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14. ABSTRACT

In this report, we will demonstrate that we have a) replicated some pilot findings presented in the initial grant application, b) completed some of the proposed research in specific aims #2 and #3, and c) started research proposed in specific aim #4. We have been unable to replicate our initial SPS effect on social avoidance and freezing induced by trimethlylthiazoline (i.e. predator odor), but are still interested in exploring SPS effects on social behavior (specific aim #2) and regulation of defense behavior (specific aim #3), because we believe these are relevant to the etiology of post traumatic stress disorder. Because we now have to use different animal paradigms to address our specific aims, we have altered our statement of work to reflect this. To date, the research from this grant application has resulted in two peer reviewed journal publications, nine poster presentations, and four manuscripts that are currently being prepared for submission.

15. SUBJECT TERMS
PTSD, SPS, anxiety, fear, conditioning, prefrontal cortex, hippocampus, amygdala

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Introduction

In second annual report to the Department of Defense (DoD), we presented the current status of our research for this research program. We stated that we had not replicated our initial SPS effect on fear extinction and extinction recall, but there was a large amount of data that was not analyzed at the time the report was due. We did not accurately communicate in our second annual report, that the report reflected an, 'on going process', and was not a final conclusion reached by my laboratory. This has been made clear in this revised report. Also, many of the new experiments we designed were consistent with the specific aims, but this also was not made clear. For example, the effects of stress on extinction recall are often investigated by looking at the effect of stress on cued conditioned freezing (i.e. freezing induced by a specific fear conditioned stimulus). However, it is sometimes informative to change the dependent variable of a paradigm and see if experimental effects are still observed. Thus, when we stated that, 'we explored the effect of SPS on conditioned discrimination', this does not represent a fundamental change in our specific aims, but is simply another means of exploring the effects of SPS on fear extinction and extinction recall. Instead of using freezing as our dependent variable, we are using operant responding. We hope in this revised submission, these kinds of changes have been made clear. We also want to make clear that we have not deviated greatly from the specific aims proposed in the grant, although we have now employed different ways of addressing certain specific aims. As a result, a change in the statement of work is needed (mainly to the stated hypotheses and methods). We hope that this submission is now clearer, the relation of our research to the specific aims are easily understood, and changes we made to the statement of work acceptable.

The overall aim of this research proposal is to investigate stress-induced changes in neural processes that lead to aberrant psychological processes that are of relevance to post traumatic stress disorder (PTSD). By doing this, we hope to increase our understanding of the trauma-induced anxiety disorder PTSD. PTSD's clinical manifestation includes three key sets of symptoms that lead to significant disability and poor overall functioning: recurrent and intrusive recollections of the traumatic event, avoidance of normal social interactions, and the perception that emotions like fear, anger, and anxiety are beyond the control of the patient (i.e. deficit in regulation of aversive emotions). Neurobiological research has implicated abnormalities in medial prefrontal cortex (mPFC), as well as altered hypothalamus-pituitary-adrenal (HPA) axis function in PTSD; however, the mechanisms that link PTSD symptom generation, mPFC function, and stress axis abnormalities have not been established. In the last decade our laboratory has developed an animal model of PTSD that induces PTSD specific, HPA axis changes and behavioral arousal in rodents: Single Prolonged Stress (SPS).

Our preliminary data initially demonstrated that SPS induces deficits in fear extinction and extinction recall. We believed that this offered an outstanding opportunity to study potential neurobiological mechanisms involved in the generation of a key set of PTSD symptoms in a valid animal model. After many experiments, however, we were unable to consistently replicate the initial SPS-induced change in fear extinction, but have consistently observed SPS-induced changes in extinction recall after changing some of our conditioning parameters. In order to induced fear conditioning we previously used 30 s tones that co-terminated with a 0.5 s 1 mA footshock. Our new procedure uses 10 s tones that co-terminate with a 1 s 1 mA footshock. We have been advised that such small changes in fear conditioning parameters should not fundamentally change the outcome of an experiment. Nevertheless, it has and if we use the new fear conditioning procedure, we consistently observed SPS-induced deficits in extinction recall.

This was not initially reported in the second annual report, because the data had not been analyzed. We were previously using visual scoring of freezing behavior, which is tedious and time consuming. We have recently obtained a video program that automatically scores freezing behavior. This has greatly increased data analysis of freezing behavior and the overall productivity of the lab.

In the initial grant proposal, we reported that SPS disrupted cued extinction recall. We have expanded this finding by demonstrating SPS also disrupts contextual extinction recall. Extinction recall is suppressed when extinction is tested outside of the extinction context. For example, if rats are fear conditioned in one context (e.g. Context A), then conditioned responding is extinguished in another context (e.g. Context B), good extinction recall is typically only observed in Context B. If rats a returned to Context A or tested in a new context (e.g. Context C), extinction recall is impaired. This is referred to as fear renewal. Thus, when we previously stated that SPS enhanced fear renewal, essentially, we were further demonstrating that SPS produces an extinction recall deficit. Thus, our research has demonstrated that SPS produces, a) cued extinction recall deficits, b) contextual extinction recall deficits, and c) deficits in extinction recall when this is tested outside of the extinction context (i.e. enhanced fear renewal). These findings will be submitted for publication very soon.

In our first annual report to the DoD we indicated that we could not replicate the effects of SPS on social interactions, using the social interaction test. The finding that SPS induces deficits in extinction recall, without fear conditioning or fear extinction deficits, suggests that SPS rats have difficulty controlling fear behavior, but do not necessarily show exaggerated fear and anxiety responses. As a result, we explored whether certain kinds of social interactions can attenuate SPS-induced changes in arousal and HPA axis reactivity. This research has demonstrated that a) increasing maternal behaviors during the neonate stage of development reverses SPS enhancement of acoustic startle reactivity and b) glucocorticoid receptor (GR) expression in the prefrontal cortex.

We also proposed to investigate the importance of the infralimbic cortex (IL), prelimbic cortex (PL), and basolateral complex of the amygdala (BLA) in anxiety stemming from social interactions. While we now know SPS has no effects in the social interaction test, demonstrating the importance of these brain regions in mediating anxiety stemming from social encounters may still increase our understanding of neural processes critical for social avoidance in PTSD. Furthermore, these experiments have never been performed and will significantly add to the scientific literature.

We also could not replicate our initial SPS effects on defense behavior regulation of freezing induced by the predator odor trimethylthiazoline (TMT). Indeed, we could not replicate the initial defense behavior regulation in control rats. However, from this specific aim, we have obtained a number of very significant findings in that have direct relevance to PTSD. These include the findings that a) the prelimbic cortex (PL) is critical for inhibition of unconditioned freezing, b) the expression of unconditioned fear is enhanced in an appetitive context, and c) enhanced TMT-induced freezing in an appetitive context is mediated by decreased neural activity in the medial prefrontal cortex (mPFC). All of these results have either been submitted for publication or will be submitted soon and are relevant to understating neural circuitry that may be critical to the etiology of PTSD.

We have also demonstrated that SPS sensitized locus coeruleus (LC) activity and started our behavioral pharmacology experiments looking at the effects of antikindling drugs on fear renewal and extinction recall.

Reviewer's comments

Concern 1: The PI was unable to replicate several key experiments from her pilot studies, therefore alternative experiments were conducted instead, which resulted in deviation of the tasks listed in the approved SOW.

Response 1: Further examination of SPS-induced behavioral changes was indeed an important component of planned research. The specific deficits that were present in pilot data had to be replicated and additional aspects of behavior had to be tested to fully understand the extant of SPS effects on social interactions, fear behaviors, etc. The SPS induced decreases in social interactions within the social interaction test, were not replicated. Better understanding of the discrepancies between the two sets of data is needed prior to proceeding with specific studies. SPS modulation of freezing induced by the non-specific predator odor trimethylthiazoline was not replicated, however more specific deficits in learned fear behavior were demonstrated. These are particularly important to our understanding of stress-induced changes in psychological processes that are relevant to PTSD. As a result, significant progress was made in understanding the neurobiology of SPS as well as the opportunity to test specific PTSD deficits in this animal model. As a result of these findings and the reviewer's comments, we have now made the appropriate changes to the statement of work.

