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13. SUPPLEMENTARY NOTES

14. ABSTRACT The hypothesis addressed by this project is that early life exposure to stress or glucocorticoids produces a distinct neurochemical and behavioral phenotype characterized by life-long vulnerability to stressors that trigger PTSD. Last year we reported that the PTSD-like traumatic stress model we had adopted from the literature was deficient in several ways. Thus, over the past year, we have developed a new model of traumatic stress which has both construct and face validity as a PTSD-precipitating stressor. Moreover, we have developed an improved measure of fear conditioning and memory for the extinction of conditioned fear. We found that prenatal stress programs a sensitization to conditioned fear in the adult offspring, and a resistance to the extinction of conditioned fear. We also found that prenatal stress programs a unique neurochemical and hormonal phenotype that suggests possible mechanisms by which it can sensitize adult conditioned fear and impair extinction of conditioned fear. Specifically, we found 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, we will now focus on noradrenergic corticosterone interactions in future studies of the role of early life stress in increased vulnerability and reduced resilience to stressors that precipitate PTSD.

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 epigenetic 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 produces a distinct neurochemical and behavioral phenotype characterized by life-long vulnerability to stressors that trigger PTSD. Moreover, we hypothesize that the susceptibility to PTSD is programmed epigenetically by early life trauma and can be reversed in adult offspring by treatments reported to reverse the epigenetic changes, such as SSRI treatment. To address this hypothesis, we proposed the following specific aims: 1. To generate and characterize four animal models of early life stress: prenatal stress; perinatal stress; prenatal dexamethasone; and perinatal dexamethasone. 2. In each model, to determine adult predictors of vulnerability to stress: Adult offspring of the four models developed in Specific Aim 1 will be tested on behavioral, physiological, molecular and neurochemical measures. 3. To determine adult vulnerability to stress: Adult offspring of the four models developed in Specific Aim 1 will be exposed to a model of traumatic stress and then a fear conditioning paradigm. Behavioral, physiological and molecular neurochemical measures will be made. 4. To determine the effects of treatments with the SSRI, sertraline, in the four models developed in specific aim 1: Epigenetic programming of hippocampal glucocorticoid receptor expression is believed to be mediated through serotonergic mechanisms and can be reversed by SSRIs. We plan to look at both hippocampal and prefrontal cortex glucocorticoid receptors because the prefrontal cortex is necessary for the memory of fear conditioning. Osmotic minipumps will be used to chronically deliver the SSRI sertraline to the four rat models and their respective controls. Behavioral, physiological and molecular/neurochemical measures of PTSD-like phenotypes will be made.

B. BODY:

During this second funding period, 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 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. 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 last year's annual report, and according to the instructions, we will not repeat the results here. 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 last year's 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: Last year 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 this year have been 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 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.

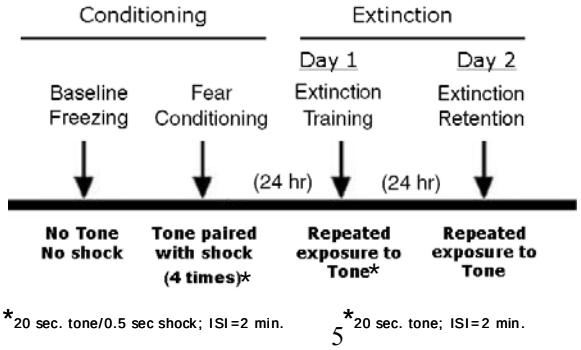

Figure 1. Procedure for measuring fear conditioning and extinction.

Figure 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.

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.

