing at least 400 g, were used as defeaters. They were housed, together with an ovariectomized female, in large resident cages ($80\times55\times40$ cm^3) in a separate room on the same 12/12 h light cycle. All experiments were conducted during the light phase. All procedures were conducted according to NIH guidelines for the care and use of laboratory animals and were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio. All efforts were made to minimize animal pain, suffering or discomfort, and to minimize the number of rats used.

Prenatal stress treatment

After 1 week in the housing facility, half of the pregnant females were immobilized daily for 1 h, from day 14 of pregnancy until birth (8–9 days). Immobilization involved taping the rat's torso and limbs gently but snugly in a prone position on a flat platform, allowing no movement. Unstressed control pregnant females were left undisturbed during this same time period.

Shock-probe defensive burying test

At PD 41–43, a subset of the offspring ($n=60$) from both groups were tested in the shock probe defensive burying test. This was to evaluate potential differences in active and passive behavioral stress-reactivity as a consequence of the prenatal stress treatment prior to any exposure to adult stress. The rats were placed into a modified cage containing 5 cm of bedding, with a shock probe protruding 6 cm into one end of the cage. The probe was set to deliver 2 mA of current when the probe was touched. After the rat made contact with the probe and received a shock, the current was shut off and the 15 min test began. Behavior was recorded using a CCD camera mounted above the cage and stored to video files for offline scoring and analysis. The dependent measures analyzed were the amount of time spent immobile and the amount of time spent engaged in actively burying the probe. After the shock-probe defensive burying test, these animals were individually housed. Likewise, rats not tested in the shock-probe defensive burying test were also individually housed at this same time point.

Adult stress treatment: chronic plus acute prolonged stress

Beginning between PD 46 and 54, half of the rats that received prenatal stress ($n=35$) and half that did not ($n=34$) received CAPS treatment, which consisted of 2 weeks of chronic intermittent cold stress followed by a single 1-h session of acute prolonged stress on day 15. For cold stress, rats were transported in their cage with food, water and bedding into a cold room at 4 °C for 6 h per day for 14 days. The acute prolonged stress on day 15 consisted of 20 min social defeat, followed immediately by 30 min immobilization, and then 10 min cold swim. For social defeat, the ovariectomized Long–Evans female was removed from the resident cage, and the test rat was placed into the resident cage. Typically within 10–30 s, the resident Long–Evans male rat will attack and defeat the smaller "intruder" Sprague–Dawley test rat. Once defeat occurred, with "defeat" defined as the test rat assuming a supine posture and the resident expressing a dominant posture for at least 4 s, the test rat was placed under a wire mesh cage for 20 min, thus protecting the test rat from further physical contact but allowing continued sensory exposure to the dominant rat. Immobilization was then conducted, as described above, for 30 min. Finally, cold swim was accomplished by placing the rat in a cylindrical tank (30 cm diam×60 cm) filled to a depth of 30 cm with water at 18 °C. The combination of PNS and adult CAPS treatment resulted in four treatment groups: No PNS/No CAPS (i.e. unstressed controls), PNS/No CAPS, No PNS/CAPS, and PNS/CAPS.

Neurochemical and hormonal analyses

All of the rats were sacrificed by rapid decapitation 5–10 days after the last stress day, or at the equivalent time for controls. Trunk blood was collected into tubes containing 10 mM EDTA and was centrifuged at 4000×g for 15 min at 4 °C for the separation and collection of plasma. Plasma was stored at −80 °C until use. Brains were removed, placed in a brain matrix on ice, and the structures of interest dissected. For PFC, a 2 mm coronal section was cut, extending from the frontal pole to approximately plate 8 in the atlas of Paxinos and Watson (1986). The hippocampus was then separated from the lateral margins of the remaining cortex. For the pontine region containing the LC, the cerebellum was removed and the obex located. A slab was cut 3–5 mm anterior to the obex (plates 53–60), and the dorsal half was collected. Brain samples were rapidly frozen in 2-methylbutane on dry ice, and stored at −80 °C until assay.

TH mRNA. TH mRNA was measured in the dorsal pons containing the locus coeruleus and in the adrenal medulla by qPCR. Total RNA was isolated from tissues using the RNeasy Plus Mini kit (Qiagen Inc., Valencia, CA, USA) as described by the manufacturer. The RNA concentration was measured by spectroscopy at 260 nm using the Nanodrop ND-1000 instrument (NanoDrop Technologies, Inc., Wilmington, DE, USA). To check the integrity of RNA, RNA was denatured with 50% formamide loading buffer and run on an E-Gel EX 1% agarose gel using the iBase Power System (Invitrogen Corp., Carlsbad, CA, USA) and the 18S and 28S bands visualized. In all, 250–500 ng RNA was converted to cDNA using random hexamers and TaqMan Multiscribe reverse transcriptase enzyme included in the High Capacity RNA Reverse Transcription kit (Applied Biosystems Inc., Foster City, CA, USA). Reactions included controls without the reverse transcriptase enzyme but with only RNA template, and negative control with the enzyme, but no template in 20 μl volume. After 2-h incubation at 37 °C, the RNA in the reactions was considered completely converted to cDNA. In order to check the quality of cDNA, cDNA (1 μl=5 ng) from all reactions was used as template for PCR with rat GAPDH primers and the Go-Taq Green PCR master mix (Promega Corp., Madison, WI, USA). No bands were detected either in negative control (no template) or in the samples without the reverse transcriptase enzyme, but a 200 bp band was seen in all cDNA-containing samples upon agarose gel electrophoresis.

TH gene expression was then quantified by qPCR using the TaqMan gene expression master mix and TaqMan Gene Expression assay ID Rn00562500_m1, consisting of a set of intron-spanning primers and FAM-labeled probe set for rat TH (Applied Biosystems Inc.) along with the cDNA, equivalent to 0.125–0.25 ng RNA. Assays were performed in triplicate and first validated using the Applied Biosystems Inc. PRISM 7900 Sequence Detection System in a 96-well format. Results were normalized to 18S rRNA, which was amplified simultaneously in the same samples using the primer-limited TaqMan VIC-MGB labeled 18S rRNA probe (Applied Biosystems Inc.). Real time PCR data were analyzed by the $2^{-\Delta\Delta C}{}_T$ method. The average difference in quantification cycle threshold (Cq) of the target gene and the 18S control for each sample was calculated, and the relative expression of TH in other groups was calculated with respect to the value obtained for the no-stress control samples.

TH protein. TH protein was measured in the adrenal medulla using Western blot. Samples were thawed on ice and homogenized in RIPA buffer (radioimmunoprecipitation assay buffer: 50 mM Tris–HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS with Sigma protease inhibitor cocktail (P8340, 1:100 dilution) and phenylmethylsulfonylfluoride (PMSF, 1 mM) added just prior to use). Homogenates were centrifuged at 16,000×g for 15 min at 4 °C and the supernatants transferred to fresh tubes. Protein concentration in the lysates was assessed by MicroBCA method (Pierce Inc., ThermoFisher Scientific, Rockford, IL, USA). Equal amounts of sample protein in 1× NuPage LDS sample buffer (Invitrogen) under denaturing condition were loaded on 4–12% NuPage Bis–Tris SDS gels (Invitrogen) and electrophoresed at 175 V for ~1 h. Proteins separated on the gel were transferred to polyvinylidene fluoride membranes using the iBlot transblot apparatus (Invitrogen). Membranes were probed by simultaneous addition of specific monoclonal antibodies to TH (Sigma, St. Louis, MO, USA) and β-actin (AbCam, Cambridge, MA, USA), followed by secondary antibody consisting of IRDye 800CW conjugated goat polyclonal anti-mouse IgG (LI-COR Biosciences, Lincoln, NE, USA), and the fluorescent signal was scanned and quantified using the Odyssey infrared imaging system (LI-COR). The ratio of TH to actin of each sample was calculated, and the relative expression of TH was computed as percentage of the no-stress control group.

GR protein levels in the hippocampus and PFC. GR protein levels were assayed in the hippocampus and the PFC using an ELISA-based TransAM kit (Active Motif, Carlsbad, CA, USA). Assays were performed according to manufacturer's instructions. Briefly, the brain tissue was homogenized in complete lysis buffer AM2, containing 1 mM dithiothreitol (DTT) and a protease inhibitor cocktail using motorized pestles in 1.5 ml microtubes (RPI, Mount prospect, IL, USA). All steps were conducted at 4 °C. After 30 min incubation on ice, the homogenates were centrifuged at 10,000×g for 10 min and the supernatants were transferred to fresh chilled tubes. Aliquots were frozen or used for protein assay using the BioRad Bradford protein assay. For the ELISA, 20 μg protein was used in a 96-well format. The homogenates, in complete binding buffer containing 1 mM DTT and herring sperm DNA, were incubated in wells coated with immobilized oligonucleotide containing a consensus GR binding site (5′-GGTACAnnnTGTTCT-3′). The bound GR was then detected using a specific GR primary antibody and an HRP-conjugated secondary antibody followed by a colorimetric step for quantification by spectrophotometry in a plate reader. The absorbance at 450 nm after suitable blank correction was used to determine GR levels. The A450 values for each

stress group were calculated as percentage of the no-stress control group.

Plasma ACTH and CORT levels. Levels of circulating ACTH and CORT were analyzed by radioimmunoassay. ACTH was determined from duplicate 100 μl plasma aliquots according to the manufacturer instructions (ImmuChem double antibody hACTH assay, MP Biomedicals, Orangeburg, NY, USA). The detection limit of the assay is 6 pg/ml, and the inter-assay variability was 10%. CORT was measured in diluted plasma samples according to manufacturer instructions (ImmuChem Double antibody, corticosterone assay, MP Biomedicals). The assay detection limit was 8 ng/ml, and inter-assay variability was 8%.

Behavioral measures

Fear conditioning and extinction. One day after the termination of CAPS treatment (or the comparable control period), a subset of rats in each group (n=98; 23–25/grp) was habituated to the two fear conditioning and extinction contexts for 15 min each. Context A is a 30.5×25.4×30.5 cm^3 square conditioning chamber (Coulbourn Instruments, Whitehall, PA, USA; model # H10-11R-TC) with metal walls and a grid shock floor attached to a shock generator (Coulbourn, # H13-15). Context B is a modified chamber with black and white vinyl walls forming a circular enclosure and a smooth green vinyl floor placed over the shock grid. Both contexts are enclosed in a 58.4×61×50.8 cm^3 sound-attenuating chamber (Coulbourn, # H10-24T). Twenty-four hours after habituation, the rats received cued fear conditioning in Context A. Each rat was placed into the chamber and, after a 5 min acclimation period, experienced four pairings of a tone (10 kHz, 75 dB, 20 s) co-terminating with a shock (0.7 mA, 0.5 s). The average inter-trial interval (ITI) was 120 s.

Seventy-two hours after fear conditioning, the rats experienced fear extinction training consisting of exposure to 10 trials of the tone alone (average ITI 120 s, for a total 27 min extinction session). Then, 24 h later, the rats were tested for retention of fear extinction during exposure to 10 trials of the tone alone. To avoid any contextual effects, all extinction and retention testing was conducted in Context B.

Freezing behavior was recorded and analyzed using the FreezeFrame and FreezeView software (ActiMetrics Software; Coulbourn Instruments # ACT-100). Freezing was defined as all behavior which fell below the motion index threshold of 10 and lasted at least 1 s. Freezing was measured during each 20-s tone presentation on the conditioning, extinction training and extinction retention days. The time spent freezing was then expressed as a percentage of each 20-s sampling period.

Open field exploration. One day after the termination of CAPS treatment (or comparable control period), the remaining rats (n=43; 10–12/group) were tested for anxiety-like and exploratory behavior in an open field (60×60×40 cm^3) under normal ambient laboratory lighting. The floor of the test arena was marked in a grid pattern of 36 squares, 10×10 cm^2 each. The test rat was placed into the center of the open field and behavior was recorded for 5 min. The number of line crossings and time spent in the center zone (i.e. the inner 16 squares) were measured.

Social interaction. Twenty-four hours after open field testing, the rats were tested in the same arena for social interaction with a novel male con-specific, weight-matched to within ±5 g of the test rat. The con-specific "stimulus rats" had all been previously habituated to interacting in the arena with other stimulus rats so that their behavior would be constant during testing. The amount of time that the test rat spent engaged in social behaviors (sniffing, chasing, climbing, etc.) was measured during the 5-min test.

Statistical analyses

For the shock-probe defensive burying data, differences in immobility time and in active burying time between rats in the two prenatal stress conditions (prenatally stressed and controls) were analyzed by t-tests. All neurochemical and plasma hormone measures were analyzed by two-way ANOVA (prenatal stress×adult stress). Similarly, for the open field and social interaction tests, group differences in the number of line crossings, time spent in the center zone of the open field, and social interaction time were each analyzed by a two-way ANOVA (prenatal stress×adult stress). For the fear-conditioning and extinction tests, group differences in percent freezing were analyzed for each session by a three-way ANOVA (prenatal stress×adult stress×tone) with repeated measures over tone. To determine if there were differences in the retention of conditioned fear, freezing levels in response to the first tone presentation on the extinction training day were analyzed by two-way ANOVA (prenatal stress×adult stress). Likewise, tone 1 was analyzed in the same way on the extinction retention day as a measure of retention of extinction that occurred the day before (see Milad et al., 2004; Vidal-Gonzalez et al., 2006; Muigg et al., 2008). Following the primary ANOVA, in order to better assess and compare the rate of extinction across groups, a non-linear regression analysis was performed. An exponential decay function was best-fit to the freezing data for each rat, from which the rate constants (k) were derived. The rate constants for each treatment group were then compared by two-way ANOVA (prenatal stress×adult stress). On the extinction training day, an increase in freezing was always observed from tone 1 to tone 2. Thus, in order to capture the true rate of extinction from the peak level of freezing, and also to obviate any potential group differences in extinction attributable solely to different starting points on tone 1, tone 1 was not included in the regression analysis. Further, cases for which a line could not be fit to the data were excluded from the regression analysis, resulting in the exclusion of one to two animals per group. Upon examination of these cases, no consistent pattern could be discerned, and the data from these cases were included in the primary ANOVA for freezing data. In all analyses, significance was determined at $P<0.05$. Sources of significant main effects or interactions were then determined by analysis with the Newman–Keuls post hoc test.

RESULTS

Shock-probe defensive burying test

Prior to administering the adult stress treatment, there were no significant differences between rats that received prenatal stress and rats that did not in either time spent immobile (Table 1; t_{58}=0.381, $P>0.05$), nor in time spent burying the shock probe (t_{58}=0.599, $P>0.05$).

TH expression in the locus coeruleus and adrenal medulla

Prenatal stress significantly reduced TH mRNA expression in homogenates of rostral pons containing the LC, irrespective of exposure to CAPS (Fig. 1; $F_{(1,66)}$=9.745,

Table 1. Shock probe defensive burying behavior before adult stress

	Controls	PNS
Immobilization time (s)	150.9±31.95	166.2±24.99
Burying time (s)	97.77±20.11	81.56±18.24

Data expressed as mean±SEM, n=28–32/group.

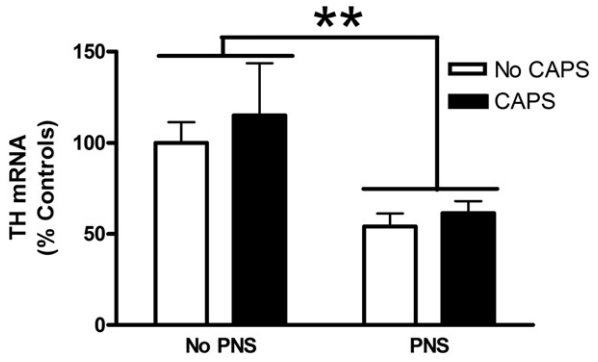

Fig. 1. Tyrosine hydroxylase mRNA expression in the rostral pons containing the locus coeruleus. Prenatal stress produced a significant decrease in TH mRNA expression in the LC region. ** main effect of PNS, $P=0.003$. Data expressed as mean percent of controls±SEM, $n=17–19$/group.

$P<0.01$). There was no main effect of CAPS ($F_{(1,66)}=0.488$, $P>0.05$) nor an interaction between PNS and CAPS ($F_{(1,66)}=0.061$, $P>0.05$). However, effects in the adrenal medulla were different (Fig. 2). For TH mRNA, there was no main effect of PNS (Fig. 2A; $F_{(1,33)}=2.606$, $P>0.05$) nor was there an effect of CAPS ($F_{(1,33)}=0.343$, $P>0.05$), but there was a significant PNS×CAPS interaction ($F_{(1,33)}=6.06$, $P<0.05$) such that PNS alone induced

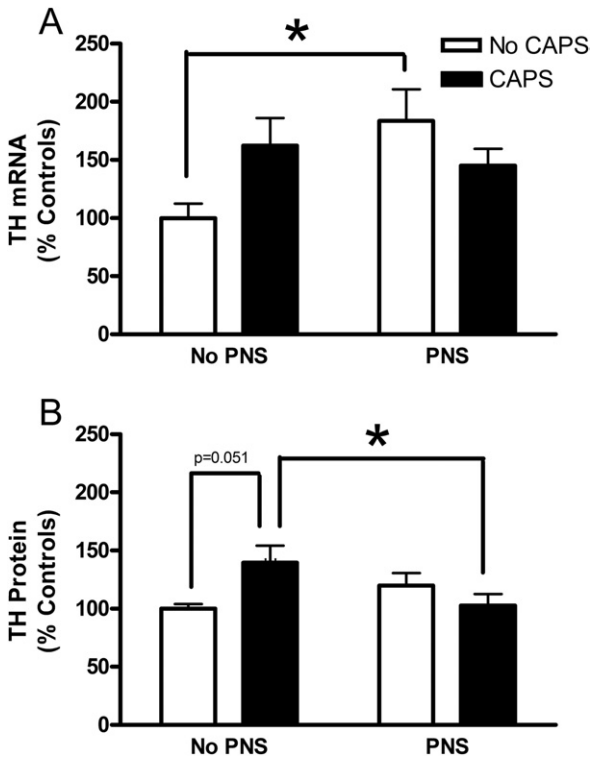

Fig. 2. Tyrosine hydroxylase mRNA and protein expression in the adrenal medulla. (A) PNS alone significantly increased TH mRNA expression in the adrenal medulla compared to unstressed controls, * $P=0.03$ by Newman–Keuls post hoc comparisons, $n=8–10$/group. (B) CAPS alone significantly increased TH protein levels compared to both PNS/CAPS (* $P=0.04$) and unstressed controls ($P=0.051$). Data expressed as mean percent of controls±SEM, $n=13$/group.