Concern 2: For example, in Specific Aim 1, instead of measuring fear extinction and extinction recall, the PI conducted experiments on fear renewal, reinstatement, contextual fear conditioning, and conditioned discrimination.

Response 2: We realize now that, in the second annual progress report, we did not explain in sufficient detail our SPS experiments of fear memories. All the experiments listed are indeed components of learning, recall and recovery of learned fear, and are consistent with the original statement of work, as these phenomena all represent different ways of looking at fear and extinction memory. This research represents a more detailed examination of specific sub-processes involved in fear and extinction learning. For example, fear renewal is generated by examining extinction outside of the extinction context, while fear reinstatement involves studying extinction recall following an unexpected footshock presentation. The studies on contextual conditioning allowed us to extend our findings to contextual extinction recall, as opposed to just cued extinction recall. Finally conditioned discrimination is a fear conditioning paradigm where operant responses, instead of freezing, are used to measure conditioned fear extinction.

Concern 3: …..in Specific Aim 2, the PI studied the effects of SPS on social buffering instead of social interactions. The PI also reported several experiments which were not in the scope of the approved SOW.

Response 3: Indeed our findings of the effects of social buffering supported our overarching hypothesis of interactions between SPS induced changes and social functioning.

It is our hope that we have properly communicated how all our research to date has been in keeping with the specific aims of the grant. Because we have changed the order of some of the experiments, and are now addressing specific aims using somewhat modified experimental paradigms, we have modified the statement of work to reflect this.

Body

Below are the four key research accomplishments for the second year of this research program. These are 1) SPS disrupts extinction recall, 2) enhancing social interactions attenuates certain SPS effects, 3) unconditioned fear is enhanced in an appetitive context and is mediated by deactivation of the mPFC, 4) SPS enhancement of fear renewal is attenuated by chronic administration of the antikindling drug phenytoin, 5) SPS alters locus coeruleus (LC) activity. The research conducted to date has compelled us to change some of our hypotheses and/or the experiments we will use (or have used) to test different hypotheses. As a result, we have made changes to the statement of work. These changes can be conceptualized as key changes as a result of key research accomplishments. Changes to the statement of work (underlined) and reasons why we made specific changes are contained here. The entire original and revised statements of work are presented in Appendix I.

Key Research Accomplishments

1) SPS disrupts extinction recall.

Prior to addressing specific aim #1, it is important to demonstrate that SPS induces deficits in extinction recall. Below, we demonstrate that SPS induces deficits in extinction recall under a number of circumstances (Figures 1 – 3). These findings will soon be submitted for publication. In all experiments, rats were first conditioned, extinguished, and then extinction recall tested.

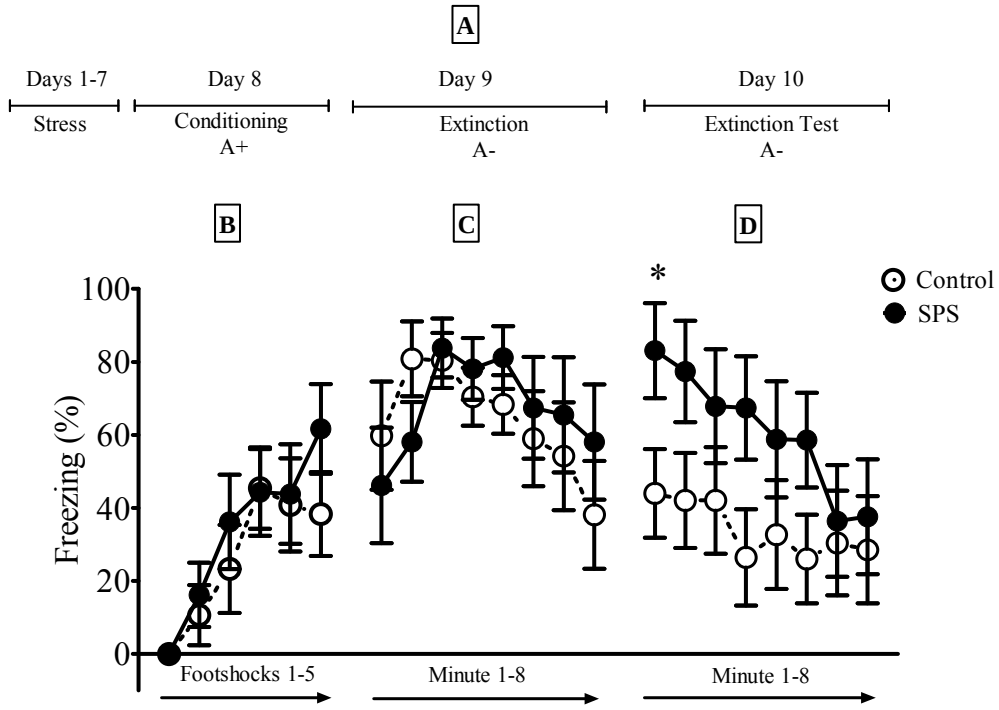

Figure 1. SPS induced deficits in contextual extinction retrieval. (A) Diagram illustrates experimental design used in this study. The two character (e.g. A+) symbol describes conditioning and extinction parameters. First letter denotes context and second character denotes the presence or absence of footshocks. (B) SPS had no effect on freezing during conditioning or (C) extinction, (D) but disrupted contextual extinction retrieval.

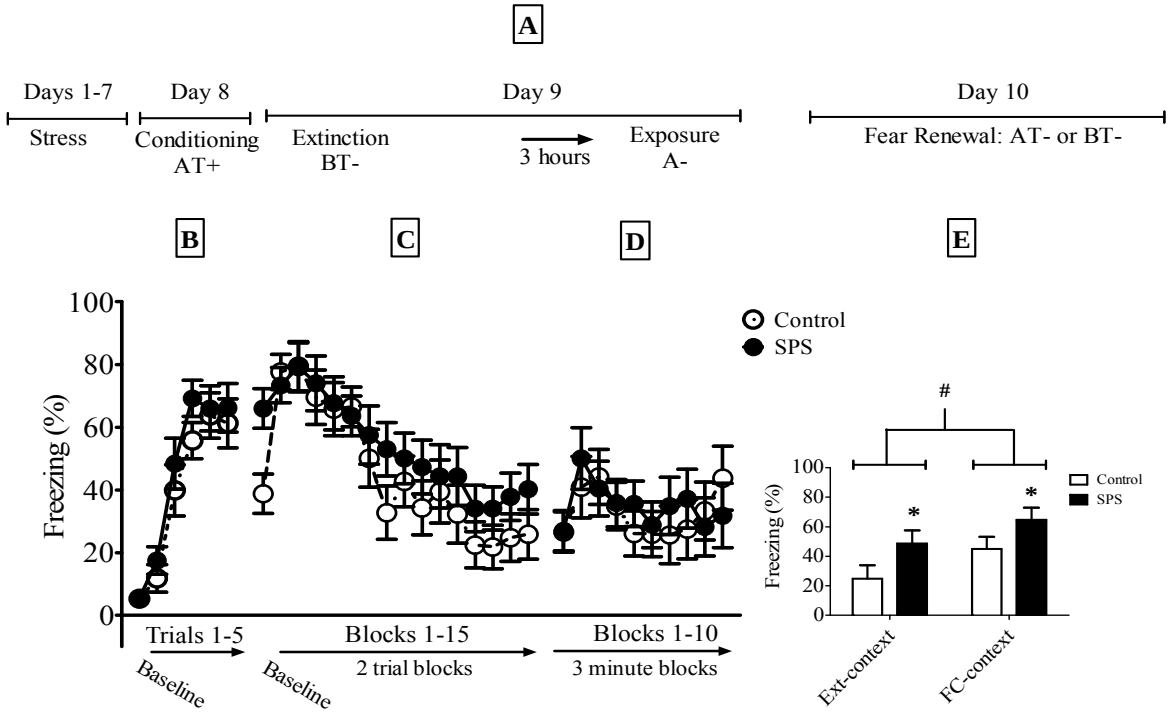

Figure 2. SPS disrupted cued extinction recall and enhanced fear renewal. (A) Illustrates the experimental design used in this study. T denotes tone presentation. (B) SPS had no effect on freezing during conditioning (C) extinction, or (D) re-exposure to the conditioning context. (E) SPS disrupted extinction recall irrespective of the context in which extinction was tested (i.e. disrupted extinction recall and enhanced fear renewal).