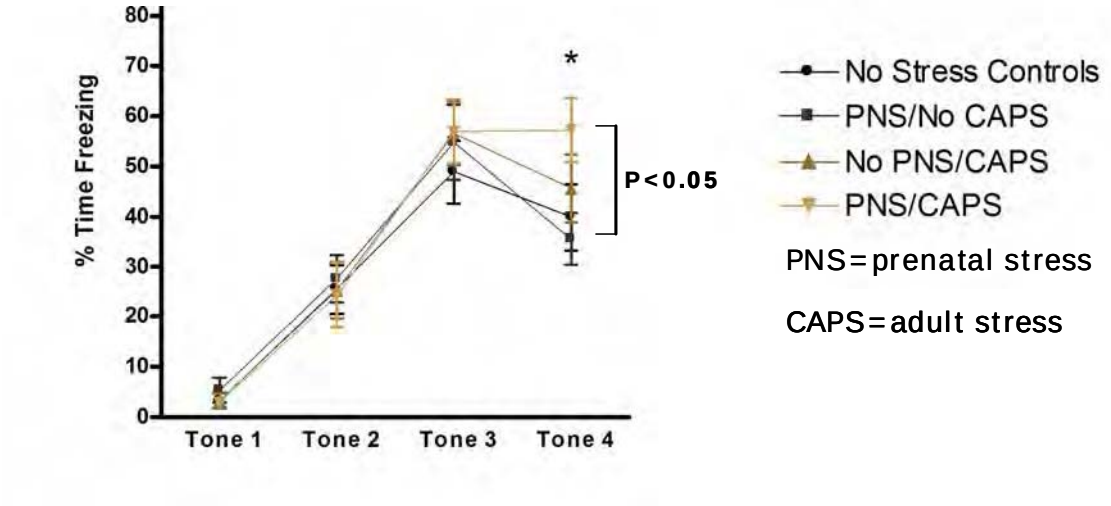

Figure 2. The effect of prenatal stress and adult stress on acquisition of conditioned fear
The data represent the mean ± SEM for 23 to 25 subjects. Data were analyzed by a 3-way ANOVA followed by post-hoc tests of significance of differences between individual means.

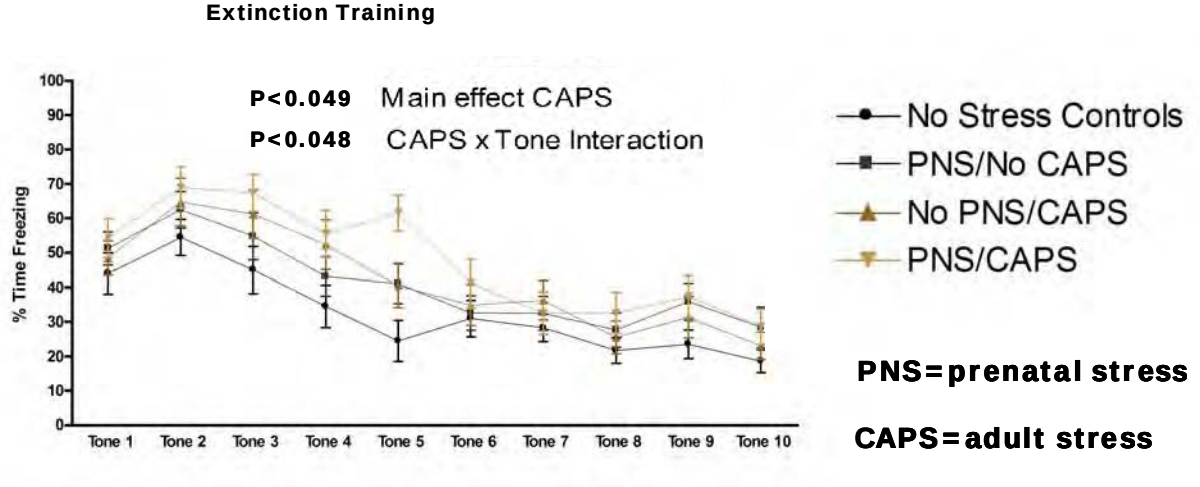

Figure 3. The effect of prenatal stress and adult stress on acquisition of conditioned fear extinction The data represent the mean ± SEM for 23 to 25 subjects. Data were analyzed by a 3-way ANOVA.

Figure 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.