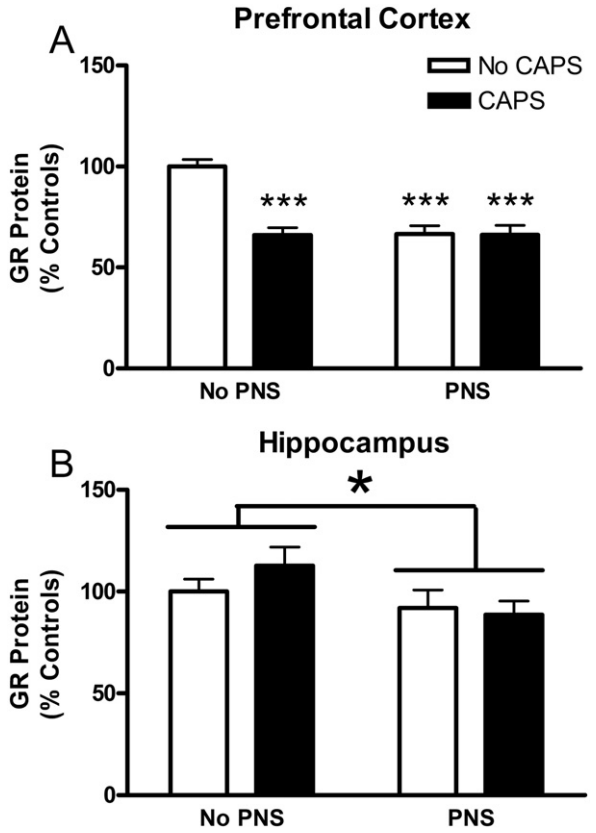

Fig. 3. Effects of PNS and CAPS on glucocorticoid receptor (GR) expression. (A) There was significantly reduced GR protein expression in the prefrontal cortex after either PNS or CAPS alone, as well as the combined PNS/CAPS treatment. *** $P=0.0001$ compared to unstressed controls, $n=18–20$/group. (B) PNS modestly but significantly reduced GR protein expression in the hippocampus. * Main effect of PNS, $P=0.04$. Data expressed as mean percent of unstressed controls±SEM, $n=14–16$/group.

significantly higher TH mRNA expression compared to controls receiving no PNS. There was also a non-significant elevation of TH mRNA in the group that received CAPS alone ($P=0.09$). Likewise, for TH protein, there was no significant main effect of PNS (Fig. 2B; $F_{(1,48)}=0.664$, $P>0.05$) nor of CAPS ($F_{(1,48)}=1.107$, $P>0.05$), but a significant PNS×CAPS interaction ($F_{(1,48)}=7.272$, $P<0.01$). Post hoc analyses reveal that rats exposed to CAPS alone had more adrenal TH protein than did control rats (P approaching significance at 0.051) or rats exposed to PNS plus CAPS.

GR levels in the mPFC and hippocampus

In the prefrontal cortex, both PNS and CAPS significantly decreased GR protein (Fig. 3A; $F_{(1,70)}=16.906$, $P<0.0001$ for PNS; $F_{(1,70)}=18.177$, $P<0.0001$ for CAPS) with no apparent additive effect of the two treatments, resulting also in a significant interaction of PNS and CAPS ($F_{(1,70)}=17.171$, $P<0.0001$). Post hoc analyses revealed that the three stress conditions (PNS, CAPS, PNS/CAPS) did not differ from each other. By contrast, in the hippocampus, only a main effect of prenatal stress was evi-

dent (Fig. 3B; $F_{(1,55)}=4.368$, $P<0.05$), causing a significant decrease in GR protein, but no main effect of CAPS ($F_{(1,55)}=0.37$, $P>0.05$) nor an interaction of PNS and CAPS ($F_{(1,55)}=1.06$, $P>0.05$).

Plasma ACTH and CORT

There were no effects of PNS or CAPS on basal plasma ACTH ($F_{(1,100)}=0.091$ for PNS, $F_{(1,100)}=0.003$ for CAPS, $P>0.05$), nor was there a significant interaction ($F_{(1,100)}=1.692$, $P>0.05$). However, prenatal stress induced a significant, long-term elevation in basal CORT (Fig. 4; $F_{(1,99)}=5.068$, $P<0.05$). Adult stress had no effect on basal CORT ($F_{(1,99)}=0.702$, $P>0.05$) nor was there a significant interaction between PNS and CAPS ($F_{(1,99)}=0.123$, $P>0.05$), probably because sacrifice and trunk blood collection for plasma measures took place 5–10 days after the termination of the adult stress treatment.

Fear conditioning and extinction

On all 3 days, there was the expected main effect of Tone ($F_{(3,276)}=107.712$, $P<0.001$ for conditioning; $F_{(9,828)}=38.875$, $P<0.001$; $F_{(9,828)}=21.271$, $P<0.001$ for extinction training and retention, respectively), confirming that the conditioning and extinction protocols were effective. Rats exposed specifically to PNS plus CAPS displayed enhanced fear conditioning and impaired extinction. For fear conditioning, there was a significant interaction between CAPS and Tone (Fig. 5; $F_{(3,276)}=2.813$, $P<0.05$). Post hoc analyses revealed that the PNS/CAPS group expressed significantly higher levels of freezing than did the PNS group on tone 4. There were no other main effects or interactions during conditioning.

On the test day, freezing in response to tone 1 alone was first analyzed to determine if there were any differences in the retention of conditioning from the previous day, and there were not. There were no main effects of PNS ($F_{(1,92)}=1.317$, $P>0.05$), or CAPS ($F_{(1,92)}=0.4607$, $P>0.05$), nor an interaction ($F_{(1,92)}=0.1656$, $P>0.05$). Next, the analysis of the subsequent course of extinction

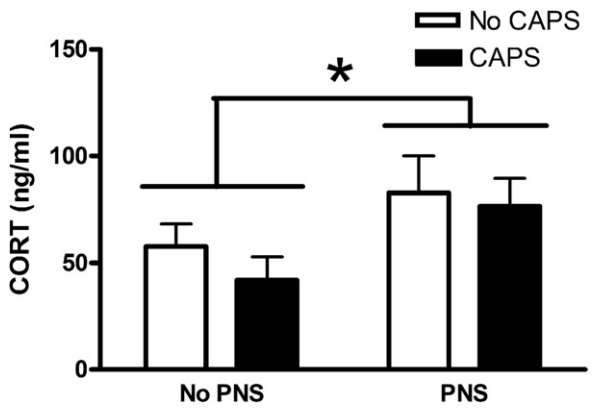

Fig. 4. Effects of PNS and CAPS on basal corticosterone. PNS-treated rats had significantly higher basal levels of circulating corticosterone. * Main effect of PNS, $P=0.03$. Data expressed as mean±SEM, $n=25$–27/group.

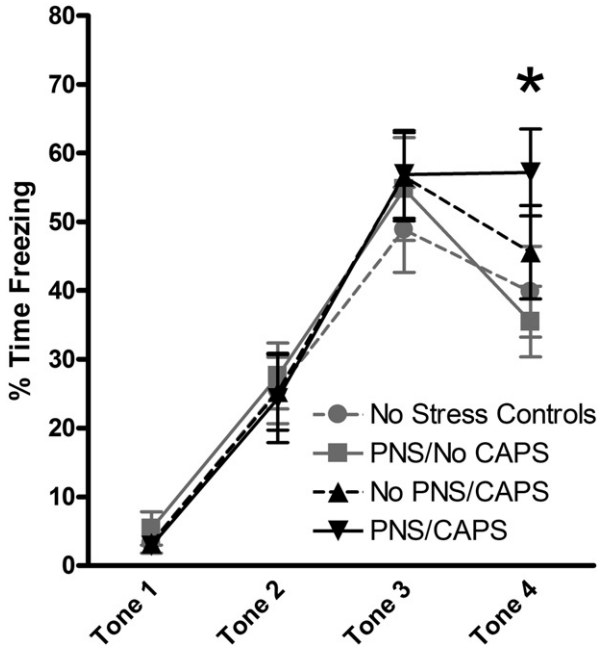

Fig. 5. Combined PNS/CAPS treatment enhanced the expression of learned fear. Rats that received PNS/CAPS displayed enhanced freezing on tone 4 of fear conditioning. * PNS/CAPS vs. PNS/No CAPS, $P=0.009$. Data expressed as mean±SEM, $n=23$–25/group.

training showed that there was a significant effect of CAPS (Fig. 6A; $F_{(1,92)}=3.97$, $P<0.05$) and a significant interaction between CAPS and Tone ($F_{(9,828)}=1.9$, $P<0.05$). Additionally, the main effect of PNS approached significance ($F_{(1,92)}=3.18$, $P=0.078$). Post hoc analyses revealed that the effect of CAPS was manifest as a delay in extinction (i.e. more tones required for extinction), as CAPS-treated rats had significantly higher freezing on tones 3–5 compared to unstressed controls (Fig. 6A). Subsequent post hoc comparisons between groups indicated that this was driven largely by a delayed extinction profile specifically in the combined PNS/CAPS group, in which freezing behavior remained elevated longer than in the other groups. The PNS/CAPS group had significantly more freezing than unstressed controls on tones 3–5, and more than both the PNS-only and CAPS-only groups on tone 5 (Fig. 6A, B). To further assess differences specifically in the rate of extinction, an exponential decay function was fit to each rat's freezing data, and the resulting rate constants (k) were compared by two-way ANOVA. Confirming the results of the primary ANOVA, there was a significant effect of CAPS ($F_{(1,89)}=7.522$, $P<0.01$), reflecting a slower rate of extinction, that was especially evident in the PNS/CAPS group. Post hoc analyses showed that the PNS/CAPS group had significantly slower rates of extinction than both the unstressed control group and the PNS only group (Fig. 7A, B). These analyses suggest that the extinction deficit induced by CAPS, and most prominently in the PNS/CAPS group, was driven primarily by a decrease in the rate of extinction. By contrast, also as seen in Fig. 7, the effect of PNS was primarily to elevate freezing (i.e. increased fear) without affecting the rate of extinction.

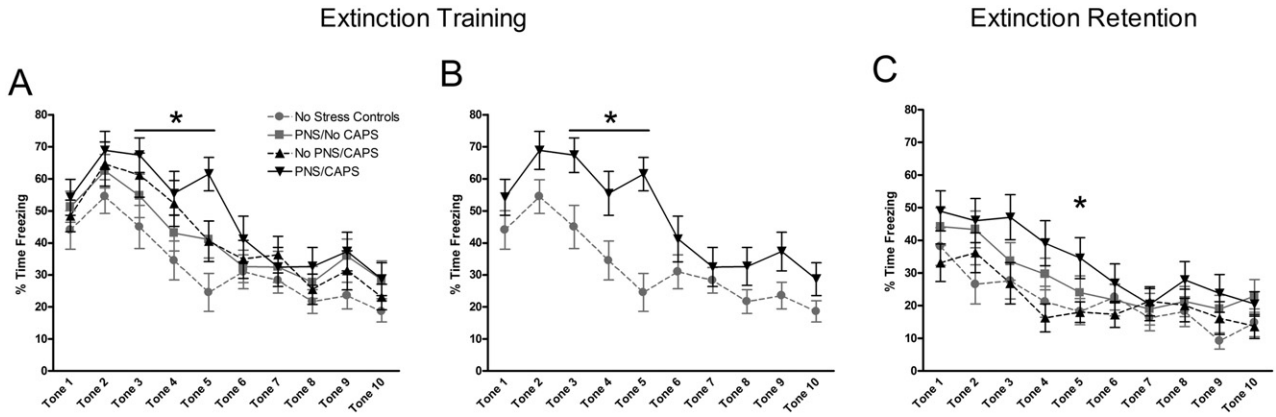

Fig. 6. Combined PNS/CAPS treatment impaired extinction. (A) CAPS caused a delay in extinction, as freezing in the CAPS-treated rats remained significantly elevated during tones 3–5 compared to non-CAPS-treated rats (* $P<0.05$, post hoc comparisons by Newman–Keuls). (B) Specific comparison of extinction in the PNS/CAPS and control groups showed that freezing in the PNS/CAPS group also remained significantly elevated during tones 3–5 compared to unstressed controls (* $P<0.05$, post hoc comparisons by Newman–Keuls), and during tone 5 compared to both the No PNS/CAPS and PNS/No CAPS groups (for clarity, only the PNS-CAPS and control groups are shown). (C) During extinction retention testing on the following day, overall freezing was elevated by PNS alone (* main effect of PNS, $P<0.05$). Although the PNS/CAPS group again appeared to be the most affected, that specific comparison was not significant ($P<0.10$). Data in all panels expressed as mean±SEM, $n=23$–25/group.

PNS treatment also elevated freezing during extinction retention, tested on the following day (Fig. 6C). Freezing in response to tone 1 was first analyzed, as above. There was no significant effect of CAPS ($F_{(1,92)}=0.0002$, $P>0.05$) nor an interaction between PNS and CAPS ($F_{(1,92)}=0.6936$, $P>0.05$). However, the main effect of PNS approached significance ($F_{(1,92)}=3.505$, $P=0.064$), suggesting that rats exposed to PNS had modestly elevated freezing even at the outset of the extinction retention day. Subsequent analysis of the full course of extinction retention by ANOVA revealed only a significant main effect of PNS ($F_{(1,92)}=5.798$, $P<0.05$). The PNS/CAPS group once again appeared to be most affected (Fig. 6C), although this specific comparison was not significant ($P<0.10$). That PNS induced an overall elevation in freezing without affecting the trajectory of extinction on retention day was confirmed by analyzing the rate constants derived from the exponential decay curves fit to the extinction retention data, for which there were no significant differences (Fig. 7C, D). Thus, unlike the effect of CAPS, and especially of PNS/CAPS, on the rate of extinction during training, the effect of PNS on extinction retention was an overall elevation in freezing, evident from trial 1 on.

Open field exploration and social interaction

In the open field test, there were no significant main effects of PNS or CAPS on number of line crossings (Table 2; $F_{(1,39)}=0.057$ for PNS, $F_{(1,39)}=0.028$ for CAPS, $P>0.05$) or time spent in the center zone ($F_{(1,39)}=2.058$ for PNS, $F_{(1,39)}=0.119$ for CAPS, $P>0.05$). Likewise, there was no significant interaction between PNS and CAPS on either measure ($F_{(1,39)}=0.195$, $P>0.05$ for line crossings; $F_{(1,39)}=3.303$, $P>0.05$ for center time). Similarly, in the social interaction test, there were no significant main effects or interactions of PNS and CAPS on time spent interacting with a novel conspecific (Table 2; $F_{(1,39)}=0.261$ for PNS, $F_{(1,39)}=0.578$ for CAPS, $F_{(1,39)}=0.041$ for PNS×CAPS, all $P>0.05$).

DISCUSSION

The hypothesis tested in this experiment was that prenatal stress produces a vulnerability to severe stressors in adulthood, such that those rats that experienced prenatal stress would exhibit a greater detrimental behavioral effect following adult stress, which might be accounted for by specific neurochemical changes in the brain and/or periphery. We found that PNS induced stable baseline alterations on several neurochemical parameters independent of adult stress exposure, and also induced a greater sensitivity to adult stress in some, but not all of the neurochemical and behavioral measures. We found that adult stress alone impaired extinction, and that effect was exacerbated by PNS. Thus, we conclude that prenatal stress exposure induces long term and stable changes in brain and peripheral stress response systems that represent a potential vulnerability to subsequent adult stress.

The rats were stressed during the last week of pregnancy (from E14 to birth), a critical period in development of the fetal HPA system, and also of potential sensitivity to maternal glucocorticoids. GR and mineralocorticoid receptor (MR) receptors are expressed in the developing rat brain at E13–E16 (Kitraki et al., 1996; Diaz et al., 1998), and fetal production of CORT, as well as maternal CORT concentrations, increase from E16 to E19 (Dupouy et al., 1975). Further, placental expression and activity of the enzyme 11β-hydroxysteroid dehydrogenase 2, which protects the fetus from maternal CORT by metabolizing it to inactive 11-dehydrocorticosterone, decreases from E16 to birth (Waddell et al., 1998). Functionally, a comparison of stress exposure during the second and third weeks of gestation found a lasting change in HPA regulation only in

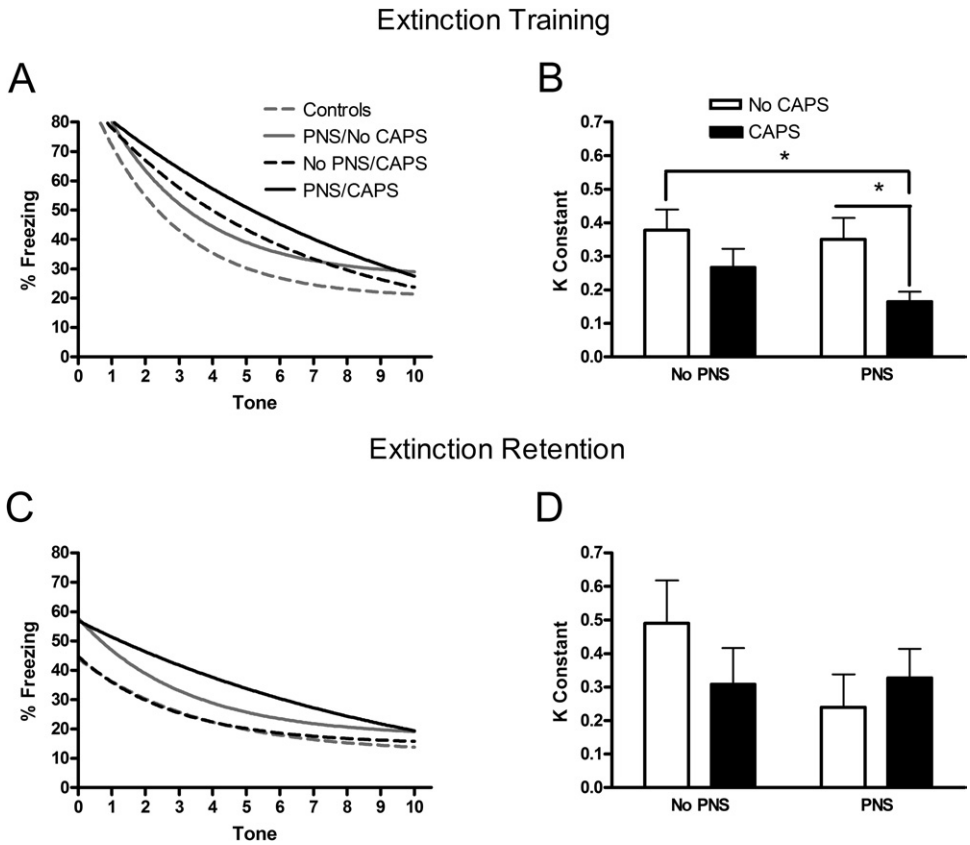

Fig. 7. Effects of PNS and CAPS on rates of extinction. Exponential decay curves were fit to each rat's freezing data, providing a rate of decay (k constant) for each animal. The k-values were then compared by two-way ANOVA to determine group differences in the rate of extinction. (A) For the purposes of illustration, exponential decay curves were fit to each group's mean data, representing the average rate of extinction for each group on training day. (B) In the analysis of k, CAPS produced a slower rate of extinction during training, with the PNS/CAPS rats displaying a significantly slower rate compared to the PNS alone and unstressed control groups (* $P<0.05$ by Newman–Keuls post hoc comparison). (C) Exponential decay curves were fit to each group's mean data to illustrate the average rate of extinction on retention day. (D) There were no significant effects of PNS or CAPS on k, the rate constant for extinction on the retention day. Data expressed as mean±SEM, $n=23$–25/group.

the offspring of mothers stressed during the third week (Koenig et al., 2005).