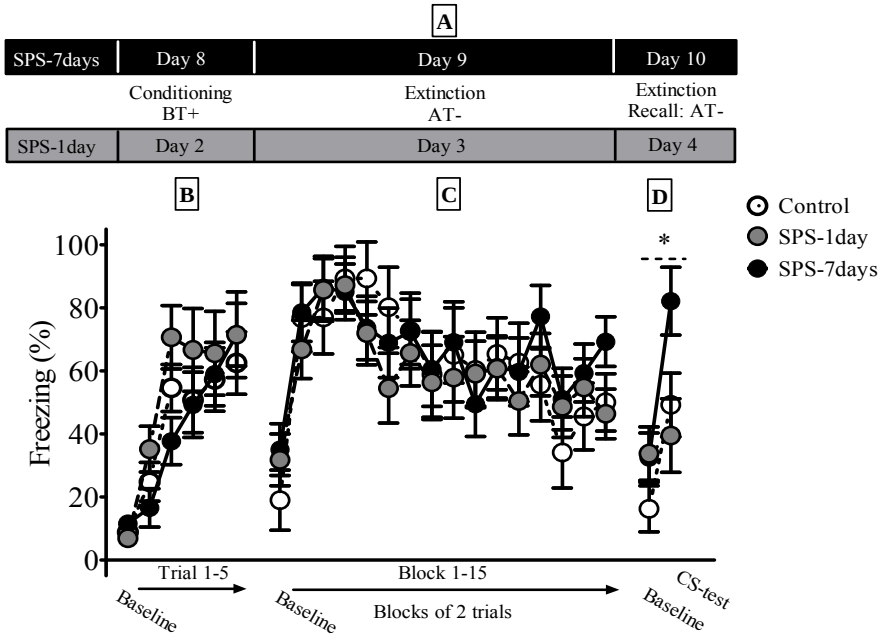

Figure 3. The effect of stress on extinction recall requires a post-stress incubation period. (A) Illustrates the experimental design used in this experiment. (B) Neither the SPS-1 day nor SPS-7 days rats displayed different freezing levels during conditioning or (C) extinction. (D) Extinction recall was impaired in the SPS-7 days group, but not SPS-1 day group.

After demonstrating conclusively that SPS disrupts extinction recall, we can now proceed with the experiments proposed in specific aims #1.

2) The protective effect of mother-pup social interactions attenuates some, but not all, SPS behavioral and physiological effects.

PTSD is characterized by deficit in social interactions both developmentally and as a result of symptoms development. In our grant proposal, we reported **interaction between SPS and social behavior** such that SPS reduced the number of social interactions in the social interaction test, and interpreted this to mean that SPS enhances anxiety from social encounters. We tested this phenomenon in a number of independent samples and did not replicate this specific finding (reported in the first annual DoD report). We studied however other developmental aspects of social interaction and SPS – mother-pup interaction, and demonstrated (see below) that this interaction indeed present – such that social interactions modulate SPS development. This allowed us to continue to pursue the question of target neurocircuitry involved in these interactions. The purpose of specific aim #2 is to, a) determine the importance of the IL and BLA in mediating social interactions and b) determine if these brain regions are critical for SPS changes in fear and anxiety stemming from social interactions. SPS has no effect on social interactions within the social interaction test, but we can still determine the importance of the IL and BLA in mediating anxiety behavior within the social interaction test. The results from these experiments will help us identify brain regions that may be of relevance to enhanced social avoidance observed in PTSD. As a result, we have modified hypotheses #2a and #2b (see below).

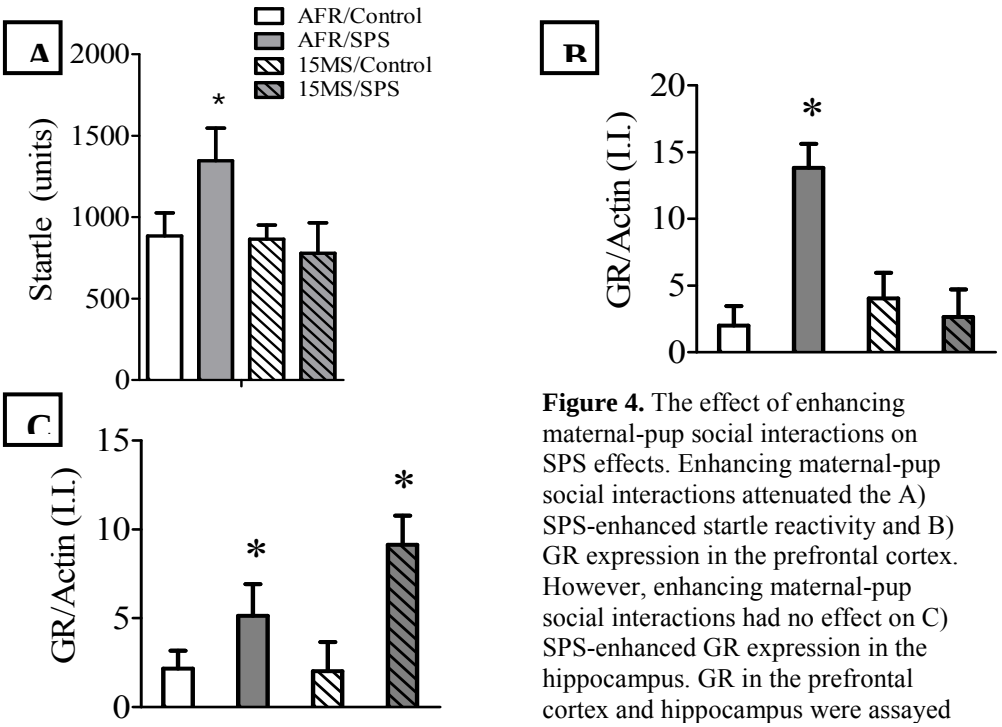

Figure 4. The effect of enhancing maternal-pup social interactions on SPS effects. Enhancing maternal-pup social interactions attenuated the A) SPS-enhanced startle reactivity and B) GR expression in the prefrontal cortex. However, enhancing maternal-pup social interactions had no effect on C) SPS-enhanced GR expression in the hippocampus. GR in the prefrontal cortex and hippocampus were assayed

The finding that SPS induces deficits in extinction recall, but not fear conditioning, suggests that SPS rats have deficits in regulating fear behavior and stress reactivity, but not necessarily exaggerated fear or stress responses. Based on this reasoning, we propose to

investigate whether SPS rats are resistant to the positive effects of social interactions (i.e. social buffering) in a number of different paradigms. This is now stated in hypothesis #2c and #2d. Indeed, we have already started and completed one experiment designed to test hypothesis #2c and #2d. In this experiment, mother-pup social interactions were enhanced by briefly separating pups from their mother over a three week period during the neonatal phase of development. Previous research has demonstrated that this enhances mother-pup social interactions (Cirulli, Capone et al. 2007) and reduces anxiety behavior and stress when pups become adults (Lehmann and Feldon 2000). We then subjected rats to either SPS or control procedures, and evaluated startle reactivity in all rats. After this, brains were extracted and glucocorticoid receptor (GR) expression assayed in the prefrontal cortex and hippocampus.