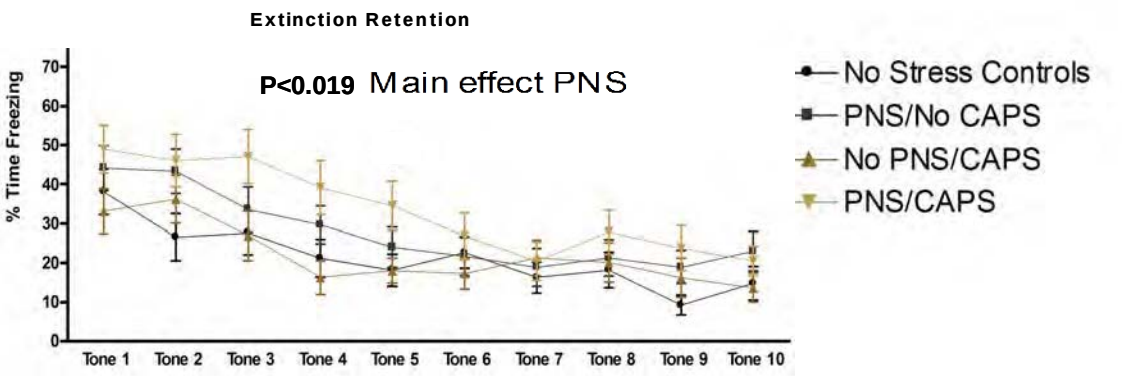

Figure 4. The effect of prenatal stress and adult stress on retention of conditioned fear extinction The data represent the mean ± SEM for 23 to 25 subjects. Data were analyzed by a 3-way ANOVA.

As shown in **Figure 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 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).

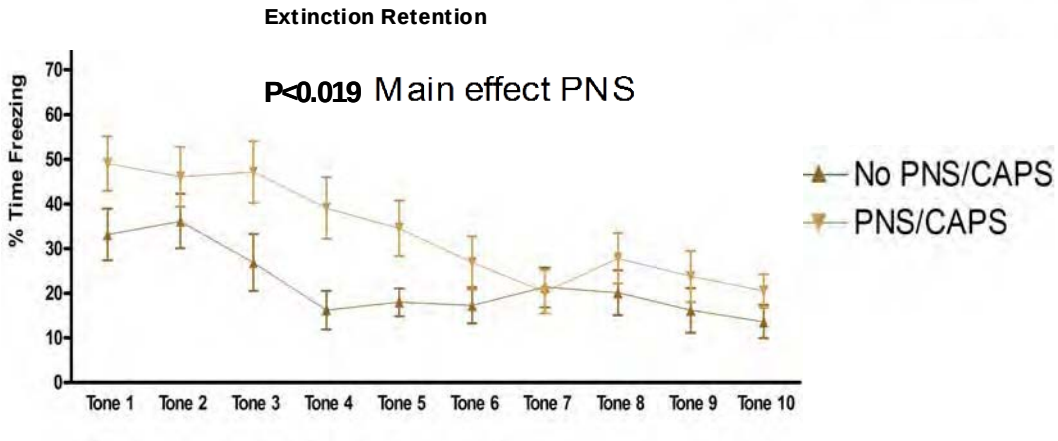

Figure 5. The effect of prenatal stress and adult stress on acquisition of conditioned fear extinction The data represent the mean ± SEM for 23 to 25 subjects. Data were analyzed by 3-way ANOVA.

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 (Tasks 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 Task .