In the present study, PNS induced a 50% reduction in TH mRNA in the dorsal pons, including noradrenergic neurons in the LC and subcoeruleus. This effect was specific to the brain, as there was, if anything, a modest elevation of TH mRNA and/or protein in the adrenal medulla after either PNS or CAPS. PNS also produced stable, long-term changes in the HPA axis. Adult offspring exposed to PNS had significantly elevated basal circulating CORT and reduced GR expression in the hippocampus and PFC, suggesting potential impairment of HPA negative feedback mechanisms. These neurochemical changes are consistent with effects of PNS reported previously in the literature. For example, PNS has been shown to affect regulation of the HPA axis, including alterations in circadian rhythm (Koehl et al., 1999), elevated CORT, and delayed recovery after mild stress (Weinstock et al., 1992; Barbazanges et al., 1996; Valle et al., 1997; Koenig et al., 2005; Fan et al., 2009). PNS has also been shown to decrease neurogenesis (Lemaire et al., 2000), and the expression of both the GR and the MR in the hippocampus (Barbazanges et al., 1996; Koehl et al., 1999). As in the present study, these effects were all observed in adult offspring of dams that had been stressed during the final week of pregnancy, suggesting that the changes are lifelong and could thus alter the response to subsequent chronic or traumatic stress in adulthood.

Table 2. Open field exploration and social interaction

	Controls	PNS alone	CAPS alone	PNS/CAPS
Open field line crossings	84.5±14.76	74.3±15.88	80.4±12.28	83.45±16.27
Open field center zone time (s)	8.23±2.05	9.07±1.51	11.47±3.49	4.32±1.2
Social interaction time (s)	67.3±7.85	70.07±10.16	58.67±5.58	65.07±10.99

Data expressed as mean±SEM, n=10–12/group.

Both PNS and CAPS also impacted behavioral responses to fear conditioning and extinction, although in different ways, and rats exposed to PNS plus CAPS had the greatest behavioral deficits. First, PNS/CAPS rats displayed slightly elevated freezing at the end of fear conditioning, due primarily to a failure to decrease freezing on the fourth trial. We have frequently observed that after a certain number of tone-shock pairings during fear conditioning, rats can start to shift to a more active coping response, exhibiting active escape behavior and less passive freezing, which was just beginning to occur on tone 4 in the other groups. Thus, the pattern of response seen on the last conditioning trial may indicate that the PNS/CAPS rats persisted in maintaining a more passive coping response to the mild acute stress.

Next, the detrimental effects of adult stress on extinction were manifest as a delay in the rate of extinction. PNS alone modestly elevated the overall level of freezing, whereas CAPS-treated rats had a significantly slower rate of extinction, and this was enhanced by prior PNS exposure. It is noteworthy that on the extinction training day, despite clear differences in the rate of extinction learning, all groups eventually achieved extinction by the end of the session. Having thus achieved extinction, there were no residual effects of CAPS treatment alone on the retention of extinction 1 day later. However, during testing for the retention of extinction, the PNS-treated rats, particularly the combined PNS/CAPS-treated rats, continued to display elevated levels of freezing, which was evident even on tone 1, and was maintained across trials, but with no differences in the extinction rate constants on the retention day. Thus, even though all groups reached equivalent levels of extinction on the training day, PNS-treated rats maintained an elevated level of fear on the following day. These results suggest that severe adult stress exposure alone can have a transient detrimental effect on the rate of extinction, which may be evident in the short-term consequences of traumatic stress exposure, but that these deficits can eventually be resolved with sufficient extinction training, until full extinction is achieved. However, a predisposing history of PNS exposure not only exacerbated the detrimental effect of adult stress on the process of extinction, but in itself it also induced a modest but persistent propensity to exhibit enhanced fear, seen as an increase in freezing during both extinction learning and retention, thus acting as a vulnerability factor.

A similar effect of PNS was reported previously (Markham et al., 2010). In that study, PNS-treated male rats displayed less freezing during conditioning, but more freezing during extinction training and retention compared to control male rats. The different effects observed during conditioning as compared to the present study may be due to differences in the PNS protocol. In the Markham study, PNS involved a variable stress procedure, whereas we used a repeated homotypic stressor (immobilization). Interestingly, in the Markham study, no effects of PNS were seen in females, in either conditioning or extinction. Thus, the factors involved in PNS-induced vulnerability may be particularly relevant to human males facing combat exposure.

Impairments in the rate of extinction of conditioned fear are relevant to human neuropsychiatric disorders in which conditioned fear is a prominent component, such as PTSD, panic disorder, and phobias. In these disorders, there is an inability to extinguish a fear reaction and reinforcement of the fear response. For example, PTSD patients show impairments in extinction (Blechert et al., 2007; Wessa and Flor, 2007) similar to those found in our study, and they have difficulty suppressing fear responses in the presence of safety signals, despite awareness of the safety signal and its meaning (Jovanovic et al., 2009). Further, exposure therapy, a form of extinction training, is effective for approximately 50% of PTSD patients (Bradley et al., 2005), although it is considered one of the most successful treatments for PTSD. In the present study, initial impairments in extinction were seen in the CAPS-treated rats, but successful “recovery” was maintained once extinction was achieved. This may reflect the success of exposure therapy in a proportion of trauma-exposed humans. On the other hand, PNS exposure induced a long-term vulnerability to adult stress, reflected by enhanced impairment of extinction learning and retention, and elevated fear even after extinction. This may reflect the fact that, in humans, certain vulnerable individuals remain impaired and/or resistant to exposure therapy. Further, the differences in the effects of CAPS alone versus PNS plus CAPS may reflect the transition from an acute and transient, perhaps even adaptive response to stress, into a long-term PTSD-like state in vulnerable individuals.

The neurochemical systems in which changes were observed after PNS may provide clues to potential mechanisms underlying the subsequent vulnerability to adult CAPS exposure, and the resulting impairments specifically in the extinction of conditioned fear. TH is the rate-limiting step in catecholamine synthesis. Chronic down-regulation of TH in the forebrain-projecting noradrenergic neurons in the LC, including the sole source of NE input to both PFC and hippocampus, could reflect a reduced capacity for sustained NE release in these forebrain targets in the face of chronic or severe stress. Reduction in brainstem TH, and presumably in NE release, could be one mechanism underlying impaired extinction learning and retention after PNS/CAPS. NE neurotransmission has been implicated in both fear conditioning and extinction. During conditioning, NE release in the amygdala is correlated with the retention of fear memories (Galvez et al., 1996; Quirarte et al., 1998; McIntyre et al., 2002), which is impaired by β-adrenergic receptor blockade (Fu et al., 2008). Likewise, NE levels increase in the mPFC in response to emotionally salient stimuli (Feenstra et al., 2001; Mingote et al., 2004; Hugues et al., 2007), and extinction retention is also impaired by blockade of β-adrenergic receptors, and enhanced by administration of yohimbine, an α_2-adrenergic autoreceptor antagonist that increases NE levels, prior to extinction training (Mueller et al., 2008; Cain et al., 2004).

Glucocorticoids also affect learning and memory, although in a more complex manner (see de Quervain et al.,

2009). Acute CORT administration immediately after training in a novel object recognition task enhanced consolidation and recognition (Roozendaal et al., 2006). By contrast, injections of GR agonist into the hippocampus immediately before retention testing in a Morris water maze impaired spatial memory (Roozendaal et al., 2004), and both of these effects were dependent on convergent noradrenergic signaling. Further, either chronic low-dose CORT or acute GR antagonists given after the first extinction exposure impaired extinction (Gourley et al., 2009). Thus, chronically elevated CORT, together with GR down-regulation and reduced NE release capacity in PFC may have all contributed to impaired extinction after PNS/CAPS.

Monoamines, including NE, have been implicated in both the stress response and in stress-related affective disorders, and antidepressants that affect the monoamines, serotonin, and NE, are the most effective pharmacological treatments for depression and anxiety disorders. However, while drugs targeting NE are effective therapeutically, evidence suggesting dysregulation of noradrenergic signaling in mood and anxiety disorders is less consistent, including alterations in NE levels and adrenergic receptor expression in depression, and reduced NE metabolite levels during the depressive phase of bipolar disorder (see Muscettola et al., 1984; Schatzberg et al., 1989; Ressler and Nemeroff, 1999; Strawn and Geracioti, 2008), although the profile is far from clear. It has been suggested that variability in such measures may be related to the expression of different symptoms, or to different subtypes of depressive disorder (Gold and Chrousos, 1999).

There is more convincing evidence of catecholaminergic dysregulation and increased response sensitivity in PTSD (Strawn and Geracioti, 2008). Elevated plasma NE levels have been correlated with greater symptom expression (Yehuda et al., 1992; Lemieux and Coe, 1995). Peripheral NE release in response to traumatic reminders is enhanced (Blanchard et al., 1991), and administration of yohimbine induces symptoms in PTSD patients (Southwick et al., 1993). Increased peripheral NE activity is consistent with the modestly elevated TH levels we observed in the adrenal medulla of stressed rats. By contrast, a limited study of post-mortem brain tissue from soldiers showed an approximate 50% reduction in the number of LC-NE neurons in the probable-PTSD group compared to controls (Bracha et al., 2005). A similar profile was seen in suicide victims, suggesting reduced NE signaling capacity in depression (Arango et al., 1996). These central changes are similar to the reduction of brainstem TH in PNS rats in the present study, and may thus be more indicative of reduced stress coping capacity and a predisposition to stress vulnerability than to the overt expression of stress-induced illness per se.

The HPA axis and glucocorticoids have also been implicated in affective disorders. In some depressed patients, there is evidence of elevated basal cortisol secretion (Board et al., 1956; Gold et al., 1986; Souetre et al., 1988; Arborelius et al., 1999), elevated corticotropin releasing hormone in cerebral spinal fluid (Nemeroff et al., 1984), blunted diurnal rhythms (Souetre et al., 1988), and impaired negative feedback by dexamethasone (reviewed in Handwerger, 2009). Likewise, anxiety disorders are also associated with alterations in HPA axis activity, but there is considerable inconsistency in the literature. Of particular relevance to the present study, HPA dysregulation has been reported in PTSD, although the exact nature of the dysregulation remains a matter of debate. Some studies have shown elevated basal urinary and plasma cortisol levels in PTSD patients compared to controls (Hoffman et al., 1989; Pitman and Orr, 1990; Lemieux and Coe, 1995), while others have shown lower urinary and plasma cortisol (Mason et al., 1986; Yehuda et al., 1990, 1993, 1995; Boscarino, 1996). Again, discrepancies may be related to the nature or duration of the trauma, or to the expression of specific symptoms (de Quervain et al., 2009; Handwerger, 2009).

Sensitized fear and impaired extinction may be indicative of a more general cognitive deficit related specifically to hypoactivity in the mPFC. Noradrenergic signaling in the mPFC is implicated in tasks requiring cognitive flexibility (Lapiz and Morilak, 2006; Aston-Jones et al., 1999), and extinction learning is a form of cognitive flexibility that is dependent upon the functional integrity of the mPFC (Morgan et al., 1993). Cognitive dysfunction, including cognitive inflexibility and perseveration, is an important component of stress-related psychiatric disorders, and individuals with depression, obsessive-compulsive disorder, or PTSD perform poorly on tests of executive function and cognitive flexibility (Sutker et al., 1995; Fossati et al., 1999; Koenen et al., 2001; Moritz et al., 2002; Kangaratnam and Asbjørnsen, 2007). Such cognitive deficits are often manifest in the form of negative biases, contributing to disordered thinking about self-worth, life stressors, and/or fear-provoking events (Coles and Heimberg, 2002; Elzinga and Bremner, 2002). It has been hypothesized that a hypoactive mPFC and associated cognitive deficits may not only contribute to the symptoms of affective disorders, but may also be a consequence of early-life stress exposure that creates a vulnerability to develop such disorders in response to later stress or trauma (Elzinga and Bremner, 2002).

The prenatal stress model of vulnerability shares some characteristics with genetic models of stress vulnerability, including Wistar–Kyoto (WKY) rats and high responder (HR) rats. WKY rats show a number of behavioral characteristics suggesting increased stress sensitivity, including heightened neophobia and depressive-like behaviors (Paré, 1994). Our laboratory has previously reported that WKY rats exhibit differences in both the expression and regulation of peripheral and central TH mRNA. Specifically, WKY rats showed attenuated TH induction and reduced NE release in the brain in response to acute stress under basal conditions, but greatly enhanced acute NE responses after chronic stress exposure, as well as increases in freezing behavior and acute HPA reactivity (Sands et al., 2000; Pardon et al., 2002, 2003; Ma and Morilak, 2004). Thus, noradrenergic dysregulation may contribute to stress-vulnerability in the WKY genetic model as well as that produced by PNS exposure in the present study. One difference is that prenatal stress induced basal changes in TH, whereas WKY rats did not differ in basal expression, but had attenuated acute stress-evoked induction of TH. In both models, the changes in TH expression may reflect reduced capacity for acute stress adaptation and coping, in-

cluding a preference for passive coping behaviors (i.e. freezing and/or immobility), which may be maladaptive in certain conditions.

HR rats are another rat model of stress vulnerability that share some neurobiological characteristics with the PNS-exposed rats, including elevated CORT, prolonged recovery of basal CORT levels post-stress (Piazza et al., 1991; Kabbaj et al., 2000), and decreased GR mRNA expression in hippocampus (Kabbaj et al., 2000). HR rats displayed increased locomotor responses to amphetamine (Piazza et al., 1989, 1991), as reported after PNS (Koenig et al., 2005), although we saw no changes in basal locomotion in the open field in the present study. However, HR rats have also been defined by high novelty-seeking behavior and enhanced exploration of anxiogenic environments (e.g. Kabbaj et al., 2000), whereas PNS rats have been reported to avoid such environments (Weinstock et al., 1992; Valle et al., 1997; Bosch et al., 2007; Fan et al., 2009). Thus, these models may produce different but overlapping behavioral and neurobiological manifestations that each may be informative for identifying mechanisms underlying individual differences in coping and stress vulnerability. The PNS/CAPS model exemplifies an experience-based stress vulnerability, which may help us to understand aspects of disorders such as PTSD, which present only after experiencing a severe stressor later in life.

Human psychopathologies are complex and multi-dimensional disorders, involving many brain systems and neural circuits. We would not suggest that PNS plus CAPS models all characteristics of any given affective disorder, nor does it replicate any human syndrome in its entirety in rats. Rather, it models key dimensions of many stress-related affective disorders, particularly those associated with fear and acute stress-reactivity. Moreover, it appears to model a vulnerability in specific neurobiological systems that can modulate the processes of fear conditioning and extinction. Thus, this model and others like it will allow a productive investigation of the potential mechanisms underlying long-term changes in the effectiveness of stress-coping capability, and of lifelong vulnerability to stress-induced psychopathology.

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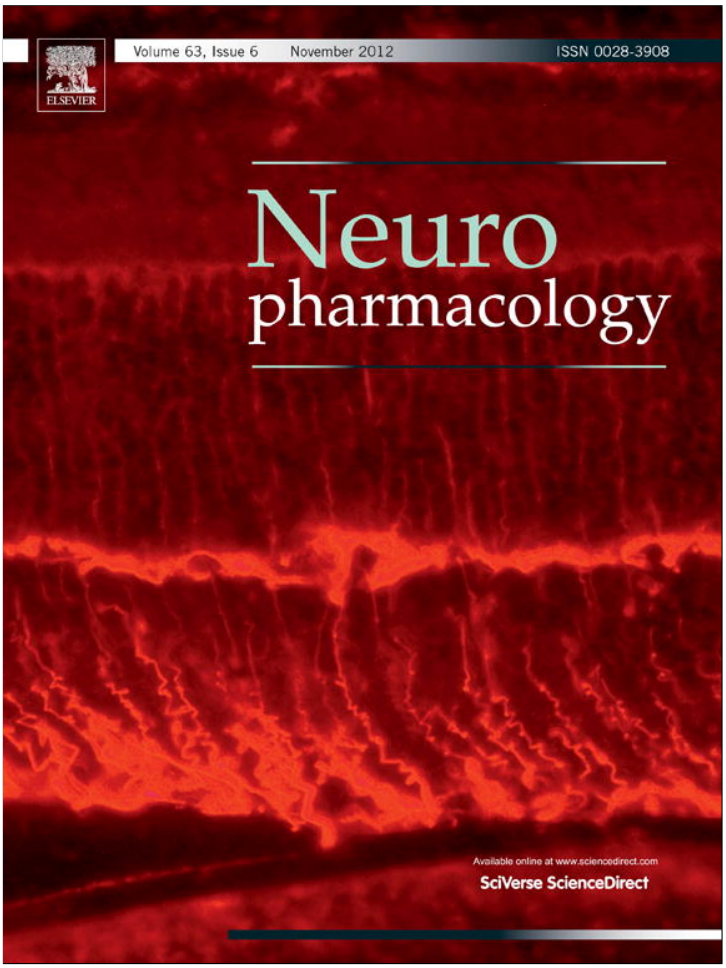

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Effects of chronic plus acute prolonged stress on measures of coping style, anxiety, and evoked HPA-axis reactivity

Megan K. Roth [a,b,1], Brian Bingham [a,b,1], Aparna Shah [a,b,1], Ankur Joshi [a,b,1,2], Alan Frazer [a,b,c,1], Randy Strong [a,b,c,1], David A. Morilak [a,b,*,1]

[a] Department of Pharmacology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Dr., San Antonio, TX 78229, USA
[b] Center for Biomedical Neuroscience, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Dr., San Antonio, TX 78229, USA
[c] South Texas Veterans Health Care Network, Audie L. Murphy Division, 7400 Merton Minter Drive, San Antonio, TX 78229, USA

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HPA-axis

ABSTRACT

Exposure to psychological trauma is the precipitating factor for PTSD. In addition, a history of chronic or traumatic stress exposure is a predisposing risk factor. We have developed a Chronic plus Acute Prolonged Stress (CAPS) treatment for rats that models some of the characteristics of stressful events that can lead to PTSD in humans. We have previously shown that CAPS enhances acute fear responses and impairs extinction of conditioned fear. Further, CAPS reduced the expression of glucocorticoid receptors in the medial prefrontal cortex. In this study we examined the effects of CAPS exposure on behavioral stress coping style, anxiety-like behaviors, and acute stress reactivity of the hypothalamic–pituitary–adrenal (HPA) axis. Male Sprague-Dawley rats were exposed to CAPS treatment, consisting of chronic intermittent cold stress (4 °C, 6 h/day, 14 days) followed on day 15 by a single 1-h session of sequential acute stressors (social defeat, immobilization, swim). After CAPS or control treatment, different groups were tested for shock probe defensive burying, novelty suppressed feeding, or evoked activation of adrenocorticotropic hormone (ACTH) and corticosterone release by an acute immobilization stress. CAPS resulted in a decrease in active burying behavior and an increase in immobility in the shock probe test. Further, CAPS-treated rats displayed increases in the latency to feed in the novelty suppressed feeding test, despite an increase in food intake in the home cage. CAPS treatment also reduced the HPA response to a subsequent acute immobilization stress. These results further validate CAPS treatment as a rat model of relevance to PTSD, and together with results reported previously, suggest that CAPS impairs fear extinction, shifts coping behavior from an active to a more passive strategy, increases anxiety, and alters HPA reactivity, resembling many aspects of human PTSD.