SPS enhanced startle reactivity and GR expression in the prefrontal cortex and hippocampus. Rats that had enhanced mother-pup social interactions when they were neonates were protected against the effects of SPS on startle reactivity and GR expression in the prefrontal cortex. However, SPS-induced changes in GR expression in the hippocampus were unaffected by enhanced mother-pup social interactions. These results are illustrated in Figure 4, and suggest social buffering during the neonatal phase of development can reduce some, but not all, SPS-induced effects. We will further explore hypothesis #2c using other experimental paradigms.

3) Unconditioned fear is enhanced in an appetitive context and is mediated by deactivation of the mPFC

In our grant proposal we presented preliminary data that suggested prior exposure to an appetitive context attenuates trimethylthiazoline (TMT)-induced freezing in control rats, but not SPS rats. We interpreted this preliminary finding to mean that freezing behavior is decreased in an appetitive context (i.e. defense behavior regulation) and this process is disrupted in SPS rats. We did not replicate these findings in subsequent studies, and came to the general conclusion that TMT-induced freezing is enhanced in an appetitive context and SPS has no effect on this enhancement (reported in the first DoD annual report). Three of these specific findings have relevance to PTSD. 1) The expression of fear can be modulated by the context in which fear is expressed. In human clinical practice it is recognized that the expression of fear is modulated by the context in which fear is evoked. Many animal reports have demonstrated that extinction is modulated by context, but this has not been demonstrated for unconditioned fear until now. 2) The concept that the IL region of the mPFC is critical for inhibition of unconditioned fear is held by most researchers, but an inhibitory role for the mPFC in unconditioned freezing (used to model fear and anxiety) has not been demonstrated until now. 3) mPFC, but not amygdala, neural activity seems critical for contextual modulating unconditioned fear. This suggests mPFC modulation of other caudal substrates (e.g. dorsal periacqueductal gray) may have a role than in modulating unconditioned fear. These experiments are briefly described below.

Summary of hypothesis #3a: In this experiment, we explored the effect of infralimbic cortex (IL) and prelimbic cortex (PL) inactivation on TMT-induced freezing. While there is a lot of research investigating the role of the mPFC in conditioned fear and extinction, the role of these brain regions in unconditioned fear requires further investigation. Rats were equipped with guide cannulas aimed at either the PL or IL. These regions were then temporarily inactivated prior to TMT-induced freezing and the effect of inactivation documented. Temporary inactivation of the PL enhanced predator odor-induced freezing. Temporary inactivation of the IL had no effect on freezing. This is illustrated in Figure 6.

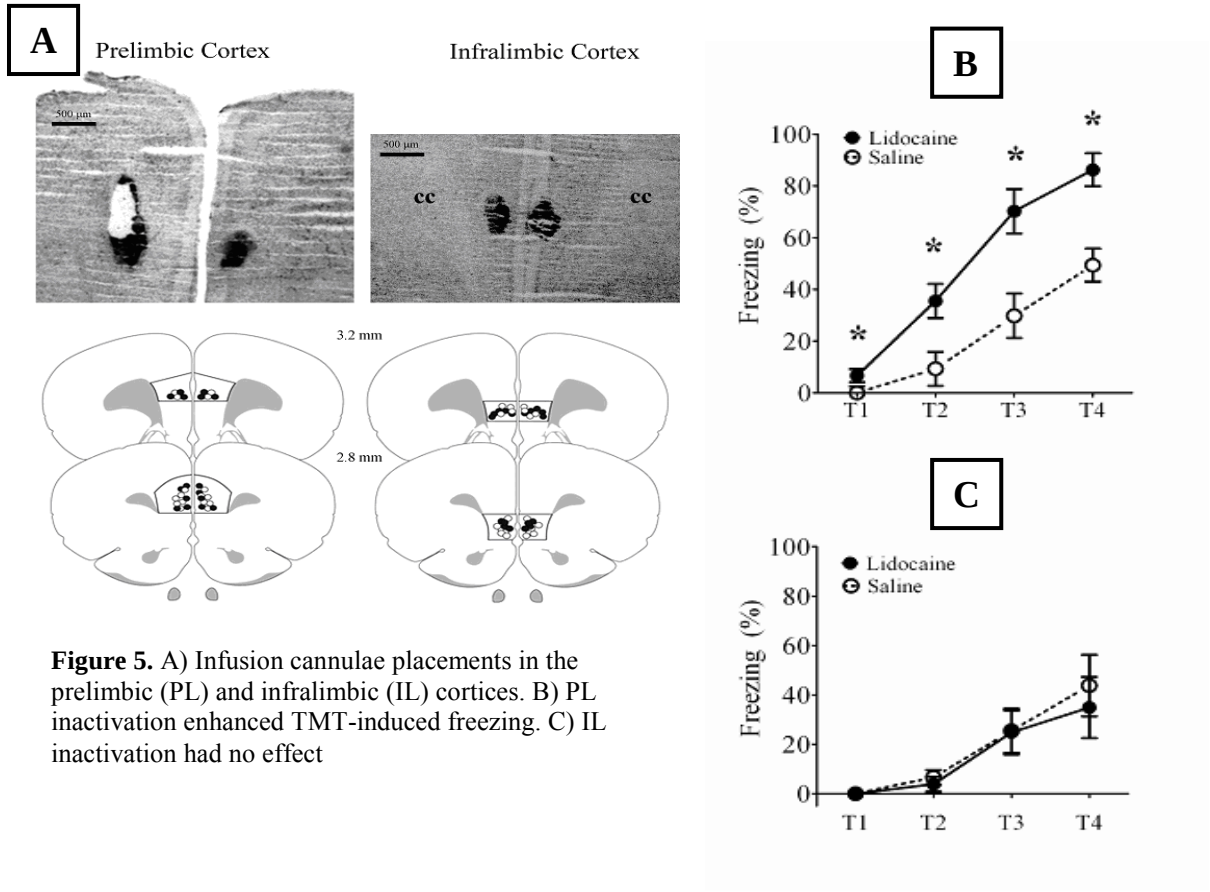

Figure 5. A) Infusion cannulae placements in the prelimbic (PL) and infralimbic (IL) cortices. B) PL inactivation enhanced TMT-induced freezing. C) IL inactivation had no effect

Summary of hypothesis #3b & c: Having demonstrated the PL inactivation enhances TMT-induced freezing, we wanted to see if enhanced TMT-induced freezing in an appetitive context results from deactivation of neural activity in the PL and enhanced neural activity in the amygdala. Rats were tested for TMT-induced freezing in a novel, familiar, or appetitive context. Thirty minutes after the start of the test rats were euthanized, brains extracted, and c-fos mRNA (used to measure neural activity) assayed in the mPFC and amygdala. As shown in Figure 6, TMT-induced freezing was enhanced in the appetitive context. This enhancement was accompanied by decreased neural activity in the PL and IL. However, no change in neural activity was observed in the amygdala. The results demonstrate that deactivation of the mPFC (specifically the PL) is a mechanism by which TMT-induced freezing is enhanced in an appetitive context. However, mPFC inhibition of amygdala activity does not appear critical for this effect, since changes in TMT-induced amygdala neural activity did not correspond to contextual modulation of TMT-induced freezing behavior (i.e. enhanced freezing in the appetitive context).