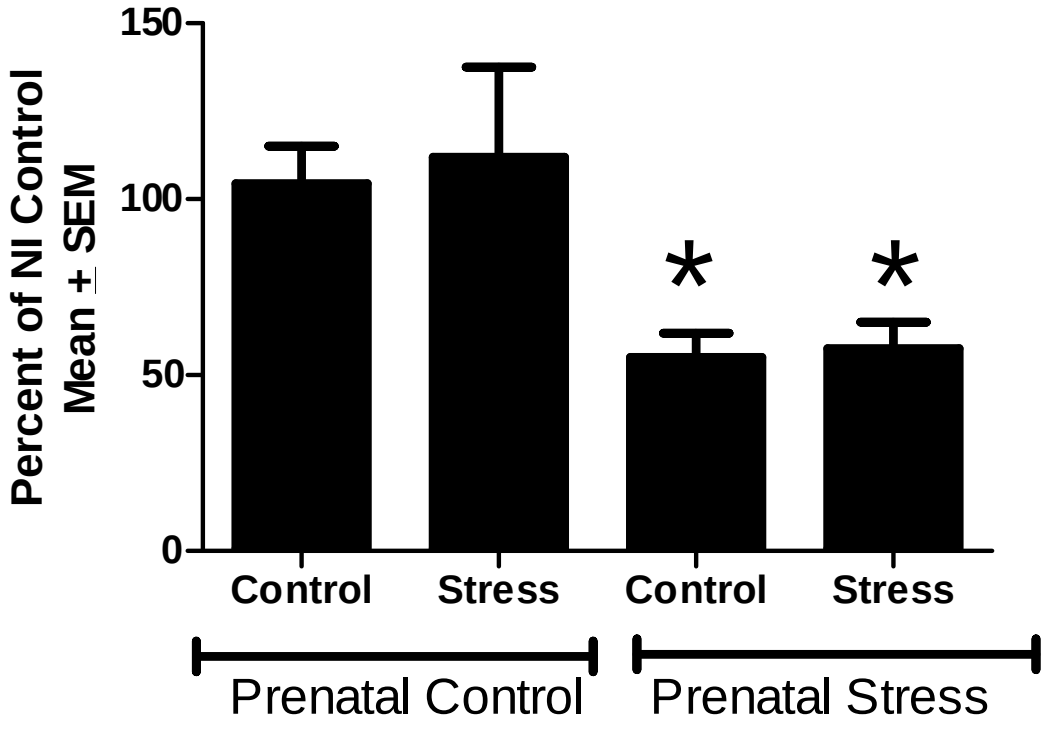

Figure 6: Effect of prenatal stress on glucocorticoid receptor protein in prefrontal cortex. The data represent the mean ± SEM for 17-18 animals. *, $p<0.05$, significantly different from non-prenatal stress controls. NI=no prenatal immobilization.

Figure 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. In future studies, we will follow-up on this finding and measure norepinephrine release by microdialysis in prefrontal cortex during extinction of rats exposed to prenatal and adult stress.

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 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.

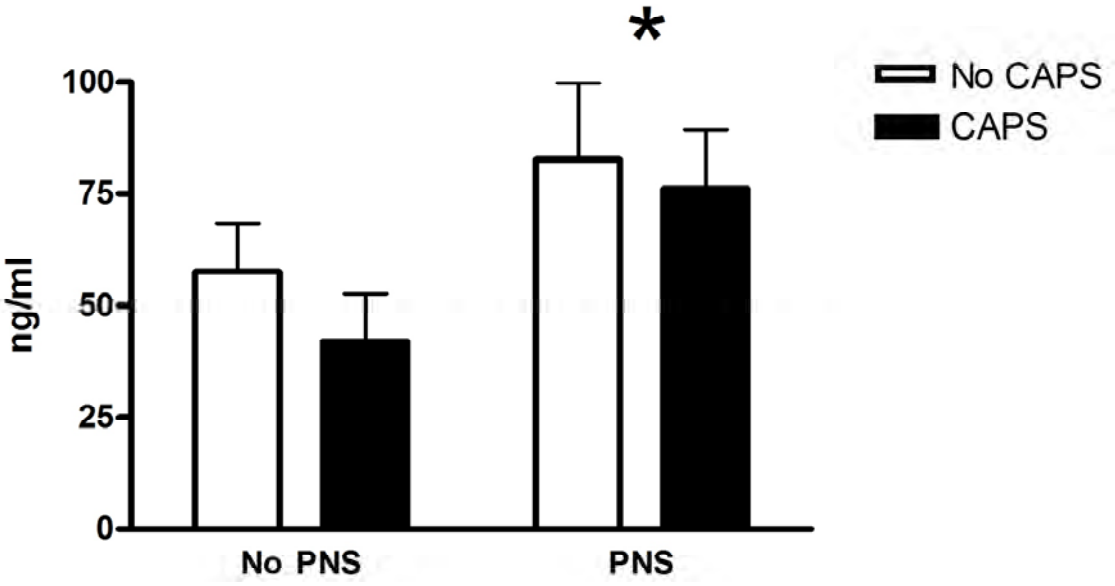

Figure 7: Effect of prenatal stress and adult stress on plasma corticosterone. The data represent the mean ± SEM for 5 animals per group. *, $p<0.05$, significantly different from non-prenatal stress groups.