1. Introduction

Post-traumatic stress disorder (PTSD) is a disabling illness that occurs after exposure to a severe stress, e.g., a life-threatening event or witnessing such an event. PTSD is characterized by three classes of symptoms: re-experiencing, avoidance, and hyper-arousal (American Psychiatric Association, 2000). Re-experiencing involves intrusions of vivid memories and dreams, and even dissociations, related to the traumatic event. Avoidance of situations or stimuli that serve as reminders of the traumatic event may also be manifest as a general emotional and social detachment. Hyper-arousal is expressed as elevated anxiety, enhanced startle, irritability, sleep disturbance, and difficulty concentrating. Rape, physical attacks or abuse, threats with a weapon, and combat are some of the events typically associated with PTSD (Kessler et al., 1995). Chronic PTSD represents a significant health concern, not only because of the disabling nature of the symptoms, but also because of the long-term consequences on physical health, including higher rates of chronic disease, such as cardiovascular disease, diabetes, asthma, and obesity, as well as higher rates of

Abbreviations: ACTH, adrenocorticotropic hormone; CAPS, chronic plus acute prolonged stress; CORT, corticosterone; GR, glucocorticoid receptor; HPA, hypothalamic–pituitary–adrenal; mPFC, medial prefrontal cortex; NSFT, novelty suppressed feeding test; PD, postnatal day; PTSD, post-traumatic stress disorder; SPS, single prolonged stress.

* Corresponding author. Department of Pharmacology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Dr., San Antonio, Texas 78229-3900, USA. Tel.: +1 210 567 4174; fax: +1 210 567 4300.
E-mail address: morilak@uthscsa.edu (D.A. Morilak).

[1] For the STRONG STAR Consortium.

[2] Present address: Center for Neuroscience, University of Pittsburgh, A210 Langley Hall, Pittsburgh, PA 15260, USA.

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substance abuse (Centers for Disease Control and Prevention, 2006; Sareen et al., 2005). Chronic stress is also a risk factor, and possibly a causal factor, in the development of depressive and anxiety disorders (Breslau et al., 1999; Gilmer et al., 2005; Jordanova et al., 2007; Kendler et al., 1999; Koenen et al., 2007, 2002).

The complex nature of stress is particularly salient in wartime situations, in which there is a chronic state of environmental stress punctuated by intense, acute traumatic events. To model this, we developed a stress treatment that we have termed Chronic plus Acute Prolonged Stress (CAPS; not to be confused with the "CAPS" assessment used in human PTSD research). CAPS treatment combines 14-days of exposure to a chronic mild environmental stressor (chronic intermittent cold stress), followed on day 15 by a single session of intense acute stressors adapted from the Single Prolonged Stress (SPS) model (Yamamoto et al., 2009).

We have shown previously that CAPS treatment impaired fear extinction (Green et al., 2011), arguably an important component of human PTSD that may contribute to treatment resistance. For instance, PTSD patients show impairments in extinction (Blechert et al., 2007; Wessa and Flor, 2007), and they are incapable of suppressing fear responses in the presence of a safety signal, despite awareness of the safety signal and its meaning (Jovanovic et al., 2009). We also showed that CAPS treatment resulted in a down-regulation of glucocorticoid receptors (GR) in the medial prefrontal cortex (mPFC) (Green et al., 2011). This could have contributed to the impairments observed during extinction testing, as glucocorticoids are known to be involved in learning and memory, including fear and extinction learning (Gourley et al., 2009; de Quervain et al., 2009; Roozendaal et al., 2004, 2006). The mPFC, particularly the infralimbic cortex, is a key region involved in extinction learning (Milad and Quirk, 2002; Milad et al., 2004; Morgan et al., 1993; Quirk et al., 2000; Sutker et al., 1995). Furthermore, humans with PTSD display dysregulated hypothalamic–pituitary–adrenal (HPA) axis activity, although the nature of this dysregulation remains unclear (Boscarino, 1996; Hoffman et al., 1989; Lemieux and Coe, 1995; Mason et al., 1986; Pitman and Orr, 1990; Yehuda et al., 1995, 1993, 1990).

Having defined some key components of PTSD in this model, in the present experiments, we continued to explore the effects of CAPS on other measures of PTSD-like symptomotology, including coping style/defensive behavior and generalized anxiety, as well as acute HPA stress reactivity. In our previous study, we observed that CAPS, particularly when combined with early life stress, resulted in persistent freezing during fear conditioning, at a point when other rats were shifting to a more active escape strategy (rearing and jumping) (Green et al., 2011). Thus, using the shock probe-defensive burying test in the present experiment, we tested the hypothesis that CAPS would produce a shift from an active coping strategy (burying) to a passive coping strategy (immobility). Likewise, we examined if CAPS would increase anxiety-like behavior in the novelty suppressed feeding test (NSFT). Finally, we tested if CAPS treatment produced changes in the HPA response evoked by an acute stressor.

2. Experimental procedures

2.1. Animals

In total, 103 adult male Sprague-Dawley rats were used in these experiments. The rats were born in our animal facility, and after weaning, they were pair-housed with a same-sex littermate until postnatal day (PD) 46–60, depending on the experiment, at which time they were singly housed prior to starting the adult stress or unstressed control treatments. The rats were housed in Plexiglas cages (25 × 45 × 15 cm) on a 12/12 h light–dark cycle (lights on at 07:00) with food and water available *ad libitum*. In addition, for the social defeat procedure, 12 adult male Long-Evans rats (Harlan, Indianapolis, IN), weighing at least 400 g, were used as defeaters. They were housed, together with an ovariectomized female, in large resident cages (60 × 60 × 35 cm) in a separate room on the same 12/12 h light cycle. All experiments were conducted during the light phase. All procedures were conducted according to NIH guidelines for the care and use of laboratory animals and were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio. All efforts were made to minimize animal pain, suffering or discomfort, and to minimize the number of rats used.

2.2. CAPS

CAPS treatment consisted of 2 weeks of chronic intermittent cold stress followed by a single 1-h session of acute prolonged stress on day 15. For cold stress, rats were transported in their home cage with food, water and bedding into a cold room at 4 °C for 6 h per day for 14 days. The acute prolonged stress on day 15 consisted of 20 min social defeat, followed immediately by 30 min immobilization, and then 10 min swim stress. For social defeat, the ovariectomized Long-Evans female was removed from the resident cage, and the test rat was placed in the cage with the resident Long-Evans male rat. Typically within 10–30 s, the resident would attack and defeat the smaller "intruder" Sprague-Dawley test rat. Once defeat occurred, defined by the test rat assuming a supine posture and the resident showing a dominant posture for at least 4 s, the test rat was placed under a wire mesh cage for 20 min, thus protecting it from further physical contact but allowing continued sensory exposure to the dominant rat. Immobilization involved taping the rat's torso and limbs gently but snugly in a prone position on a flat platform, allowing no movement, for 30 min. Finally, swim stress was accomplished by placing the rat in a cylindrical tank (30 cm diameter × 60 cm height) filled to a depth of 30 cm with water at approximately 23 °C. Control rats were handled briefly for approximately 30 s.

2.3. Experiment 1: shock-probe defensive burying test

CAPS treatment was initiated between PD 51–53 (n = 9/group). One day following the end of CAPS (or the comparable time for controls), rats were tested in the shock probe defensive burying test to evaluate potential shifts in active and passive behavioral coping strategies in response to acute stress. The rats were placed into a modified cage containing 5 cm of bedding, with a shock probe protruding 6 cm into one end of the cage. The probe was set to deliver 2 mA of current when the probe was touched. After the rat made contact with the probe and received a shock, the current was shut off and the 15 min test began. Behavior was recorded using a CCD camera mounted above the cage and stored to video files for offline scoring and analysis. The dependent measures analyzed were the amount of time spent immobile and the amount of time spent engaged in actively burying the probe. Burying was defined as behavior consisting of burrowing into the bedding with the snout and upper body, then plowing, pushing, or shoveling the bedding toward the probe, and also flicking or spraying bedding material toward the probe. Immobility was defined as a lack of movement other than that required for breathing (slight scanning movements of the head were permitted). Behavior clearly identified as resting behavior (e.g., laying on side, legs extended) was excluded from immobility measures. As a proportional measure of preferred response, the bury time ratio was calculated as (time spent burying)/(time spent burying + time spent immobile).

2.4. Experiment 2: novelty suppressed feeding test (NSFT)

CAPS was initiated on PD 47 (n = 14–15/group). Following CAPS (or the comparable time period for controls), the rats were left undisturbed for 2 days. Beginning on the 3rd day, the animals were food deprived for 48 h (water was available *ad libitum*). The test was conducted on the 5th day post-CAPS, as described by Bodnoff et al. (1988), with minor modification. The rats were transferred to the behavior room and allowed 1 h to acclimate. The rats were then individually placed into a corner of an unfamiliar black Plexiglas open field (100 × 100 × 40 cm) facing the center where food pellets were placed. The latency to begin feeding and the amount of food consumed during the 12 min test were recorded. Latency to feed was defined as the time from when the rats were placed into the open field until they began to eat the pellets (not just approach or play with them). Following the test, the rats were returned to their home cage, where food consumption was monitored for another 30 min to determine if there were any changes in appetitive behavior. Food consumption was determined by subtracting the weight of any remaining food from the total weight of food placed in the open field and the home cage.

2.5. Experiment 3: evoked HPA responses to acute immobilization stress

CAPS was initiated between PD 46–60 (n = 7–14/group). Group assignments were matched to balance the range of ages at which CAPS was initiated across all groups. Three days prior to the acute prolonged stress (Day 12 of CAPS or the comparable time for controls), all rats underwent jugular catheterization surgery. Rats were anesthetized with a mixture of ketamine 43 mg/ml, acepromazine 1.4 mg/ml, xylazine 8.6 mg/ml, administered i.m. at 1.0 ml/kg, and a catheter comprised of silastic and PE50 tubing was inserted into the jugular vein, then passed subcutaneously and exteriorized via an incision at the back of the neck and plugged. Every

3rd day until testing the catheter was flushed with approximately 0.2 ml of sterile heparinized saline (50 IU/ml) to maintain patency.

Separate groups were tested 1 or 5 days following the termination of CAPS. On the test day, the rats were transported to a quiet room, the catheter was connected via a fluid filled line to a syringe for remote blood collection without disturbing the animal, and approximately 0.1 ml of heparinized saline was administered to ensure patency. The rats were then given 90 min to acclimatize after transport. For blood sampling, 0.4 ml of blood was withdrawn via the catheter and replaced with 0.4 ml of sterile saline. Two baseline blood samples were collected 15 min apart. The rats were then immobilized for 30 min as described in Section 2.2. Two blood samples were collected during the stress, one at 5 min after the onset of stress, and one at 30 min. Following the 30 min stress sample, the rats were returned to their home cages and allowed to recover, during which time 4 blood samples were collected at 15, 30, 60, and 90 min post-stress. Blood was collected into tubes containing 10 μl of 0.5 M EDTA. Plasma was separated immediately by centrifugation at 10,000 rpm for 15 min at 4 °C, and stored at −80 °C until assayed.

Because the CAPS protocol includes a single 30-min immobilization stress, it was possible that any changes observed during the acute stress exposure on test day could be due to habituation or sensitization to the second presentation of immobilization stress, rather than an effect of CAPS specifically. Therefore, a control experiment was conducted in which 2 separate groups of rats ($n = 8–10$) were exposed to a single 30 min immobilization stress rather than the full CAPS procedure, 3 days after catheterization surgery. A third group was briefly handled but not immobilized. Then, 1 or 5 days later, all rats were exposed to an acute 30-min test immobilization, and blood samples were collected as above.

Plasma levels of ACTH and CORT were analyzed by radioimmunoassay. ACTH was determined from duplicate 100 μl samples according to the manufacturer's instructions (MP Biomedicals, Orangeburg, NY). The detection limit was 6 pg/ml, and the inter-assay variability was 10%. CORT was measured in diluted plasma samples according to the manufacturer's instructions (MP Biomedicals). Detection limit was 8 ng/ml, and inter-assay variability was 8%.

2.6. Statistical analyses

For the shock-probe defensive burying data, differences in immobility time, active burying time, and bury-time ratio were analyzed by *t*-test. Likewise, in the novelty suppressed feeding test, differences between groups in latency to feed and amount of food consumed were analyzed by *t*-test. Plasma hormone measures were analyzed by 2-way analysis of variance (ANOVA; group × sample, with repeated measures). In all analyses, significance was determined at $p < 0.05$. After ANOVA, sources of any significant main effects or interactions were determined by analysis with the Newman–Keuls *post-hoc* test.

3. Results

3.1. Experiment 1: shock-probe defensive burying test

CAPS-treated rats displayed significantly less burying behavior than control rats (Fig. 1A, $t_{(16)} = 2.258$, $p < 0.05$) and significantly more immobility (Fig. 1B, $t_{(16)} = 2.963$, $p < 0.01$). Consequently, CAPS-treated rats displayed a significantly lower bury-time ratio than control rats (Fig. 1C, $t_{(16)} = 4.169$, $p < 0.001$). This reduction in bury ratio reflects a shift from a predominantly active behavioral coping strategy to a predominantly passive coping strategy.

3.2. Experiment 2: novelty suppressed feeding test

CAPS-treated rats displayed a significantly longer latency to feed (Fig. 2A, $t_{(27)} = 2.532$, $p < 0.05$). CAPS reduced weight gain during the treatment, as these rats had lower mean body weight (281.5 ± 5.3 g) than controls (298.0 ± 5.6 g) prior to testing (Fig. 2B, $t_{(27)} = 2.135$, $p < 0.05$), as expected after chronic cold stress. Nonetheless, there was no difference in the amount of food consumed during the test period (Fig. 2C, $t_{(27)} = 1.54$, $p > 0.05$), and CAPS-treated rats consumed slightly more food than controls in their home cage (Fig. 2D, $t_{(27)} = 2.451$, $p < 0.05$). The fact that CAPS-treated rats consumed equivalent amounts of food during the test, and more food than controls in the home cage indicates that the increase in latency to feed was not due to a reduction in appetite, but to an increase in anxiety in the novel environment.

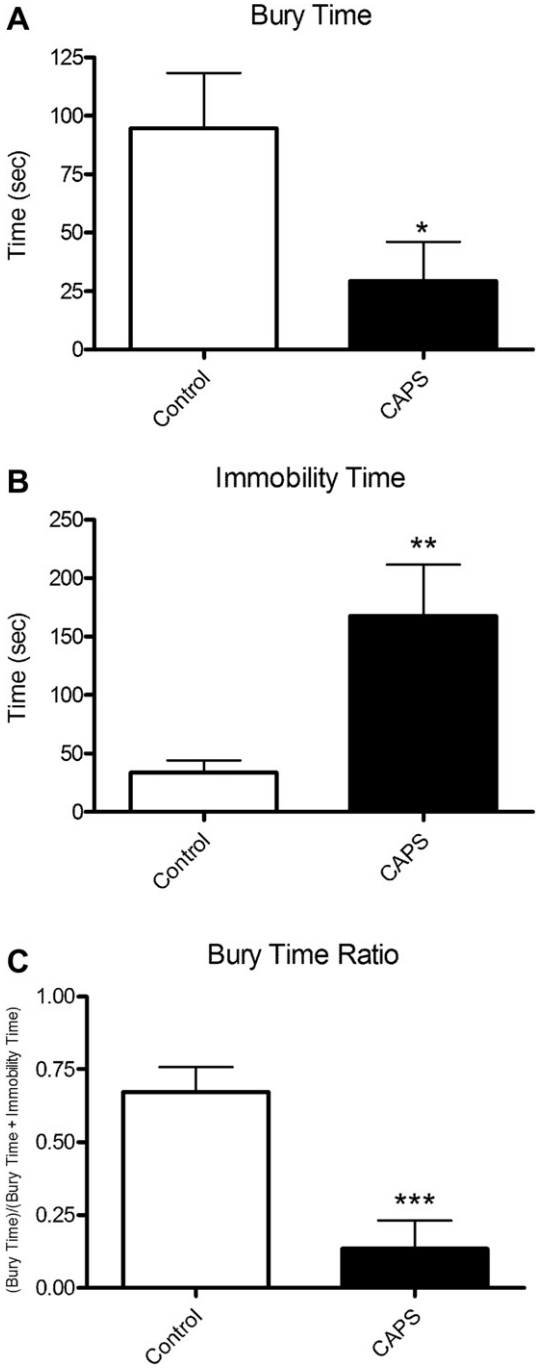

Fig. 1. Effect of CAPS on active defensive burying behavior and passive immobility in the shock-probe defensive burying test. A) On the shock-probe test, CAPS-treated rats displayed significantly less active burying behavior (29.33 ± 16.77 s) than unstressed control rats (94.67 ± 23.58 s). B) CAPS-treated rats displayed significantly more immobility (167.60 ± 43.98 s) in response to a single, brief mild shock than did the non-stressed controls (33.67 ± 10.39 s). C) Consequently, CAPS-treated rats had a lower bury ratio (0.13 ± 0.10) than control rats (0.67 ± 0.09), reflecting a shift from a predominantly active behavioral coping strategy (ratio > 0.5) in the control group to a predominantly passive strategy (ratio < 0.5) following CAPS treatment. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Data expressed as mean ± SEM, $n = 9$/group.