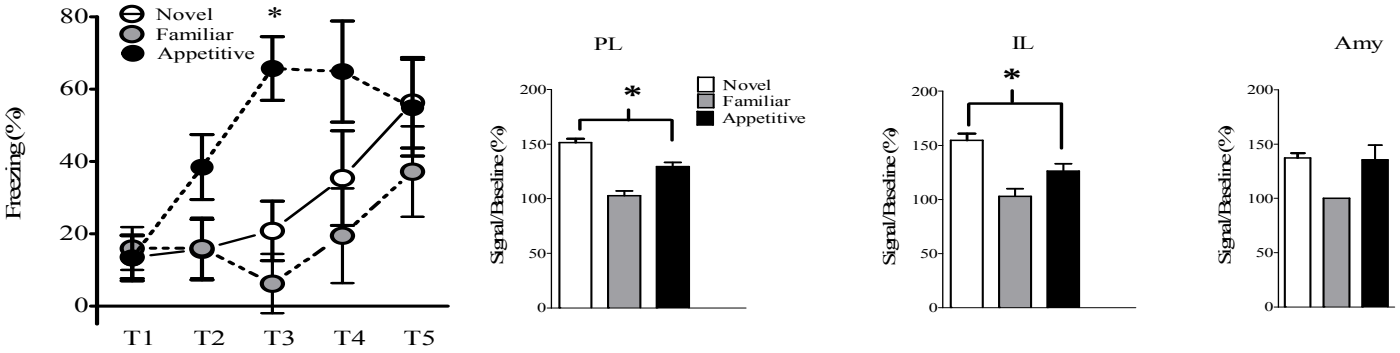

Figure 6. Effect of context on TMT-induced freezing and c-fos mRNA expression. (Left panel) TMT-induced freezing is enhanced in an appetitive context. (Second and third panels) This effect appears mediated by decreased neural activity in the PL and IL. Given that only temporary inactivation of the PL enhances TMT-induced freezing, it would appear that decreased neural activity in the PL underlies enhanced TMT-induced freezing in an appetitive context (Last panel) Amygdala neural activity does not drive contextual modulation of unconditioned freezing. C-fos mRNA intensity was scored and expressed as a percent change over baseline.

It is important to note that the initial intention of specific aim #3 was to evaluate the effect of SPS on defense behavior regulation. By using different extinction paradigms (e.g. extinction recall and fear renewal) we have demonstrated that SPS rats have difficulty in regulating conditioned fear behavior. We will still explore hypotheses #3b and #3d, using a variety of experimental methods to modulate the freezing response induced by TMT. If we are unsuccessful at demonstrating a modulated TMT-induced freezing response that is sensitive to SPS, we will use a combination of extinction recall and fear renewal paradigms to test these hypotheses.

4) Chronic antikindling drug administration attenuates SPS-induced deficits in extinction recall

In this experiment we evaluated the effect of SPS on extinction recall outside of the extinction context and determined if chronic administration of the antikindling drug Phenytoin can reverse these effects. This experiment was proposed in specific aim #4 (hypothesis #4b). SPS enhanced fear renewal without affecting fear conditioning or extinction. Systemic administration of phenytoin reversed this effect. This is shown in Figure 7.

5) SPS sensitizes LC activity

We believe that increased excitability of mPFC/amygdala activity in SPS rats is due to enhanced brain noradrenergic activity. In order to pursue this hypothesis, we investigated the effects of SPS on single unit activity in the locus coeruleus (LC, major source of norepinephrine in the brain). SPS and control rats were subjected to stereotaxic surgery and single unit activity was recorded from the LC of rats under general anesthesia. As demonstrated below, SPS attenuated baseline single unit LC activity, but enhanced evoked single unit activity (induced by pinching the paw of rats) in the LC. This is shown in Figure 8.

In another group of SPS and control rats we investigated whether SPS altered stress-induced upregulation of tyrosine hydroxylase (TH) mRNA in the LC. TH is the rate limiting enzyme in the norepinephrine biochemical cascade and changes in TH activity are correlated

with the electrophysiological response of LC neurons. SPS and control were restrained for 1.5 hours in order to investigate the effect of SPS on TH mRNA upregulation. Baseline TH mRNA levels were equivalent in SPS and control rats, but stressed-induced upregulation of TH mRNA was enhanced in SPS rats when compared to controls (Figure 9).

Taken together, these results demonstrate that SPS enhances electrophysiological and biochemical activity of LC neurons. We will continue to explore this line of research, as it relates to the research aims stated in specific aim #4.

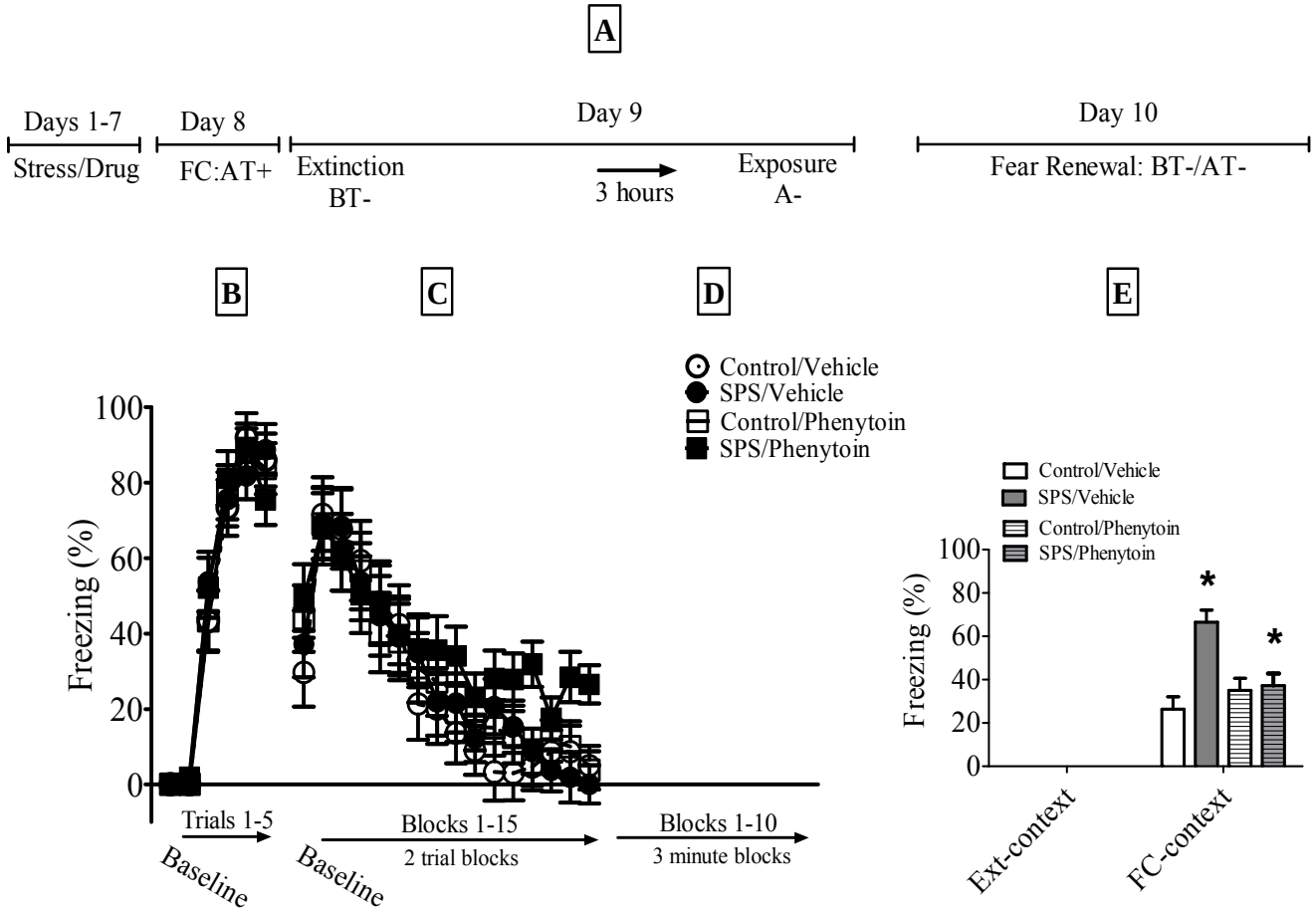

Figure 7. The effect of chronic antikindling drug administration on SPS-induced deficits in extinction recall. A) Experimental design. 40mg/kg of phenytoin or vehicle was subcutaneously administered to rats during the seven day quiescent period of SPS. Control rats received an identical drug treatment. Neither SPS nor chronic administration of phenytoin had any effect on B) fear conditioning or C) fear extinction. E) SPS enhanced fear renewal (or disrupted extinction recall) and this effect was blocked by chronic phenytoin administration. Chronic phenytoin administration had no effect on fear renewal in control rats. Data for D and extinction recall in the extinction context needs to be scored.