We then measured glucocorticoid receptor (GR) protein in the prefrontal cortex (Task 1 Step 14 and Task 2 Step 13). As shown in Figure 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.

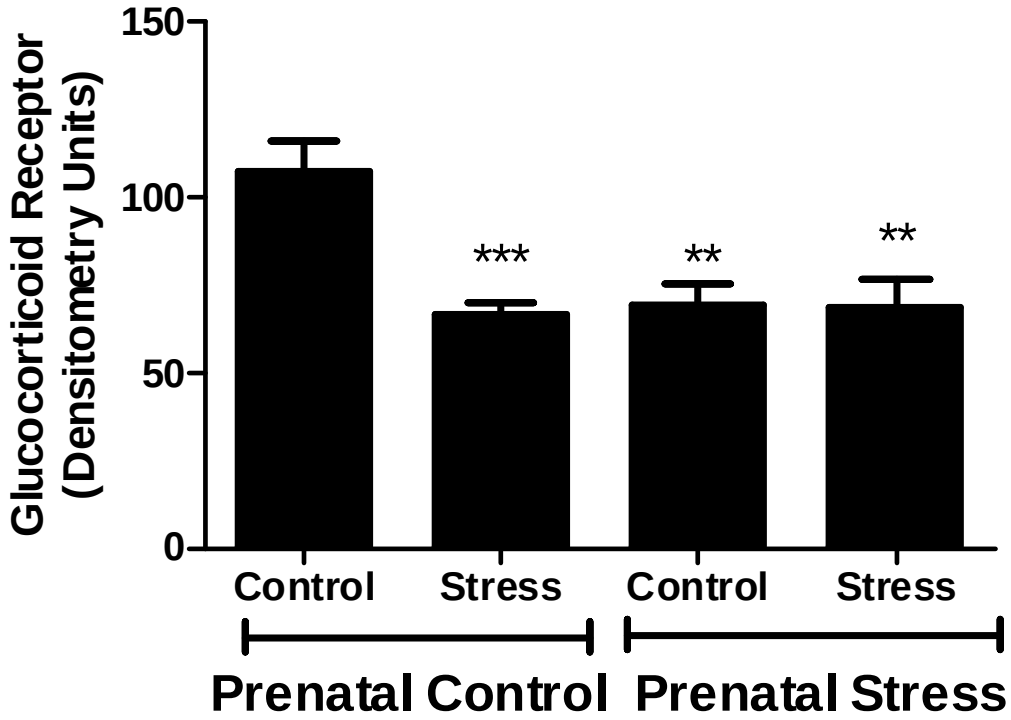

Figure 12: Effect of prenatal stress and adult stress on glucocorticoid receptors in the prefrontal cortex. The data represent the mean ± SEM for 18 to 19 animals per group . **, $p<0.01$, significantly different from non-prenatal stress. *, $p<0.001$, significantly different from non-stressed control group.

KEY RESEARCH ACCOMPLISHMENTS:

- **We have established a reliable and informative measure of fear conditioning and extinction, which is the key feature of PTSD that we would like to model.**
- **We developed a model of traumatic stress (CAPS) that does not interfere with our measure of fear conditioning and extinction. Thus, we overcame a serious technical challenge that was discussed in last year's report.**
- **We found that prenatal stress exposure programs adult sensitization to conditioned fear and resistance to the extinction of conditioned fear.**
- **We found that prenatal stress programs a unique neurochemical phenotype characterized by: reduced TH expression in noradrenergic neurons in the LC region of the brainstem; and reduced GR protein in prefrontal cortex.**
- **We also found that prenatal stress programs a unique endocrine phenotype characterized by chronically elevated plasma corticosterone. The chronically elevated corticosterone may explain the reduction in GR protein in the prefrontal cortex.**

REPORTABLE OUTCOMES:

Randy Strong, David Morilak, and Alan Frazer (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

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.

CONCLUSION: Last 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 this 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, we will now focus on noradrenergic-corticosterone interactions in future studies of the role of early life stress in increased vulnerability and reduced resilience stressors that precipitate PTSD. We will also begin to probe possible epigenetic mechanisms that control the effects of maternal stress on gene expression.

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APPENDICES:

None

SUPPORTING DATA:

Shown in the body of the report.