3.3. Experiment 3: evoked HPA response to acute immobilization stress

Acute immobilization stress induced a significant increase in both ACTH and CORT ($F_{(7,168)} = 47.8$, $p < 0.0001$; $F_{(7,182)} = 62.05$, $p < 0.0001$, respectively; significance not indicated in Fig. 3 for

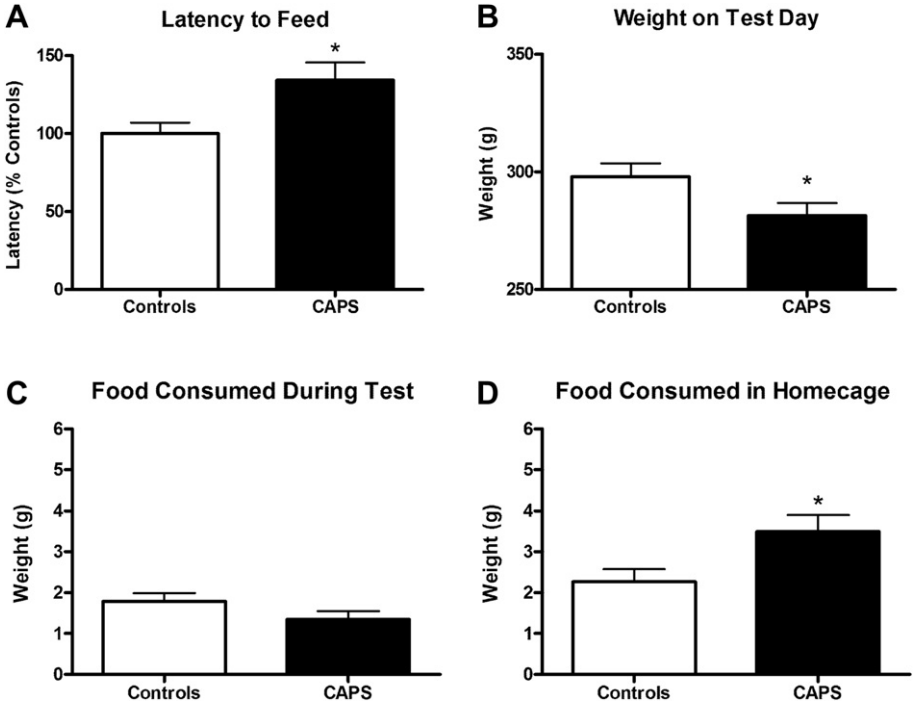

Fig. 2. Effect of CAPS on latency to feed in the novelty suppressed feeding test. A) CAPS-treated rats displayed a longer latency to begin eating (134.3 ± 11.4 % of controls, compared to 100.0 ± 6.9 %). B) As expected, CAPS-treated rats had lower mean body weight on test day (281.5 ± 5.3 g) compared to controls (298.0 ± 5.6 g). C) CAPS-treated rats and control rats consumed an equivalent amount of food during the test (1.35 ± 0.20 g and 1.79 ± 0.20 g, respectively). D) In fact, CAPS-treated rats displayed increased food consumption in the home cage after the test (3.5 ± 0.4 g) compared to control rats (2.3 ± 0.3 g), perhaps related to the reduction in body weight gain during CAPS treatment (panel B). Thus, the increase in latency to feed was not attributable to a loss of appetite, but to an increase in anxiety in the novel environment. $^{*}p < 0.05$. Latency expressed as mean percent of controls ± SEM. Feeding expressed as mean ± SEM, $n = 14$–15/group.

clarity). There was a significant main effect of Group on ACTH release (Fig. 3A, $F_{(2,24)} = 3.469$, $p < 0.05$) and a Group × Sample interaction ($F_{(14,168)} = 2.345$, $p < 0.01$). Specifically, CAPS-treated rats displayed a blunted ACTH response to acute stress. Post-hoc analyses revealed that the 1-day post-CAPS group was significantly different from the control group on the 5- and 30-min stress samples, and significantly different from the 5-day post-CAPS group on the 30-min stress sample. The effect on the 5-min stress sample for the 5-day post-CAPS group approached significance ($p = 0.0505$). Thus, the most robust effect of CAPS on the subsequent ACTH response to an acute immobilization stress occurred one day after the CAPS procedure, and by 5 days, the ACTH response was returning toward that seen in control rats. Although CORT levels in the 1-day post-CAPS group were slightly but consistently lower than in controls, this was not a significant reduction (Fig. 3B, $F_{(2,26)} = 1.542$, $p > 0.05$ for main effect of stress; $F_{(14,182)} = 1.228$, $p > 0.05$ for stress × sample interaction).

The control experiment, conducted to ensure that the blunted ACTH response in CAPS-treated rats was not due simply to habituation to a second exposure to immobilization stress, showed no effect of prior immobilization on ACTH release in response to the test immobilization stress (Fig. 4, $F_{(2,24)} = 1.162$, $p > 0.05$), nor was there a group × sample interaction ($F_{(14,168)} = 0.871$, $p > 0.05$). Because the effect of CAPS on the ACTH response in experiment 3 was seen only at 1-day post-CAPS, we also analyzed the results of the control experiment for day 1 only, excluding day 5. There was still no effect of prior immobilization ($F_{(1,17)} = 1.161$, $p > 0.05$) nor an interaction ($F_{(7,119)} = 1.224$, $p > 0.05$). Thus, these results suggest that the changes in evoked ACTH response following exposure to CAPS were due to the CAPS treatment specifically, and not due simply to a second exposure to immobilization. Because the effect of CAPS on CORT did not achieve significance in experiment 3, CORT was not analyzed in the control experiment.

4. Discussion

In the present set of studies, CAPS-treated rats displayed a shift from an active to a passive coping style, and an increase in anxiety-related behavior. These behavioral effects co-occurred with a blunted ACTH response to acute stress. In addition to these changes, we have previously reported that CAPS impaired fear extinction and reduced GR expression in the mPFC (Green et al., 2011).

4.1. Passive coping

In our previous report (Green et al., 2011), prenatal stress did not alter the preference for active coping relative to passive coping on the shock-probe defensive burying test (prior to CAPS exposure). However, after CAPS exposure, we noted a different behavioral profile during fear conditioning. After multiple tone–shock pairings, control rats begin to show a decrease in freezing, which appears to be due to a shift in behavioral response to the tone, away from the passive freezing response to more active escape behaviors, including rearing and jumping. In that study, rats exposed to prenatal stress and CAPS as adults did not display this shift. Rather, they continued to display high levels of freezing. While this observation is anecdotal, and behavior observed during fear-conditioning is not a validated measure of coping style, this led us to hypothesize that CAPS might produce a shift from active coping to a more passive coping strategy. This hypothesis was tested explicitly in the present study using the shock probe defensive burying test, in which rats can exhibit 2 qualitatively different types of behavioral responses to the shock probe in varying proportions—an active response (burying the probe) and a passive response (immobility). Control rats displayed a slight preference for active coping behavior (burying), whereas CAPS-treated rats displayed a substantial shift to a strong preference for

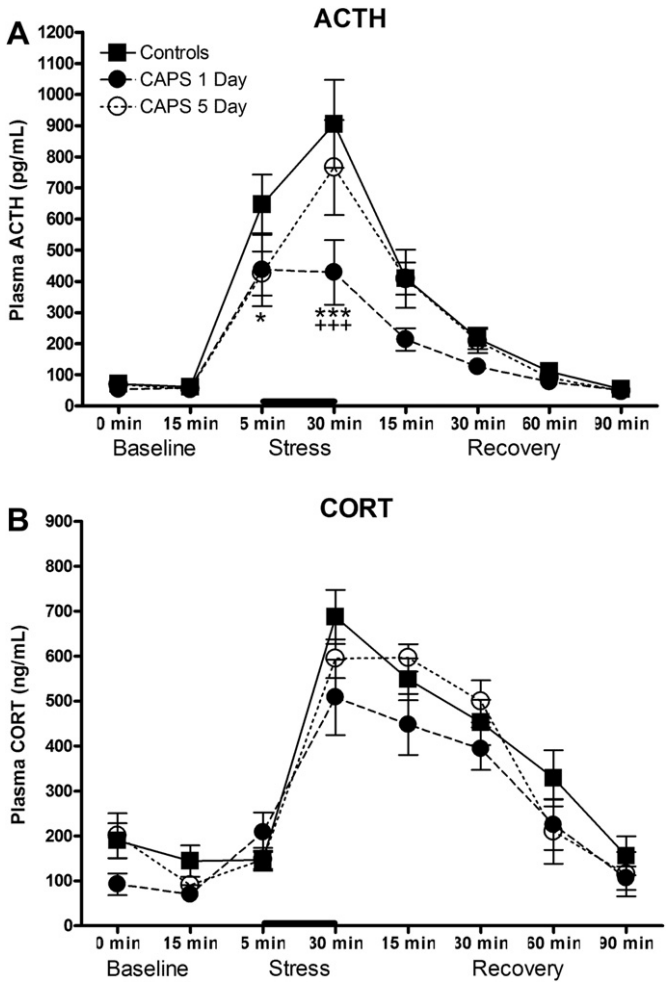

Fig. 3. Effect of CAPS on HPA stress reactivity in response to acute immobilization stress. A) CAPS-treated rats displayed a blunted ACTH response to a subsequent acute stressor (bar), particularly in the group tested on day 1 post-CAPS. B) There were no significant differences between groups on the acute CORT response to immobilization stress, although there was a modest decrease in the group tested on day 1 post-CAPS. $^{*}p < 0.05$ 1-day post-CAPS vs controls, $^{***}p < 0.001$ 1-day post-CAPS vs controls, $^{+++}p < 0.001$ 1-day post-CAPS vs 5-days post-CAPS. Data expressed as mean ± SEM, $n = 7–14$/group.

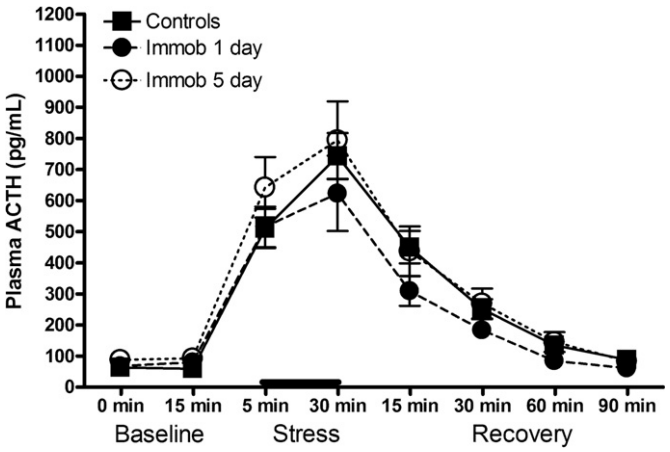

Fig. 4. Lack of effect of a single prior exposure to acute immobilization stress on the subsequent ACTH response to a second immobilization stress. There was no effect of prior immobilization on ACTH release evoked by a second immobilization stress (bar) administered either 1 or 5 days later, suggesting that the effect seen in Fig. 3 was due to CAPS exposure, specifically. Data expressed as mean ± SEM, $n = 8–10$/group.

passive coping behavior (immobility). The increase in immobility cannot be explained by an overall decrease in locomotor activity, as we previously showed that CAPS-treated rats displayed no change in exploration in an open field (Green et al., 2011).

Coping style can mitigate the physiological impact of stress, and there is evidence from both animal and human research that active coping is more adaptive. Previous research has shown that when a rat is given the option to engage in an active coping response, such as chewing on a dowel during immobilization, the stress response is reduced (e.g., Hori et al., 2004; Ono et al., 2008). By contrast, when a rat is deprived of an active response option, such as removing the bedding during the shock probe test so the rat cannot bury, the physiological stress response is increased (Bondi et al., 2007). Likewise, rats that show a low bury response have higher CORT responses during the test (for review see Koolhaas et al., 1999).

A shift to immobility in the shock probe test resembles the "learned helplessness" phenomenon described in both the human and animal psychological literature. Passive responding and failure to engage in active coping responses has long been demonstrated in a number of animal models, originally in dogs (e.g., Seligman et al., 1968) and then rodents (e.g., Maier, 1984). The learned helplessness model shares some characteristics with the CAPS model. For example, rodents exposed to inescapable tail shock display less aggression in a shock-elicited aggression test (similar to our finding of reduced active burying in the shock-probe defensive burying test) and reduced intruder attack by alpha males (Maier, 1984). These rats also display cognitive deficits, including more errors in tests involving learning contingencies (Maier, 1984) and delayed contextual fear extinction (Baratta et al., 2007). Again, this is similar to our previous finding that CAPS treatment impaired fear extinction.

Passivity may contribute to maladaptive stress responses. Consistent with this, studies with animals and humans have shown that active, stimulus-based, or problem-oriented coping styles, as opposed to more passive, emotion-based, avoidant coping styles, buffer HPA activation in response to the stressor/stimulus, increase the ability to eliminate the threat, and improve long-term mental and physical health outcomes (for review see, Koolhaas et al., 1999; Olff et al., 2005). On the other hand, in humans, the negative thought patterns related to many affective disorders often contribute to the perception that there is no way out of a stressful situation and little or no control over one's situation and environment. Emotional withdrawal is a key symptom in the diagnosis of PTSD (American Psychiatric Association, 2000), and studies have shown that individuals previously exposed to traumatic events show greater levels of introversion, social isolation, and emotional blunting (e.g., Bunce et al., 1995). A predisposition for withdrawal may actually contribute to the development of PTSD, and greater symptom expression over time. For example, traumatized individuals who show a shift toward passive coping styles, including withdrawal, are more likely to develop PTSD at 3 months post-trauma (Gutner et al., 2006). Furthermore, men who were abused as children and display high levels of introversion and withdrawal are more likely to meet thresholds for clinical diagnosis of PTSD in adulthood (O'Leary, 2009). Similarly, individuals who report peri-traumatic feelings of helplessness are more likely to develop PTSD (Beck et al., 2006; Hari et al., 2010; O'Donnell et al., 2010).

4.2. Anxiety

We previously examined the effect of CAPS on anxiety-related behavior in an open field and found no differences. In the present experiment, we examined potential anxiogenic effects of CAPS

using a more robust test of anxiety involving an approach-avoidance conflict, the novelty suppressed feeding test. In this test, food deprived rats must enter an anxiety-provoking environment to obtain food. Previous studies with the NSFT have shown that chronic stress results in longer latency to approach the food and begin eating, particularly in more passive, "low responder" rats (Stedenfeld et al., 2011), and that antidepressant treatment reduces the expression of anxiety-related behaviors in this test (Furmaga et al., 2011; Ibarguen-Vargas et al., 2009). Similarly, CAPS-treated rats displayed an increased latency to feed in the novel environment, reflecting greater anxiety, despite an increase in total food intake.

Anxiety is an important component of most animal models of human stress-related psychiatric disorders, as anxiety is a key element of such disorders, including PTSD. Further, PTSD is highly comorbid with other anxiety disorders, and also with depression (Kessler et al., 1995; Rush et al., 2005). These disorders are all notable for an extensive degree of overlap in symptomatology, including, for example, irritable mood, difficulty concentrating, and sleep disturbances. Further, antidepressants are also effective pharmacological treatment for many anxiety disorders (for review, see Morilak and Frazer, 2004). Thus, there are likely to be common neurobiological mechanisms and similar psychopathological processes underlying these shared symptoms.

4.3. Acute HPA stress-reactivity

CAPS treatment reduced the acute ACTH stress response, especially on day 1 after the termination of CAPS. The control experiment confirmed that this was not merely due to habituation to the prior exposure to immobilization stress on day 15 of CAPS treatment. There was also a slight but non-significant suppression of the CORT response to acute stress, and during the post-stress recovery period. It is not clear why the effect of CAPS on the acute CORT response was less robust than on the ACTH response. It may simply be due to differences in the temporal sensitivity of these measures. ACTH is rapidly and dynamically reactive. However, with CORT being slower to respond and slower to clear, a sample at any given time point represents a cumulative response. Thus, differences may have been obscured. On the other hand, it is possible that the adrenal glands may have been sensitized by the previous stress exposure, resulting in greater CORT release in response to ACTH, thus compensating in part for the reduction in evoked ACTH levels. Previous research has shown that chronic stress increases adrenal mass, which may contribute to such sensitization (e.g., Blanchard et al., 1998; Hauger et al., 1990). Another possibility may be related to intensity of the stress induced by immobilization. The HPA response to immobilization was very robust, and may have masked a modest difference between CORT responses in Control and CAPS-treated rats. It may be informative to employ a milder probe stimulus in future studies. Finally, it is important to note that the baseline CORT levels in this experiment were higher than those reported in our previous study (Green et al., 2011). This is likely attributable to differences in methodology. In the present study, rats were exposed to surgery, and then on the test day to handling and a novel environment, all of which can elevate baseline CORT levels, even with a period of acclimation. In the previous study, CORT levels were measured in trunk blood samples collected by rapid decapitation immediately after removal from their home cages.

The changes observed in acute HPA axis stress reactivity are interesting in light of the human PTSD literature. Evidence suggests that HPA activity is reduced in PTSD. However, the full HPA axis profile of individuals with PTSD is not clear, and there are many inconsistencies in the literature (for discussion, see de Kloet et al., 2006). Some studies have shown urinary and plasma cortisol levels to be lower in PTSD patients compared to controls (Boscarino, 1996; Mason et al., 1986; Yehuda et al., 1995, 1993, 1990), and these hormone levels may be negatively correlated with symptom severity (Olff et al., 2006). Further, individuals with lower CORT levels at the time of the post-trauma emergency room visit are more likely to develop PTSD (Delahanty and Nugent, 2006). Reduced hormonal responses may be due to a sensitized negative feedback mechanism, as PTSD patients tend to show greater ACTH suppression by dexamethasone (e.g., Duval et al., 2004). The present results are in line with these findings.

Few animal models of stress have replicated the HPA-axis characteristics of human PTSD, as the typical effect of chronic stress exposure in rodent models is sensitization of the HPA response to acute stress, if any change is observed at all. Rimanoczy et al. (2003) showed that prenatal stress exposure to morphine resulted in a suppressed ACTH response to restraint in adulthood, while maintaining a normal CORT response, similar to the effect seen in our study. Similarly, the SPS model, from which the acute component of our CAPS model was adapted, enhanced HPA suppression in response to dexamethasone treatment (Yamamoto et al., 2009). Further, rats exposed to SPS also display a blunted CORT response to a subsequent acute stressor (Harvey et al., 2006).

By comparison, varying alterations in ACTH and/or CORT have been reported in studies employing the widely-used Chronic Variable Stress (CVS)/Chronic Mild Stress (CMS) model. Most have shown either no change or an increase in basal ACTH (e.g., Choi et al., 2008a,b; Kioukia-Fougia et al., 2002; Ostrander et al., 2006) and no change or an increase in basal CORT (e.g., Choi et al., 2008a,b; Christiansen et al., 2012; Ostrander et al., 2006; Wu and Wang, 2010). Blunting of circadian cycles has been reported (Christiansen et al., 2012). Changes in HPA response to acute stress challenge after CVS/CMS are variable. One study reported an increased ACTH response to acute restraint stress, but no change in CORT response (Choi et al., 2008b). In another, an increase in ACTH response to a mild novelty stress was seen 1 day after CVS, which returned to normal on day 4 post-CVS, followed by a decrease in ACTH response on day 7, returning to baseline by day 30 (Ostrander et al., 2006). As in the present study (and in Choi et al., 2008b), the CORT response did not match the ACTH response. There was no change in the CORT response one day post-CVS, a decrease at days 4 and 7, then a return to normal by day 30. By contrast, when this same group challenged with a systemic stressor (hypoxia), the effect was similar to that seen in the present study, a decrease in ACTH response one day post-CVS, which returned to normal on day 4, with no change in CORT. ACTH then increased on day 7 post-CVS, again with no comparable change in CORT. Other factors that can affect changes in hormonal response after stress are anhedonia-like traits (Christiansen et al., 2012) and strain differences (Wu and Wang, 2010). In most chronic stress models, regardless of the nature of the change in HPA response, it is important to note that, as in the present study, effects were transient, and changes in ACTH and CORT responses are often dissociated.