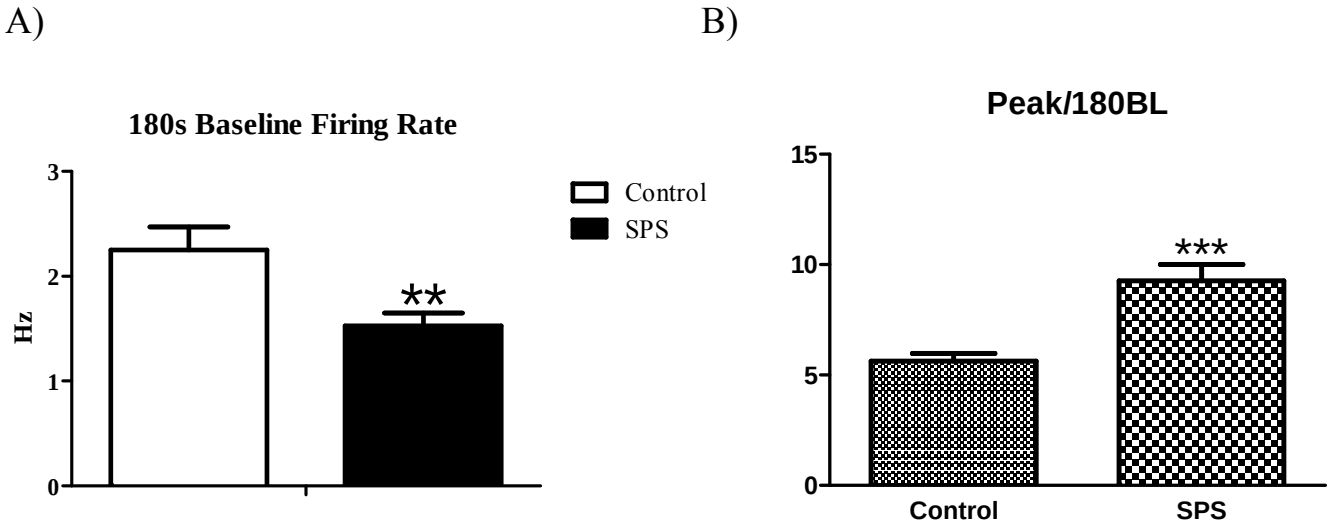

Figure 8. The effect of SPS on single unit activity. A) SPS attenuated the baseline firing rate of LC neurons, but B) enhanced stimulated LC single unit activity. Peak/180BL – peak response divided by 180 s of baseline

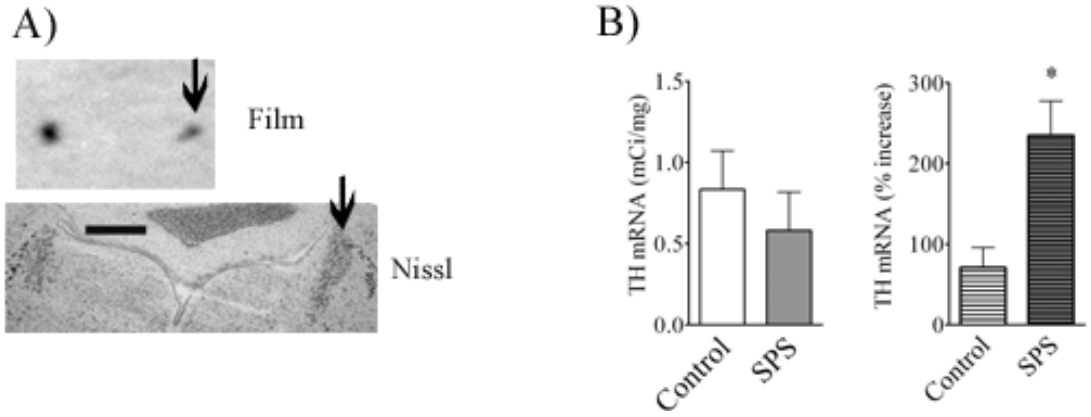

Figure 9. A) Top panel shows film from a typical in situ hybridization experiment. Dark spots correspond to TH mRNA activity in the LC. Bottom panel illustrates a Nissl stained section showing the location of the LC. B) (left panel) Baseline TH mRNA levels were not affected by SPS but (right panel) restraint stress-induced increases in TH mRNA was enhanced in SPS rats.

Reportable Outcomes

The research we have conducted within the last year has resulted in one journal manuscript publication, one journal article submission, and five poster presentations. These are listed below.

Journal articles

1) Knox, D., Perrine, S, George, S., Galloway, M., and Liberzon, I. (2010). Single Prolonged Stress Decreases Glutamate, Glutamine, and Creatine Concentrations In The Rat Medial Prefrontal Cortex. Neuroscience Letters, 480(1): 16 – 20. PMID: 20546834.

2) Fitzpatrick, C., Knox, D., Liberzon, I. (2011). Inactivation of the prelimbic cortex enhances freezing induced by trimethylthiazoline, a component of fox feces. Behavioural Brain Research: 22(1): 320 - 323. PMID:21420435

Poster presentations

1) Fitzpatrick C, Knox D, Liberzon I. Psychiatry, Univ. of Michigan, ANN ARBOR, MI. Society for Neuroscience, November 2010, San Diego. Temporary inactivation of the prelimbic but not infralimbic cortex enhances freezing induced by trimethylthiazoline, a component of fox feces

2) Knox D, Liberzon I. Psychiatry, Univ. of Michigan, ANN ARBOR, MI. Society for Neuroscience, November 2010, San Diego. A comparison of the effects of TMT exposure and restraint stress on HPA axis function and noradrenergic systems.

3) Stout S, Tan M, George S, Knox D, Stern ER, Liberzon I. Dept Psychiatry, Univ. Michigan, ANN ARBOR, MI. Society for Neuroscience, November 2010, San Diego. The effects of early life and adult stress on HPA-axis function and anxiety-like behavior.

4) George, SA., Knox, D., Fitzpatrick, C., Maren, S., Abelson, JL. Liberzon, I. Biological Psychiatry Annual Meeting, May 2010, New Orleans. The effect of Single Prolonged Stress, a rodent model of PTSD, on extinction recall and reinstatement.

5) George, SA., Knox, D., Khan, S., Maren, S., Liberzon, I.. Anxiety Disorders of America Association, March 2010, Baltimore. The effect of Single Prolonged Stress, a rodent model of PTSD, on fear conditioning, extinction and extinction recall.

6) Knox, D., George, SA., Khan, S., Maren, S., Liberzon, I. American College of Neuropsychopharmacology Annual Meeting, Dec 2009, Hollywood, FL. The effect of Single Prolonged Stress, a rodent model of PTSD, on unconditioned anxiety, fear conditioning, extinction and extinction recall.

Conclusions

Based on the results of this research program we are able to conclude that 1) SPS decreases excitatory tone in the mPFC, enhances fear recovery and induces deficits in extinction recall without affecting fear conditioning or consistently affecting fear extinction, augments the expression of GR in the prefrontal cortex and hippocampus, and sensitizes LC activity. Furthermore some of the effects of SPS can be attenuated by increasing mother-pup social interactions and chronic antikindling drug administration. We also demonstrated a number of novel findings not necessarily related to the initial goals of the grant, but nevertheless important to understanding the etiology of PTSD. These are 1) the PL is critical for inhibition of unconditioned fear, 2) TMT-induced freezing is enhanced in an appetitive context and this is mediated by mPFC deactivation.

In general we have demonstrated that SPS disrupts mPFC function and enhances glucocorticoid receptor expression in the hippocampus and prefrontal cortex. What remains to be accomplished in the remainder of this research program is to link these findings with SPS-induced changes in extinction recall and enhanced fear recovery.

References

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Knapska, E. and S. Maren (2009). "Reciprocal patterns of c-Fos expression in the medial prefrontal cortex and amygdala after extinction and renewal of conditioned fear." Learn Mem **16**(8): 486-493.

Knox, D., S. A. Perrine, et al. (2010). "Single prolonged stress decreases glutamate, glutamine, and creatine concentrations in the rat medial prefrontal cortex." Neurosci Lett **480**(1): 16-20.

Lehmann, J. and J. Feldon (2000). "Long-term biobehavioral effects of maternal separation in the rat: consistent or confusing?" Reviews in the neurosciences **11**(4): 383-408.

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Supporting Data

Most of the supporting data is contained in the Body Key Accomplishments Section. We use this section to describe the results of experiments we conducted that did not yield significant findings, but demonstrate the productivity of the lab in the second year of this research program. As such, these experiments support the assertion that we are continuously trying to address the specific aims of the research program by adopting a number of creative experimental designs. As is the case with research, changes in experimental design do not always yield significant findings.

1) The Effect Of SPS On Conditioned Discrimination

Rationale: Animal studies of fear conditioning and extinction have provided great insights into human anxiety disorders and how psychopathological avoidance may be overcome. Avoidance in both animals and humans, however, is determined by more than just fear; reward-motivated approach behavior provides a countervailing force and avoidance occurs only when fear overcomes approach tendencies. Few studies to date have attempted to examine both sides of this equation. In this study, we examined the effect of conditioned fear on the inhibition of rewarded behavior in an effort to develop an animal model that will allow study of approach-avoidance gradients and their neural underpinnings. We specifically tested the impact of trauma/stress on the learning of fear-based inhibition of rewarded responding.
Methods: Twenty one rats were trained to press a lever for a food reward and were then subjected to Single Prolonged Stress (SPS) or a control procedure. We then examined the effect of SPS on the acquisition of a Conditioned Emotional Response (CER). While animals were pressing a lever for food, a conditioned stimulus (CS+) (light or tone) was paired with a mild foot-shock, while a CS- had no consequence. The ability of the conditioned stimuli to interfere with lever pressing was measured on four consecutive days. Following this, extinction training occurred, and animals' extinction of the conditioned response was examined.
Results: SPS and control rats did not differ on their acquisition of a conditioned emotional response, demonstrating comparable levels of conditioned suppression to the CS+ [$F(1,19) = .001$, $p = .98$] and CS- [$F(1,19) = 3.66$, $p = .071$]. Similarly, all rats extinguished their conditioned response to the CS+ equally during extinction training [$F(1,19) = .53$, $p = .48$] and showed no differences in their response to CS- [$F(1,19) = .013$, $p = .91$].
Relevance to overall projects: Currently, not relevant to the grant.

7) The Effect Of SPS On Social Buffering

Rationale: Social buffering refers to attenuated expression of fear conditioned freezing when two rats are simultaneously evaluated for fear conditioned freezing. We used this experiment to compensate for the null effects regarding SPS and social interactions, which we reported in our previous annual report.
Method: Sixteen rats in total were used for this behavioral test. Rats were assigned to SPS or control conditions. Fear conditioning was conducted in a manner identical to that already described. During fear extinction, both partner and experimental rats were placed into the fear conditioning context and tones were presented. Freezing to each tone was then documented. Three rats were excluded from statistical analysis.
Results: Both SPS and control rats demonstrated robust fear conditioned freezing. There was a paradoxical stress effect with SPS rats exhibiting lower freezing in the presence of a partner rat than control rats [$F(1,10) = 10.3$, $p = 0.009$]. Given that it did not appear any buffering took

place in the control rats and the variability in the data, it is difficult to interpret this data set.
Relevance to overall project: Currently not relevant to aims of the grant.

Appendix I

Previous Statement of Work

Institution name: University of Michigan Medical School, 1500 Medical Center Dr., Ann Arbor, MI 48109;
Ann Arbor VA Healthcare Systems, 2215 Fuller Road, Ann Arbor, MI 48105.

Personnel and effort: Israel Liberzon MD, principal investigator (1.00 cal. months): Will oversee the whole project to ensure that all experiments are conducted in a timely fashion, consistent with the proposal, and in accordance with institutional guidelines and the principles of ethical use of animals in research. He will assure that all experiments are conducted with appropriate experimental rigor, and that data is published after completion of experiments. Dr. Liberzon will have primary responsibility for the interpretation of data and will primarily be responsible for writing research documents generated by this research proposal. Samir Khan PhD, co-investigator (6.00 cal. months): Will perform the bulk of experiments, data analysis, and research document preparation. Dayan Knox PhD, co-investigator (6.00 cal. months): Will perform the bulk of the experiments, data analysis, and research document preparation. Tony King PhD, co-investigator (0.60 cal. months): Will assist in developing protocols for protein and mRNA assays. Wayne Aldridge PhD, co-investigator (0.24 cal. months): will assist with electrophysiological experiments. TBA, research assistant (.60 cal. months years 1 & 2 and 6.00 cal. months years 3 & 4): will assist Drs. Khan and Knox in conducting all experiments. Stephen Maren PhD, consultant: will assist in electrophysiological and fear conditioning experiments.

General Tasks (6/1/08 – 6/31/08): 1) All equipment will be purchased within the first month of the release of funds to the University of Michigan. 2) All behavioral equipment will be purchased within the first month of the release of funds. 3) Electrophysiology equipment and molecular biology equipment required for testing hypotheses in specific aim 1 will be purchased within the first month of the release of funds to the University of Michigan.

Experimental Animals: Male Sprague Dawley rats will be used as subjects in all experiments. We anticipate an average of 15 rats/independent sample for all experiments in order to obtain statistical significance. However, this number will be adjusted from experiment to experiment used to test each hypothesis based on the difficulty of the experiments proposed, and the need for extra rats in the event that we are required to adjust the methods to deal with potential problems that may arise. In total we request 1,466 rats to complete the research proposal.

Animal protocol: All experiments will be staggered and a single animal protocol that includes all proposed experiments will be written in order to facilitate smooth transition from experiment to experiment. This protocol
will be written and submitted to the Veteran Affairs Institutional Animal Care Usage Committee within a month of notification that the University of Michigan has received the Intramural research award.

Proposed experiments: All experiments will be conducted between 6/1/08 – 5/31/12. Below we detail the experiments that we propose to conduct as they relate to each specific aim, and give time lines for the completion of these experiments. For all specific aims, the following sequence of tasks will be adhered to in order to allow for the execution of proper experimental protocols. A combination of temporary inactivation, single unit electrophysiology, pharmacological intervention, and molecular biology techniques will be used to test hypotheses.

Tasks:

1) Purchase supplies for experiment (e.g. cannulas, electrodes, infusers, antibodies)
2) Purchase animals, perform SPS and/or drug procedures, and/or surgical procedures
3) Perform behavioral protocols (e.g. fear conditioning, extinction, social interaction)
4) Sacrifice rats and prepare tissue for histology or assay (e.g. Nissl stain, Western blot)
5) Perform assay (e.g. mRNA, protein), histology, or complete electrophysiology data analysis (within two weeks of termination of a particular experiment)
6) Repeat steps 2-5 at least once in order to replicate findings.