These results would suggest that an HPA regulatory process that blunts the ACTH response to a subsequent acute stressor emerges in response to chronic stress, then dissipates over time when the stress ceases. In humans with PTSD, even after termination of the primary stressor, the cognitive process of re-experiencing may become a secondary chronic stressor on its own, maintaining the dysregulatory process. Thus, animal models may be particularly useful in revealing mechanisms by which pathological processes after traumatic stress are initiated, and in identifying unique mechanisms by which HPA responses may be inhibited in PTSD, as opposed to the hyperactive HPA axis often seen in other chronic stress-related mood disorders, such as depression.

Despite the transient effect of CAPS on the HPA response to acute stress, behavioral effects were evident at all time points. We examined the effects of CAPS on shock-probe defensive burying behavior on day 1 post-stress, comparable to when we observed the greatest ACTH suppression. However, the need for food restriction in the novelty-suppressed feeding test, and the desire to avoid confounding stress with food deprivation, necessitated testing on day 5 post-stress. In both cases, at 1 day and 5 days post-stress, we observed behavioral effects of CAPS treatment. In general, then, it appears that although the HPA effects begin to recover by day 5, the behavioral effects are evident at day 1 (increased passive coping in shock probe defensive burying), day 2 (increased freezing in fear conditioning, Green et al., 2011), and still present at day 5 (increased anxiety in NSFT, and impaired fear extinction, Green et al., 2011).

4.4. Conclusion

Valid animal models of human psychopathology must be based on a theoretical framework that shares a fundamental aspect of the human disorder, and they must show behavioral and biochemical features that resemble those in the human disorder (Willner, 1986). One requirement for a diagnosis of PTSD is experience of a traumatic event (American Psychiatric Association, 2000). This was modeled by the CAPS treatment in the present study, involving a low-level chronic "state" of stress, followed by a highly salient and intense acute stress experience, which may model the kinds of experiences that initiate PTSD, particularly in combat veterans. Chronic stress is correlated with vulnerability to PTSD (Breslau et al., 1999; Koenen et al., 2007, 2002), and in combat situations, chronic stress, punctuated by acute traumatic events, is the norm. Further, once the trauma has been experienced, an exaggerated and persistent fear response is arguably the fundamental aspect of PTSD (American Psychiatric Association, 2000), and this may be prolonged by impairments in extinction learning (Blechert et al., 2007; Wessa and Flor, 2007; Jovanovic et al., 2009). In our previous report, we showed that CAPS exposure enhanced freezing during fear conditioning and impaired extinction. Further, in the present study, CAPS resulted in other PTSD-like symptoms, including: anxiety; a shift from effective active coping to less adaptive passive coping; and HPA axis dysregulation.

The brain mechanisms that underlie these effects remain to be elucidated. CAPS is a combination of chronic metabolic stress (chronic cold) and a single session of intense acute stress that was adapted from the single-prolonged stress model (SPS; Yamamoto et al., 2009, 2010). Each of these components may have neurobiological consequences that contribute to the resulting phenotype. SPS has been shown to increase inhibitory avoidance, decrease extinction, and increase acoustic startle (Yamamoto et al., 2010; Ganon-Elazar and Akirav, 2012; Knox et al., 2012). This may be due, in part, to reduced excitatory neurotransmitter tone in the PFC and hippocampus, as SPS decreased glutamate and creatine in the PFC, and increased glycine transporter expression in the ventral hippocampus (Yamamoto et al., 2010; Knox et al., 2010). Chronic cold has been shown to impair cognitive flexibility and to decrease serotonin release in the orbital frontal cortex (Lapiz-Bluhm et al., 2009). Changes in prefrontal executive function could compromise the ability to regulate or select from among possible responses in fear- or anxiety-provoking situations. Chronic cold stress alone has been shown to sensitize the ACTH response to immobilization stress (Ma and Morilak, 2005), whereas SPS increased negative feedback inhibition of ACTH release (Liberzon et al., 1997), similar to the blunted ACTH response in the present study. Thus, the phenotype of CAPS-treated rats appears to be a combination of acute and chronic stress effects, perhaps involving changes in modulatory neurotransmission in the prefrontal cortex, consistent with our previous observations of altered GR expression following CAPS treatment (Green et al., 2011). This further suggests that drugs that modulate monoaminergic transmission, glucocorticoid activity, or excitatory amino acid signaling may represent viable strategies for treatment and symptom management of PTSD. Interestingly, it was recently reported that the SPS-induced increase in glycine transporter expression in the hippocampus was normalized with repeated extinction training, perhaps identifying a mechanism by which therapeutically effective behavioral interventions can also mitigate the effects of chronic stress (Yamamoto et al., 2010).

In sum, the CAPS model may prove useful as a valid animal model with which to investigate neurobiological mechanisms underlying pathophysiological changes associated with PTSD, or mechanisms of novel therapeutic strategies for PTSD.

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Exogenous prenatal corticosterone exposure mimics the effects of prenatal stress on adult brain stress response systems and fear extinction behavior

Brian C. Bingham [a,1], C.S. Sheela Rani [a,1], Alan Frazer [a,b,1], Randy Strong [a,b,1], David A. Morilak [a,1,*]

[a] *Department of Pharmacology and Center for Biomedical Neuroscience, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78229, United States*
[b] *Research Service, South Texas Veterans Health Care Network, Audie L. Murphy Division, 7400 Merton Minter Drive, San Antonio, TX 78229, United States*

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Stress vulnerability;
Tyrosine hydroxylase

Summary Exposure to early-life stress is a risk factor for the development of cognitive and emotional disorders later in life. We previously demonstrated that prenatal stress (PNS) in rats results in long-term, stable changes in central stress-response systems and impairs the ability to extinguish conditioned fear responding, a component of post-traumatic stress disorder (PTSD). Maternal corticosterone (CORT), released during prenatal stress, is a possible mediator of these effects. The purpose of the present study was to investigate whether fetal exposure to CORT at levels induced by PNS is sufficient to alter the development of adult stress neurobiology and fear extinction behavior. Pregnant dams were subject to either PNS (60 min immobilization/day from ED 14—21) or a daily injection of CORT (10 mg/kg), which approximated both fetal and maternal plasma CORT levels elicited during PNS. Control dams were given injections of oil vehicle. Male offspring were allowed to grow to adulthood undisturbed, at which point they were sacrificed and the medial prefrontal cortex (mPFC), hippocampus, hypothalamus, and a section of the rostral pons containing the locus coeruleus (LC) were dissected. PNS and prenatal CORT treatment decreased glucocorticoid receptor protein levels in the mPFC, hippocampus, and hypothalamus when compared to control offspring. Both treatments also decreased tyrosine hydroxylase levels in the LC. Finally, the effect of prenatal CORT exposure on fear extinction behavior was examined following chronic stress. Prenatal CORT impaired both acquisition and recall of cue-conditioned fear extinction. This effect was additive to the impairment induced by previous chronic stress. Thus, these data suggest that fetal exposure to high levels of maternal CORT is responsible for

* Corresponding author. Tel.: +1 210 567 4174, fax: +1 210 567 4300.
E-mail address: morilak@uthscsa.edu (D.A. Morilak).
[1] For the STRONG STAR Consortium.

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many of the lasting neurobiological consequences of PNS as they relate to the processes underlying extinction of learned fear. The data further suggest that adverse prenatal environments constitute a risk factor for PTSD-like symptomatology, especially when combined with chronic stressors later in life.

1. Introduction

Post-traumatic stress disorder (PTSD) is a disabling affective disorder that occurs as a consequence of a physically or emotionally traumatic experience. It is characterized by intrusive memories, a state of hyper-vigilance, and an inability to inhibit fear responses to trauma-associated cues. An estimated 9 million people in the United States suffer from PTSD, yet this is only a portion of those who experience trauma (Kessler et al., 2005). Therefore, other factors are likely to confer vulnerability to developing PTSD subsequent to traumatic stress, including experiential, environmental, or biological predispositions. Clinical studies suggest that early life stressors, such as childhood exposure to trauma, low socioeconomic status, and familial instability increase susceptibility to PTSD later in life (Breslau et al., 1999; Koenen et al., 2007). Prenatal stress (PNS) is an adverse early life event that has been associated with increased risk for anxiety, ADHD, schizophrenia, developmental delays, and hypothalamic–pituitary–adrenal (HPA) axis dysregulation in humans; however, very little is known about its role as a potential risk factor for PTSD (Davis and Sandman, 2012; Talge et al., 2007).

Animal studies suggest that PNS programs the adult stress system to create a stress-reactive phenotype, showing fear- and depression-like behaviors that resemble aspects of PTSD (Weinstock, 2008). PNS has been shown to permanently program the brain corticosteroid and brain monoamine systems, both of which are implicated in the formation and extinction of fear memories. PNS can reduce glucocorticoid receptor (GR) and/or mineralocorticoid receptor (MR) expression in adult offspring (Brunton and Russell, 2010; Green et al., 2011; Harris and Seckl, 2011; Weinstock, 2008). PNS also alters catecholamine release in brain areas associated with behavioral and cognitive components of the stress response. It has been shown to decrease basal and stress-induced norepinephrine release in the prefrontal cortex (PFC) and locus coeruleus (LC) as well as dopamine in the LC (Carboni et al., 2010; Takahashi et al., 1992). In addition to these biochemical effects, PNS causes enduring behavioral changes, including anxiety-like behavior on the elevated plus maze, an increase in freezing behavior following footshock, and compromised performance in cognitive tasks like the Morris water maze (Brunton and Russell, 2010; Kofman, 2002; Salomon et al., 2011; Takahashi et al., 1992; Weinstock, 2008).

This altered physiological and behavioral response to stress may create a state of vulnerability to chronic stressors later in life and thus increase the risk for PTSD. Indeed, after experiencing a traumatic stress, many individuals continue to endure a secondary, persistent state of chronic stress that is produced by intrusive memories, nightmares, and increased physiological stress responses to cues associated with the initial trauma. In individuals who may be impaired in their ability to cope with stress, this persistent chronic stress may facilitate the transition from acute stress disorder to chronic PTSD (Davidson and Baum, 1986; Wessa and Flor, 2007). Likewise, chronic stress may also impair the ability to extinguish trauma-associated fear memories. Preclinical studies in rodents have demonstrated that chronic stress in adulthood facilitates conditioned fear behavior and impairs the retention of subsequent extinction of conditioned fear (Farrell et al., 2010; Garcia et al., 2008). We recently showed that both PNS and adult chronic stress independently impaired acquisition of fear-extinction. These effects appeared to be additive, such that rats receiving both PNS and adult chronic stress were consistently the most impaired in their ability to extinguish fearful associations, a hallmark trait of PTSD in humans (Green et al., 2011).

Fetal exposure to maternal glucocorticoids represents one potential mechanism whereby PNS may program the adult stress response in utero. During PNS, glucocorticoids are released by the dam, and, at high concentration, can cross the placental barrier to exert direct effects on gene transcription in the fetus (Harris and Seckl, 2011; Takahashi et al., 1998). Fetal exposure to high levels of glucocorticoids results in long-term impairments in cognitive and emotional regulation (Alexander et al., 2012). Both the direct administration of glucocorticoids and the inhibition of placental barrier enzymes mimic some of the effects of PNS (Welberg et al., 2000). Likewise, maternal adrenalectomy is able to prevent some of the lasting effects of PNS (Barbazanges et al., 1996; Salomon et al., 2011). However, it is unknown if prenatal glucocorticoid exposure mimics the effects of PNS on the formation and extinction of fear memories in the adult offspring. It is also unknown whether a history of prenatal glucocorticoid exposure interacts with later stress to further impair fear extinction, i.e., creating vulnerability for a PTSD-like phenotype. To address these questions, we compared the effects of prenatal corticosterone (CORT) administration in the absence of maternal stress to those of PNS. We first determined a dose of exogenous CORT, delivered to the mother, that best mimics both fetal and maternal circulating CORT levels induced by PNS. To determine if CORT treatment mimics the neurobiological consequences of PNS, we then measured the mRNA and protein expression of the GR, corticotrophin releasing factor (CRF), brain-derived neurotrophic factor (BDNF), and tyrosine-hydroxylase (TH) in the brains of the adult male offspring. Finally, we measured the effect of prenatal CORT exposure on fear conditioning and extinction behavior in the adult offspring, with and without exposure to chronic stress. We hypothesized that prenatal CORT exposure would mimic the neurobiological effects of PNS and create an additive detrimental effect on fear conditioning and extinction behavior when combined with chronic stress later in life.

2. Methods

2.1. Animals

Timed-pregnant female Sprague-Dawley rats (Harlan, Indianapolis) arrived on embryonic day (ED) 6 and were

single-housed in standard Plexiglas cages (25 cm × 45 cm × 15 cm) on a 12/12 h light–dark cycle (lights on at 7:00 h) with food and water available ad libitum. On postnatal day (PD) 5, litters were culled to eight pups each, maximizing the number of males. Upon weaning (PD 21), male pups were housed 2–3 per cage with littermates until PD 45, at which time they were single-housed. In total, 154 adult male offspring (from 30 litters: 11 stressed, 9 CORT-treated, 10 control) were used in these experiments. An additional 32 females were sacrificed at E16 or E20 to provide maternal and fetal CORT levels. For the social defeat procedure, 12 adult male Long-Evans rats (Harlan), weighing at least 400 g, were used as defeaters. Each resident male was housed in a large cage (80 cm × 55 cm × 40 cm) with an ovariectomized female, in a separate room from the experimental colony. All experiments were conducted during the light phase. All procedures were conducted according to NIH guidelines for the care and use of laboratory animals and were reviewed and approved by the Institutional Animal Care and Use Committee of The University of Texas Health Science Center at San Antonio. All efforts were made to minimize animal pain, suffering, or discomfort, and to minimize the number of rats used.

2.2. Prenatal stress

From ED14 to ED21, stressed females were injected daily with either sesame oil vehicle (1.4 ml/kg, sc. Sigma–Aldrich) or saline vehicle (4.5 ml/kg, sc.), depending on the experiment. They were then immobilized for 60 min. They were held gently but firmly on a flat rack while the limbs, shoulders, and hips were taped to the rack. The midsection was not taped to avoid putting physical pressure on the fetuses. The animals were unable to move, but respiration was unhindered and they were not enclosed to avoid hyperthermia. Unstressed females were injected with either CORT (10 mg/kg, sc. Sigma–Aldrich), saline, or oil vehicle and returned to their home cages. This dose was established in pilot studies to approximate stress CORT levels.

2.3. Measurement of fetal corticosterone levels

Dams from each treatment group were sacrificed by rapid decapitation immediately after 1 h immobilization, or 1 h following CORT or vehicle injection on day ED16 or ED20. Maternal trunk blood was collected in chilled 15 ml conical tubes containing 100 μl of 0.5 M EDTA. Immediately thereafter (<1 min), pups were removed by Cesarean section and rapidly decapitated. For the E20 fetuses, approximately 70 μl of trunk blood was collected from each fetus (n = 1–3/litter) with a heparinized capillary tube, and deposited into ice-cold eppendorf vials. Because of the small fetal blood volume at E16, each sample (n = 1–2/litter) represents blood pooled from 3 sibling fetuses, approximately 70 μl total. After collection, blood samples were centrifuged at 4 °C (3000 × g for 15 min) and the plasma removed and stored at −20 °C. Plasma CORT levels were determined via radioimmunoassay as previously described (Roth et al., 2012).

2.4. Chronic plus acute prolonged stress (CAPS)

To investigate the interaction between prenatal CORT exposure and behavioral susceptibility to chronic stress later in life, offspring in each treatment condition were subjected to chronic plus acute prolonged stress (CAPS) treatment from PD 46–48 to PD 60–62. The chronic component of the CAPS procedure entailed 14 days of chronic intermittent cold stress. The rats were transported in their home cage, with food, bedding, and water, into a cold room (4 °C, 6 h/day) for 14 consecutive days. The acute component on day 15 consisted of 3 acute stressors administered sequentially in a single 1-h session: social defeat (20 min), immobilization (30 min), and forced swim (10 min). For social defeat, the ovariectomized Long-Evans female was removed from the resident cage, and the test rat was placed into the resident cage. After the resident Long-Evans male rat attacked and defeated the test rat, defined by the test rat assuming a submissive posture for at least 4 s, the test rat was placed under a wire mesh cage for 20 min, protecting it from further physical contact but allowing continued sensory interaction. Immobilization involved taping the torso, head, and limbs gently but firmly in a prone position on a flat platform, allowing no movement for 30 min. For swim stress, the rat was placed in a cylindrical tank (30 cm diameter × 60 cm height) filled to a depth of 30 cm with water at approximately 23 °C.

2.5. Fear conditioning and extinction

Fear conditioning and extinction were performed as previously described with minor modification (Green et al., 2011). One day after the termination of CAPS treatment (or the comparable time point for controls), rats were habituated to two contexts for 15 min each. Twenty-four hours after habituation, the rats received cued fear conditioning in Context A, a shock chamber with metal walls and a grid floor. Each rat was placed into the chamber and, after a 5 min acclimation period, experienced four pairings of a tone (10 kHz, 75 dB, 20 s) co-terminating with a shock (0.8 mA, 0.5 s, average inter-trial interval = 120 s). Extinction training occurred 3 days later. The rats were placed in Context B, a similar chamber but with smooth vinyl floors and walls to avoid contextual freezing. They were exposed to 16 trials of the tone alone, with an average inter-trial interval of 2 min. On the following day, the rats were returned to Context B, and the retention of extinction was tested by presenting them with 16 additional tones. Behavior during each stage was video-recorded and freezing behavior during each tone presentation was analyzed off-line using the FreezeFrame and FreezeView software (Coulbourn Instruments #ACT-100). Freezing was defined as behavior below a motion index threshold of 10 lasting at least 1 s.

2.6. Tissue collection

Rats were sacrificed on PD 65–67 by rapid decapitation. The medial prefrontal cortex (mPFC), hippocampus, hypothalamus, and the pontine area containing the locus coeruleus (LC) were quickly dissected using a brain matrix on ice, as described previously (Green et al., 2011). The brain samples were frozen on dry ice and stored at −80 °C until use. TH mRNA and protein were analyzed in the LC samples; mRNA and protein for GR in mPFC, hippocampus, and hypothalamus samples; mRNA and protein for CRH in hypothalamus

samples; and mRNA and protein for BDNF in hippocampus samples.

2.7. mRNA analyses

Total RNA was isolated from each brain region and converted to cDNA as described previously (Green et al., 2011). Real-time PCR was performed using the following Taqman gene expression assays (Applied Biosystems/Life Technologies, Carlsbad, CA): rat TH, Rn00562500_m1; rat GR (NR3C1), Rn00561369_m1; rat CRH, Rn01462137_m1; and rat BDNF, Rn01484924_m1. All assays consisted of intron-spanning primers and FAM-labeled probes. Results were normalized using the eukaryotic 18S rRNA endogenous control assay (4319413E) labeled with VIC dye. Assays were performed in triplicate after validating with the ABI Prism 7900 HT instrument and following the MIQE guidelines. Real time PCR data were analyzed by the $2^{-\Delta\Delta Ct}$ method. Relative expression of the gene of interest in treatment groups was expressed as percent of control.

2.8. Protein analyses

TH protein in the LC region was analyzed by Western blot as described previously (Green et al., 2011). GR protein was analyzed using an ELISA kit (TransAM GR kit, Active Motif, Carlsbad, CA) and protein levels were computed from A450 values. CRH protein levels in hypothalamus or hippocampus were assayed using an extraction-free Enzyme Immunoassay kit (Phoenix Pharmaceuticals, Burlingame, CA). Similarly, BDNF protein levels in the hippocampus were assayed using a rat BDNF ELISA kit (Syd Labs Inc., Malden, MA) following the manufacturer's protocol. All results were expressed as a percent of the oil-treated non-stressed control mean.

2.9. Data analysis and statistics

Maternal and fetal CORT measures were analyzed by ANOVA at each age, with Newman—Keuls Multiple Comparison post-tests where significant effects were revealed. All adult neurochemical measures were analyzed by ANOVA with Dunnett's post-test used for comparison to the vehicle control group. For the fear conditioning, extinction, and retention data, group differences in percent freezing were first analyzed for each session by a three-way ANOVA (prenatal CORT $\times$ adult stress $\times$ tone) with repeated measures over tone. In addition, total freezing during extinction, represented by the mean area under the extinction curve, was analyzed by 2-way ANOVA with Bonferroni post-tests for pairwise comparisons. Subsequently, to better assess and compare the rate and extent of extinction across groups, the freezing data for all animals within a group were best fit to an unconstrained, single-exponential decay function using Graphpad Prism 5. As reported previously (Green et al., 2011), freezing typically increased from tone 1 to tone 2 in the extinction session. Therefore, tone 1 was not included in the regression analysis, so that the extinction rate could be calculated from the point of maximum freezing. From the resulting regression equations, the decay constant (k), plateau value, and their standard errors (SE) were derived for each group. Differences between groups were analyzed using an extension of Cochran's Q methodology (Cochran, 1954) which partitioned the overall Chi-square (df = 3) into independent factor components according to a 2 (prenatal CORT exposure) $\times$ 2 (adult stress) design. The Q statistics were then transformed to F values as described (Cochran, 1954).

3. Results

3.1. Maternal and fetal corticosterone levels

Both PNS and CORT treatment significantly increased maternal CORT levels (Fig. 1A) measured at E16 ($F_{(3,15)} = 58.07$; $p < 0.01$) and E20 ($F_{(3,15)} = 60.89$; $p < 0.01$) when compared to the unstressed saline and oil-treated controls ($p < 0.01$). At E16, there were no differences in the plasma CORT levels of PNS dams compared to CORT-treated dams; however, at E20, the PNS-induced plasma CORT level was slightly higher than that of the CORT-treated dams ($p < 0.01$). There were no differences in CORT levels between saline- and oil-treated dams at either age. As with the dams, PNS and CORT treatment also increased fetal CORT levels (Fig. 1B) at E16 ($F_{(3,25)} = 18.71$; $p < 0.01$) and E20 ($F_{(3,39)} = 10.41$; $p < 0.01$) when compared to the respective unstressed saline- and oil-treated control groups at both ages ($p < 0.01$). There were no differences in fetal plasma CORT between saline- and oil-treated groups, or between PNS and CORT groups at either age. As expected, fetal CORT increased from

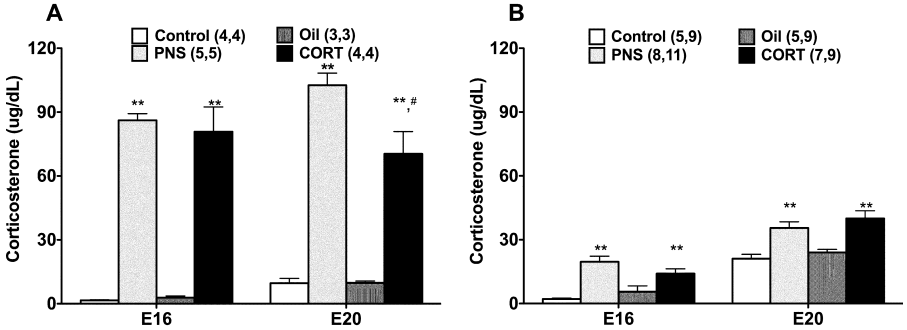

Figure 1 Maternal (A) and fetal (B) plasma CORT levels following a 60 min immobilization stress or 60 min after acute CORT injection on embryonic day ED16 or ED20. Both PNS and CORT increased plasma CORT compared to the respective control treatments in both the dams and fetuses at each day (**$p < 0.01$). On E20, PNS induced a slightly larger increase than CORT treatment in the dams ($^{\#}p < 0.01$); however, there were no differences in the corresponding fetal levels. (n) = number of samples per group.

Table 1 Effects of PNS and CORT on litter size, percent males, and maternal weight gain during treatment.

Treatment group	Litter size (pups)	% Male (pups/litter)	Maternal weight gain E14–21 (g)
Oil	9.7 ± 1.5	57.6 ± 8.8	71.1 ± 6.9
PNS	12.3 ± 1.1	54.0 ± 5.3	55.1 ± 5.2[a,b]
CORT	11.6 ± 1.1	55.2 ± 4.8	86.3 ± 3.9

[a] $p < 0.05$ vs. oil.
[b] $p < 0.05$ vs. CORT.

E16 to E20, as the fetal adrenal glands began producing endogenous CORT (Dupouy et al., 1975). This is also reflected in the slight elevation of CORT in control dams from E16 to E20.

As there were no differences in CORT levels between unstressed saline- and oil-injected controls in the first experiment, only oil-injected animals were used as controls in subsequent experiments. Neither PNS nor CORT treatment altered litter size or the percentage of male pups per litter. PNS did, however, result in a significant decrease in maternal weight gain from E14 to E21, i.e., during the period of daily stress treatment, when compared to both the oil- and CORT-treated dams (Table 1).

3.2. GR mRNA and protein expression in the adult mPFC, hippocampus and hypothalamus

Adult expression of GR mRNA (Fig. 2A–C) and protein (Fig. 2D–F) were measured in the mPFC, hippocampus, and hypothalamus. One-way ANOVA for mRNA expression revealed significant treatment effects in all three brain regions (mPFC: $F_{(2,33)} = 5.76$, $p < 0.01$; hippocampus: $F_{(2,31)} = 8.74$, $p < 0.01$; hypothalamus: $F_{(2,35)} = 3.53$, $p < 0.05$). Post-hoc analyses showed that, in the mPFC and hypothalamus, GR mRNA was reduced in the CORT-treated group but not in the PNS group (Fig. 2A and C). In the hippocampus, both PNS and prenatal CORT exposure resulted in a significant reduction in GR mRNA expression compared to controls (Fig. 2B). GR protein levels were also significantly affected by the prenatal treatment (for mPFC: $F_{(2,21)} = 24.27$; for hippocampus: $F_{(2,30)} = 26.85$; for hypothalamus: $F_{(2,37)} = 15.61$; all $p < 0.01$). Further, post-hoc comparisons showed that GR protein expression was significantly reduced in all three brain regions by both PNS and CORT treatment compared to controls (Fig. 2D–F).

3.3. CRH expression in the adult hypothalamus

Both PNS and prenatal CORT treatment resulted in a significant reduction in CRH mRNA expression in the hypothalamus ($F_{(2,35)} = 7.36$; $p < 0.01$; Fig. 3A), accompanied by a 40–60% decrease in CRH protein ($F_{(2,39)} = 23.14$; $p < 0.01$; Fig. 3D).

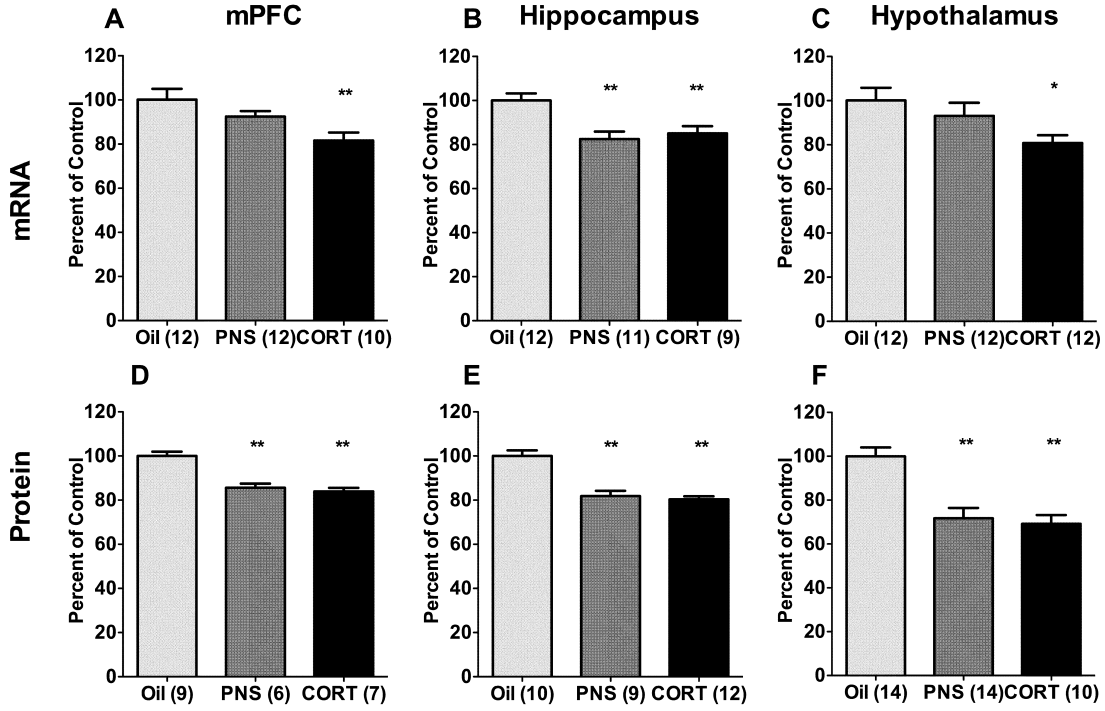

Figure 2 Expression of GR mRNA and protein in the adult offspring of control, PNS, and CORT treated dams. Prenatal CORT treatment decreased GR mRNA expression in the mPFC (A), hippocampus (B) and hypothalamus (C). PNS decreased GR mRNA in the hippocampus only. Both PNS and CORT treatment decreased GR protein in all three regions (D–F). *$p < 0.05$, **$p < 0.01$ vs. control, (n) = number of samples per group.

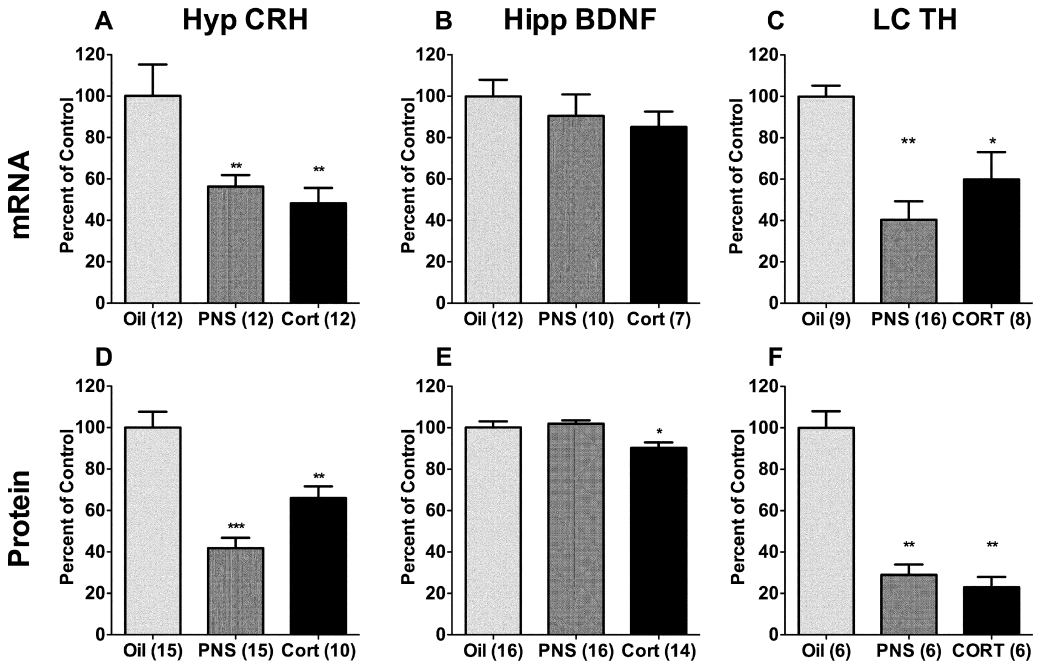

Figure 3 Expression of CRH, BDNF, and TH in the adult offspring of control, PNS, or CORT treated dams. Both PNS and CORT treatment decreased CRH and TH mRNA and protein content in the hypothalamus (A and D) and LC (C and F), respectively. CORT treatment decreased hippocampal BDNF protein with no effect on mRNA (B and E). *$p < 0.05$, **$p < 0.01$ vs. oil control, (n) = number of samples per group.

We also measured CRH mRNA expression in the adult hippocampus and found no change in either of the prenatal treatment groups compared to controls (data not shown).

3.4. BDNF expression in the adult hippocampus

No significant changes in BDNF mRNA expression were seen in the adult hippocampus in either treatment group ($F_{(2,28)} = 0.69$; $p = 0.5$; Fig. 3B). However, a modest but significant reduction in BDNF protein level was seen in the adult hippocampus of animals exposed to prenatal CORT treatment, but not to PNS ($F_{(2,45)} = 6.14$; $p < 0.01$; Fig. 3E).

3.5. TH expression in the adult locus coeruleus

ANOVA revealed a significant difference in the expression of TH mRNA ($F_{(2,33)} = 9.67$; $p < 0.01$; Fig. 3C) and protein ($F_{(2,17)} = 48.27$; $p < 0.01$; Fig. 3F) in the region of pons containing the LC. Post-hoc analyses indicate that both PNS and CORT treatment decreased TH expression when compared to control.

3.6. Fear conditioning and extinction

Having established that prenatal CORT treatment reproduces many of the effects of PNS on several neurobiological measures in adults, we next determined the impact of prenatal CORT treatment on fear conditioning and extinction behavior following adult CAPS stress (Fig. 4). CAPS stress alone increased freezing behavior during fear conditioning with no significant effect of prenatal CORT (Fig. 4A). A three-way ANOVA with repeated measures indicated main effects only for CAPS ($F_{(1,54)} = 5.99$; $p < 0.05$) and Tone ($F_{(3,162)} = 82.64$; $p < 0.01$).

By contrast, both CAPS and prenatal CORT significantly delayed the extinction of conditioned fear. A three-way ANOVA for freezing behavior during extinction learning (Fig. 4B) indicated main effects of prenatal CORT ($F_{(1,54)} = 5.22$; $p < 0.05$), CAPS ($F_{(1,54)} = 10.03$; $p < 0.01$), and Tone ($F_{(15,810)} = 37.13$; $p < 0.01$) with no significant interactions. The extinction learning impairment was also evident in the total amount of freezing displayed during the extinction procedure, measured by the area under the extinction curve (AUC). Two-way ANOVA for AUC again revealed main effects of prenatal treatment ($F_{(1,54)} = 4.77$; $p < 0.05$) and CAPS ($F_{(1,54)} = 10.65$; $p < 0.01$) with no interaction (Fig. 4C). Further, to better assess the rate and final degree of extinction, freezing behavior across tones was fit by non-linear regression analysis to a single exponential decay curve for each group (Fig. 4D). The decay constant (k) and plateau value were then analyzed. CAPS treatment significantly reduced the decay constant ($F_{(1,87)} = 5.25$; $p < 0.05$) while prenatal CORT tended to reduce the decay constant ($F_{(1,87)} = 2.67$; $p = 0.11$) (Fig. 4E). Neither factor significantly altered the plateau parameter (Fig. 4F).

Combined, these analyses indicate that CAPS and prenatal CORT independently impair acquisition of extinction learning by increasing total freezing during training and decreasing the rate of extinction learning without impacting the final level of asymptotic freezing behavior reached at the end of the extinction learning session.

One day after extinction learning, the animals were tested for their ability to recall the extinction training from the day before (Fig. 5). CAPS had no significant effect on extinction retention, whereas prenatal CORT treatment significantly impaired it (Fig. 5A). A three-way ANOVA indicated main effects of prenatal CORT ($F_{(1,54)} = 7.26$; $p < 0.01$), Tone ($F_{(15,810)} = 11.49$; $p < 0.01$), and a CORT by Tone interaction

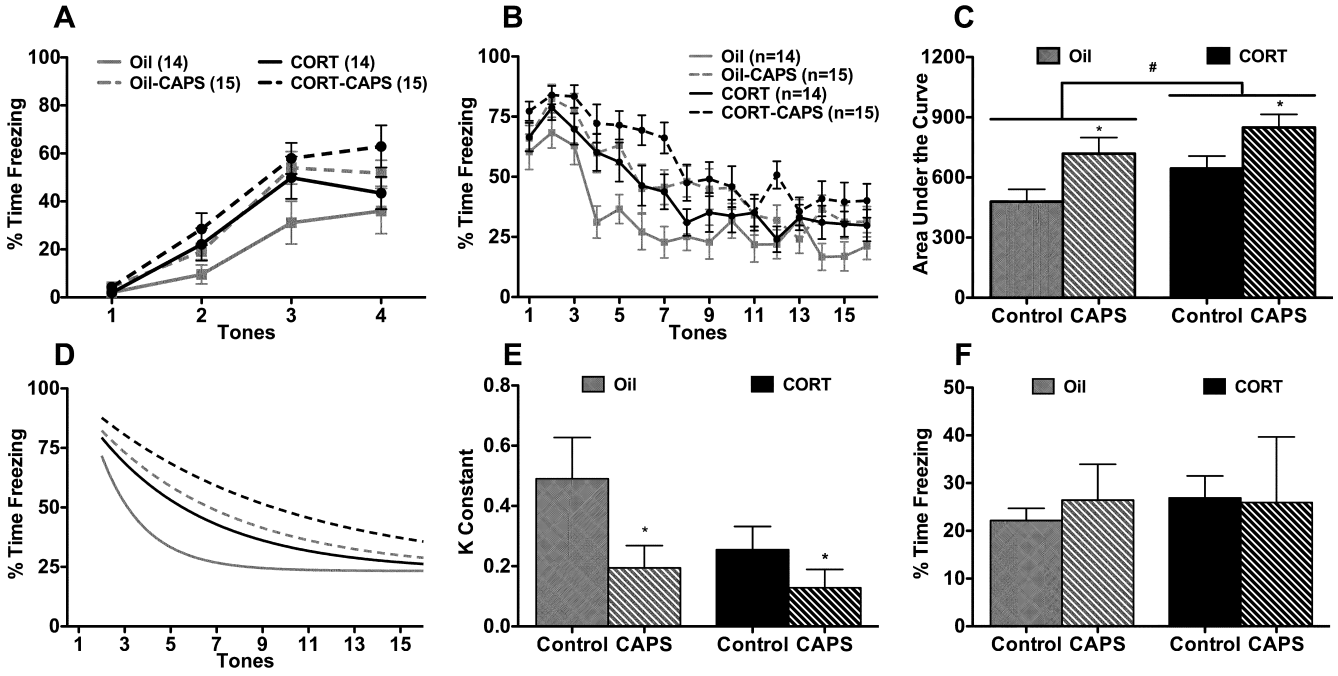

Figure 4 Fear conditioning and extinction learning following prenatal CORT and adult CAPS treatment. (A) CAPS stress (dashed lines) significantly increased freezing behavior during fear conditioning. (B) Both prenatal CORT and adult CAPS treatment significantly delayed the extinction of cue-conditioned fear 3 days after conditioning. (C) Both PNS and CAPS increased total freezing behavior during extinction as measured by area under the curve. (D) Extinction data fitted to a single-exponential decay curve for each group. (E) Analysis of the decay constants (k) derived from the regression lines in (D) indicates that both prenatal CORT and adult CAPS stress reduced the rate of extinction learning. (F) By contrast, analysis of the plateau value indicates no effect of either prenatal CORT or CAPS on the final level of freezing behavior displayed at the end of the extinction learning session. $^{*}p < 0.05$ main effect of CAPS vs. control. $^{\#}p < 0.05$ main effect of prenatal CORT vs. control. (n) = number of subjects per group.

($F_{(15,810)} = 2.14$; $p < 0.01$). Animals treated prenatally with CORT also demonstrated significantly higher freezing in response to the first retention tone presentation (Fig. 5B), analyzed by 2-way ANOVA ($F_{(1,54)} = 6.45$; $p < 0.05$). Analysis of AUC as a measure of total freezing during extinction retention also showed a significant effect of prenatal CORT ($F_{(1,54)} = 7.17$; $p < 0.01$; Fig. 5C). When freezing behavior across tones was fit to a single exponential decay function (Fig. 5D), there were no significant effects of either CORT or CAPS on the rate constant; however, there was a main effect of prenatal CORT treatment on the plateau ($F_{(1,87)} = 15.27$; $p < 0.01$).

Combined, these analyses indicate that CORT-treated animals are able to re-extinguish at the same rate as controls; however, they are impaired in their initial extinction recall, and they are ultimately unable to extinguish to the same extent as controls (Fig. 5E and F).

4. Discussion

We previously demonstrated that PNS decreases GR and TH expression and impairs the extinction of learned fear (Green et al., 2011). The current study tested the hypothesis that fetal exposure to stress-relevant CORT levels is sufficient to recreate the programming effects of PNS on brain stress systems that also mediate fear learning and extinction. Pregnant dams were subjected to either PNS or daily injections of CORT, titrated to match the fetal CORT levels induced by PNS. The offspring were then allowed to grow to adulthood for neurochemical and behavioral testing. The results confirmed and extended our previous findings. Prenatal CORT exposure was sufficient to recapitulate PNS-induced decreases in GR expression in the mPFC, hippocampus, and hypothalamus of the adult offspring. Both prenatal treatments also decreased hypothalamic CRH and TH in the LC, whereas prenatal CORT alone decreased hippocampal BDNF. Prenatal CORT also mimics the previously described effect of PNS by impairing fear extinction and retention, alone and in combination with subsequent adult stress. With both prenatal stress and exogenous CORT treatment, circulating fetal CORT levels did not reach those measured in the dams, most likely due to placental metabolism of some proportion of maternal CORT before it could reach the fetus, by the enzyme, 11β-hydroxysteroid dehydrogenase type 2 (Seckl and Meaney, 2004). Maternal corticotrophin-binding globulin (CBG) may also have a role in regulating the amount of circulating CORT available to impact the fetus, and prenatal stress can decrease maternal CBG levels (Takahashi et al., 1998). However, this would, if anything, increase the amount of free CORT to which the fetuses could be exposed during stress, but is unlikely to have altered the relative levels of circulating CORT after exogenous administration. At any rate, similar fetal CORT levels were achieved in both conditions, and the results indicate that excessive CORT exposure during fetal development is sufficient for many of the neurochemical and behavioral consequences of PNS, including those related to associative fear extinction.

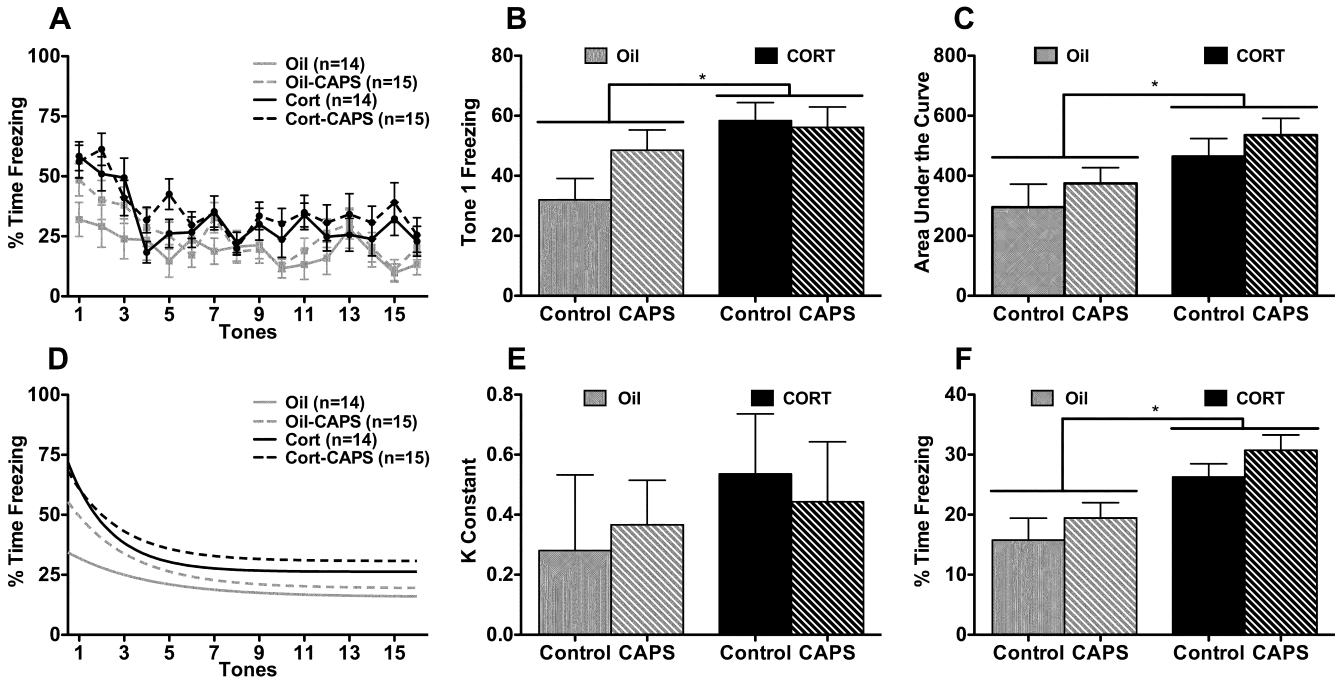

Figure 5 Effects of prenatal CORT and adult CAPS treatment on the retention of extinction. (A) Prenatal CORT (black lines) significantly increased freezing behavior during extinction retention testing 24 h after extinction training. (B) Prenatal CORT significantly increased freezing behavior in response to the first retention tone presentation, indicating impaired recall of previous extinction training. (C) Only prenatal CORT increased total freezing behavior during extinction retention, as measured by area under the curve. (D) Extinction retention data fitted to a single-exponential decay curve for each group. (E) Analysis of the decay constants (k) derived from the regression lines in (D) indicates that all groups showed equivalent rates of extinction re-learning. (F) By contrast, analysis of the plateau term indicated that rats exposed to prenatal CORT treatment were unable to re-extinguish to the same final level of freezing behavior as controls. *$p < 0.05$ main effect of CORT vs. oil-treated group, (n) = number of subjects.

4.1. Long-term changes in gene and protein expression in the brain

Both PNS and CORT suppressed the expression of GR in multiple areas of the brain, providing evidence that fetal CORT exposure is the likely mechanism underlying the long-term programming effect of PNS on brain GR expression. PNS and prenatal CORT also decreased CRH mRNA and protein in the hypothalamus. As most hypothalamic CRH-containing neurons reside in the paraventricular nucleus (PVN), it is likely that the decrease occurred primarily in those HPA-related neurons. We previously found that PNS increased basal CORT secretion in the adult offspring (Green et al., 2011). Therefore, one potential interpretation of these data is that brain GR and CRH levels are down-regulated as a consequence of life-long exposure to elevated basal glucocorticoids. Alternatively, it is possible that the decreases in GR and CRH are established through direct epigenetic programming in utero and that the increase in basal CORT is a compensatory response. While changes in HPA regulatory proteins are not always noted with PNS or prenatal glucocorticoids, others have also found that PNS causes a decrease in negative feedback capability and an increase in basal or evoked glucocorticoid release (Barbazanges et al., 1996; Weinstock, 2008).

In human subjects, this pattern of CORT release and putative glucocorticoid sensitivity is more in line with a depressive-like phenotype than the traditional PTSD-like phenotype (Yehuda et al., 1991). However, two factors are important to consider. First, depression and PTSD are highly comorbid, and the associated HPA activity is often dependent on gender, trauma, and developmental stress history (Shea et al., 2005). Second, reductions in GR expression in the mPFC and hippocampus may have effects on stress-related learning and memory that transcend HPA regulation. GR agonist administration in the prelimbic subregion of the mPFC increases inhibitory avoidance memory and systemic administration of a GR antagonist blocks the reconsolidation of fear memory (Pitman et al., 2011; Roozendaal et al., 2009). GR activation increases the surface expression of both NMDA and AMPA receptors in the mPFC, facilitating short-term memory (Yuen et al., 2009). Activation of the mPFC, specifically the infralimbic (IL) subregion, also facilitates extinction learning and retention (Morgan et al., 1993; Vidal-Gonzalez et al., 2006). Therefore, it is possible that a decrease in GR expression within the mPFC and hippocampus may be more relevant to functional impairment of extinction learning and retention than to HPA regulation.

We also found that prenatal CORT caused a small but significant decrease in BDNF in the hippocampus. Hippocampal BDNF has been shown to play an important role in extinction learning (Peters et al., 2010). Thus the decrease in BDNF is consistent with the impairment in extinction retention in rats exposed to prenatal CORT. However, the reduction in BDNF was relatively modest, and there was no effect in PNS animals. Thus, while reduced BDNF expression may have contributed to the extinction deficit, it may not be

the primary mechanism. Further, the differential effects on BDNF expression highlight the fact that prenatal CORT exposure is only one component of prenatal stress. It is unlikely that prenatal CORT can account for all of the long-term consequences of PNS.

Consistent with our previous study (Green et al., 2011), both PNS and prenatal CORT induced a long-term decrease in pontine TH mRNA and protein levels. This brain region contains the LC, the primary source of norepinephrine (NE) innervation of the forebrain, and the sole source of NE in the prefrontal cortex. Others have demonstrated a decrease in NE content, basal release, and nicotine-evoked release in the mPFC as a consequence of PNS (Carboni et al., 2010; Takahashi et al., 1992). However, the mechanisms underlying the long-term regulation of TH by prenatal CORT remain unclear. The TH gene contains a composite GRE/AP-1 site (Rani et al., 2009) and is responsive to regulation by glucocorticoids; however, this regulation is age- and brain region-specific. While direct comparison of fetal brain development in rats and humans varies by brain region, the period of maternal stress in this study (E14—E21) corresponds roughly to weeks 6—16 of human fetal brain development (Weinstock, 2001). A recent study demonstrated that glucocorticoids given to rats at days E18—E21 induced a marked *increase* in brainstem TH expression, TH activity, and brain NE content; however, when given postnatally, glucocorticoids had no effect (Kalinina et al., 2012). In adult animals, adrenalectomy blocked the stress-induced increase in TH expression in the nucleus of the solitary tract, whereas in the LC, exposure to CORT either had no effect or inhibited the stress-induced increase in TH expression (Makino et al., 2002; Núñez et al., 2009; Smith et al., 1991). Thus, the long-lasting reduction of TH expression may be due to epigenetic modification of transcriptional regulatory elements by prenatal CORT exposure, rather than direct transcriptional effects of glucocorticoids. Functionally, the long-term decrease in TH expression in the LC may reduce the capacity for NE release in the mPFC during extinction training.

4.2. Fear conditioning and extinction

Prenatal CORT and adult CAPS stress both altered fear conditioning and extinction in distinct patterns. CAPS stress increased freezing during fear conditioning, and delayed the acquisition of fear extinction, but had no further effect on the retention of extinction learning. By contrast, prenatal CORT exposure had no significant effect on freezing behavior during fear conditioning, but it also impaired extinction learning. Unlike CAPS stress, prenatal CORT also significantly impaired the recall of extinction learning and the final asymptotic level of freezing behavior that the animals achieved. The impairment of extinction by both prenatal CORT and adult CAPS stress, the lack of significant interaction between these factors, and differential effects on conditioning and extinction retention all suggest that the effects of prenatal CORT and adult stress are additive and independent. This is similar to our previous findings with PNS, in which CAPS and PNS both impaired extinction independently and additively with no interaction (Green et al., 2011). Hence, the combination of prenatal CORT exposure and adult stress creates an impaired phenotype that is greater than that created by either factor alone. These findings suggest that chronic or traumatic stress in humans, when superimposed on a history of high prenatal glucocorticoid exposure, can create an additive impairment on fear extinction — even in contexts that are distinct from the initial trauma. This type of extinction deficit is a prominent and defining feature of PTSD.

Effective extinction learning occurs, in part, through activity in both the IL subregion of the mPFC and the lateral amygdala. Within the lateral amygdala, extinction learning depends on NMDA receptor-mediated synaptic plasticity involving the glutamate NR2B receptor (Sotres-Bayon et al., 2007). In contrast, IL facilitation of extinction learning does not appear to be dependent on NMDA receptor activity (Santini et al., 2001). However, inactivation of the IL immediately prior to extinction training also results in impaired extinction learning and retention, indicating that it has an activity-dependent role in extinction learning (Sierra-Mercado et al., 2011). Therefore, it is possible that CAPS stress and/or prenatal CORT exposure impairs extinction learning either through a decrease in extinction-evoked IL activity or a decrease in NR2B-mediated plasticity in the lateral amygdala. In support of this hypothesis, a history of elevated CORT exposure has been shown to decrease both NMDA and AMPA receptors in the vmPFC and impair contextual extinction learning (Gourley et al., 2009).

In contrast to our model of chronic stress-induced impairments in extinction learning, others have found that acute single prolonged stress (SPS) impairs extinction learning and retention and is associated with an increase in GR expression within the prefrontal cortex and hippocampus (Knox et al., 2012). Although the authors did not distinguish between subregions of the PFC, similar behavioral outcomes might be achieved through differential modulation of GR expression patterns across PFC subregions that mediate the adaptive responses to specific types or durations of stress (Sotres-Bayon and Quirk, 2010).

The deficit in extinction retention that we previously described after PNS and currently describe in rats exposed to prenatal CORT may also be related to the reduced expression of TH in the LC and subsequent dysregulation of noradrenergic transmission. Specifically, such a deficit may be related to a reduced capacity for noradrenergic facilitation of extinction consolidation. NE is released during emotional arousal and helps mediate the consolidation of emotionally salient stimuli (Berlau and McGaugh, 2006). During extinction training, NE is released in the mPFC and promotes the consolidation of extinction memory through the activity of adrenergic β_1-receptors in the infralimbic (IL) region of the mPFC (Mueller et al., 2008). Release of NE during extinction facilitates the establishment of long-term potentiation such that upon subsequent extinction recall, the IL inhibits fear expression through projections to the basolateral (BLA) and intercalated (IC) nuclei of the amygdala (Amano et al., 2010; Knapska and Maren, 2009; Sierra-Mercado et al., 2011). Therefore, a putative decrease in extinction-induced release of NE in the IL, as a result of prenatal CORT exposure, could impair consolidation of the extinction memory. Norepinephrine has also been shown to interact with glucocorticoids in the mPFC to establish emotionally salient memories (Barsegyan et al., 2010). Therefore, the deficits in extinction learning and retention, noted especially in the prenatal CORT—CAPS animals, may be due to a decrease in mPFC GR acting in concert with a putative decrease in NE release

capability. Because we previously demonstrated that CAPS stress alone has no effect on TH expression, this may also explain why CAPS stress alone is insufficient to impair extinction retention. As such, these animals may be delayed in acquiring extinction, but once acquired, they have sufficient NE release to consolidate that learning. Deficits in extinction learning and retention after other types of adult stress have also been associated with an IL-specific retraction of apical dendrites and a switch from IL-driven long-term depression to long-term potentiation within the BLA (Izquierdo et al., 2006; Maroun, 2006), either of which could potentially alter IL-mediated inhibition of the BLA and impair extinction of learned fear.

It is possible that PNS could have altered post-natal maternal behavior toward the pups, to account for some of the long-term behavioral changes in the adult offspring, although this is less likely for prenatal CORT treatment alone. Others have shown that PNS decreased nursing time and increased the time the dam spent away from the nest, but had no effect on more consequential licking and grooming behaviors (Bourke et al., 2013). Cross-fostering PNS pups to non-stressed mothers reversed some of the long-term behavioral and neurobiological effects of PNS, although these findings are complicated by effects of early cross-fostering itself, independent of PNS (Maccari et al., 1995). Prenatal CORT administration has been shown to alter maternal behavior, but only at doses much higher (40 mg/kg) than those used in the current study, whereas CORT at the dose we gave (10 mg/kg) had no effect (Brummelte and Galea, 2010).

5. Conclusion

We have demonstrated that prenatal CORT exposure, at levels comparable to those achieved during prenatal stress, significantly altered the long-term expression of several proteins crucial to the adult stress response in stress-relevant brain regions, replicating and expanding upon previously demonstrated effects of prenatal stress. Further, prenatal CORT exposure also reproduced the previously reported effects of PNS on the extinction of cue-conditioned fear behavior. These results suggest potential mechanisms that link the molecular programming effects of prenatal CORT exposure within components of the brain stress response system to changes in fear-related learning and plasticity. The manner in which two independent risk factors (e.g., prenatal CORT exposure and adult stress) combine to dysregulate fear responding may inform our understanding of the mechanisms underlying susceptibility and development of stress-related psychiatric disorders such as PTSD. Further, the resulting inability to extinguish learned fear responses may exacerbate exposure to secondary stressors associated with the trauma as susceptible individuals continue to respond to fear-inducing cues. This may facilitate the transition from acute stress disorder to chronic PTSD, and limit the subsequent efficacy of extinction-based therapies. This research further highlights the need for effective interventions following traumatic stress, particularly for individuals who may be at risk due to early life history.

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Conflict of interest

Dr. Frazer has served on advisory boards for Lundbeck and for Takeda Pharmaceuticals International, Inc. and Eli Lilly and Co. Dr. Morilak has received research funding from Forest Labs and Lundbeck. None of these activities represents a conflict with the present work. All other authors declare that they have no conflict of interest.

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