Specific Aim 1): ***Examine the roles of altered mPFC function and expression of brain glucocorticoid receptors in the development of SPS induced extinction deficits (as a model of PTSD intrusive symptom cluster).***	**No. of Animals**	**dates**
Hypothesis #1a: Temporary inactivation of the IL will lead to deficits in fear extinction in control rats, and this effect will be attenuated in SPS exposed rats. Methods used – cannula infusion, single prolonged stress, and fear conditioning	75	6/1/08 – 8/31/08
Hypothesis #1b: SPS exposure induces extinction deficits by altering neural activity in the IL. Methods used - Single unit electrophysiology, single prolonged stress and fear conditioning	45	9/1/08 – 1/31/09
Hypothesis 1c: SPS exposure induces extinction deficits by altering brain glucocorticoid receptor expression. Methods used - Western Blotting, in situ hybridization, reverse transcriptase polymerase chain reaction, and single prolonged stress.	90	2/1/09 – 5/31/09

Specific Aim 2): ***Examine the roles of altered mPFC/amygdala function and of HPA/glucocorticoid receptor function in the development of SPS induced avoidance of social interactions (as a model of PTSD social avoidance cluster)***	**Number of Animals**	**dates**
Hypothesis #2a: Temporary inactivation of the IL will lead to avoidance of social interactions, similar to that seen in SPS animals. Methods used – cannula infusion, single prolonged stress, and social interaction test.	75	6/1/09 – 8/31/09
Hypothesis #2b: Temporary inactivation of the BLA will increase social interactions. Methods used – cannula infusion, single prolonged stress, and social interaction test.	75	91/1/09 – 11/30/09
Hypothesis #2c: SPS exposure induces avoidance of social interactions by altering neural activity in the IL/BLA. Methods	45	12/1/09 – 4/30/10

used - Single unit electrophysiology, single prolonged stress and social interaction		
Hypothesis 2d: SPS induced changes in avoidance of social interactions are mediated, in part, by changes in HPA axis/glucocorticoid function. Methods used - Western Blotting, in situ hybridization, reverse transcriptase polymerase chain reaction, and single prolonged stress.	90	5/1/10 – 7/31/10

Specific Aim 3): ***Examine the role of altered mPFC/amygdala function and of altered HPA/glucocorticoid function in the development of SPS induced deficits in defensive behavior regulation (as a model of emotional dysfunction in PTSD).***	**Number of Animals**	**dates**
Hypothesis #3a: Temporary inactivation of the IL will lead to deficits in defense behavior regulation similar to that observed in SPS animals. Methods used – cannula infusion, single prolonged stress, and predator induced freezing	75	8/1/10 – 10/31/10
Hypothesis #3b: Temporary inactivation of the BLA will attenuate the defense behavior regulation deficit induced by SPS. Methods used – cannula infusion, single prolonged stress, and predator induced freezing	75	11/1/10 – 01/31/11
Hypothesis #3c: SPS exposure induces deficits in regulation of defensive behavior by altering neural activity in the IL/BLA. Single unit electrophysiology, single prolonged stress and predator induced freezing	45	2/1/11 – 4/30/11
Hypothesis #3d: SPS induced changes in defense behavior regulation are mediated, in part, by altered HPA axis/glucocorticoid function. Methods used - Western Blotting, in situ hybridization, reverse transcriptase polymerase chain reaction, and single prolonged stress.	90	5/1/11- 7/31/11

Specific Aim 4): *Examine the ability of SSRI and antikindling drug administration to alleviate SPS induced extinction deficit, social avoidance, and defensive behavior regulation deficits; and the role of mPFC/amygdala activity and HPA/glucocorticoid function in this process.*	**Number of Animals**	**dates**
Hypothesis #4a. SSRI administration will attenuate extinction deficits and social avoidance by altering IL/BLA electrophysiological activity in SPS animals and by reversing changes in glucocorticoid receptor and mRNA expression in the prefrontal cortex, hippocampus, and hypothalamus. Methods used - Single unit electrophysiology, single prolonged stress, fear conditioning, social interaction, predator induced freezing, western blotting, in situ hybridization, and reverse transcriptase polymerase chain reaction.	359	8/1/11 – 1/31/12
Hypothesis# 4b. Antikindling/mood stabilizer administration will	327	2/1/12 – 5/31/12

attenuate SPS induced defensive behavior regulation deficits by modulating neural activity in the IL/BLA. (two different drugs will be tested). Methods used - Single unit electrophysiology, single prolonged stress, and predator induced freezing.		

Revised Statement of work

Institution name: University of Michigan Medical School, 1500 Medical Center Dr., Ann Arbor, MI 48109;

Ann Arbor VA Healthcare Systems, 2215 Fuller Road, Ann Arbor, MI 48105.

Personnel and effort: Israel Liberzon MD, principal investigator (1.00 cal. months): Will oversee the whole project to ensure that all experiments are conducted in a timely fashion, consistent with the proposal, and in accordance with institutional guidelines and the principles of ethical use of animals in research. He will assure that all experiments are conducted with appropriate experimental rigor, and that data is published after completion of experiments. Dr. Liberzon will have primary responsibility for the interpretation of data and will primarily be responsible for writing research documents generated by this research proposal. Samir Khan PhD, co-investigator (6.00 cal. months): Will perform the bulk of experiments, data analysis, and research document preparation. Dayan Knox PhD, co-investigator (6.00 cal. months): Will perform the bulk of the experiments, data analysis, and research document preparation. Tony King PhD, co-investigator (0.60 cal. months): Will assist in developing protocols for protein and mRNA assays. Wayne Aldridge PhD, co-investigator (0.24 cal. months): will assist with electrophysiological experiments. TBA, research assistant (.60 cal. months years 1 & 2 and 6.00 cal. months years 3 & 4): will assist Drs. Khan and Knox in conducting all experiments. Stephen Maren PhD, consultant: will assist in electrophysiological and fear conditioning experiments.

General Tasks (6/1/08 – 6/31/08): 1) All equipment will be purchased within the first month of the release of funds to the University of Michigan. 2) All behavioral equipment will be purchased within the first month of the release of funds. 3) Electrophysiology equipment and molecular biology equipment required for testing hypotheses in specific aim 1 will be purchased within the first month of the release of funds to the University of Michigan.

Experimental Animals: Male Sprague Dawley rats will be used as subjects in all experiments. We anticipate an average of 15 rats/independent sample for all experiments in order to obtain statistical significance. However, this number will be adjusted from experiment to experiment used to test each hypothesis based on the difficulty of the experiments proposed, and the need for extra rats in the event that we are required to adjust the methods to deal with potential problems that may arise. In total we request 1,466 rats to complete the research proposal.

Animal protocol: All experiments will be staggered and a single animal protocol that includes all proposed experiments will be written in order to facilitate smooth transition from experiment to experiment. This protocol

will be written and submitted to the Veteran Affairs Institutional Animal Care Usage Committee within a month of notification that the University of Michigan has received the Intramural research award.

Proposed experiments: All experiments will be conducted between 6/1/08 – 5/31/12. Below we detail the experiments that we propose to conduct as they relate to each specific aim, and give time lines for the completion of these experiments. For all specific aims, the following sequence of tasks will be adhered to in order to allow for the execution of proper experimental protocols. A combination of temporary inactivation, single unit electrophysiology, pharmacological intervention, and molecular biology techniques will be used to test the hypotheses.

Tasks:

1) Purchase supplies for experiment (e.g. cannulas, electrodes, infusers, antibodies)
2) Purchase animals, perform SPS and/or drug procedures, and/or surgical procedures
3) Perform behavioral protocols (e.g. fear conditioning, extinction, social interaction)
4) Sacrifice rats and prepare tissue for histology or assay (e.g. Nissl stain, Western blot)
5) Perform assay (e.g. mRNA, protein), histology, or complete electrophysiology data analysis (within two weeks of termination of a particular experiment)
6) Repeat steps 2-5 at least once in order to replicate findings.

Specific Aim 1): ***Examine the roles of altered mPFC function and expression of brain glucocorticoid receptors in the development of SPS induced extinction deficits (as a model of PTSD intrusive symptom cluster).***	**No. of Animals**	**dates**
Hypothesis #1a: Temporary inactivation of the IL will lead to deficits in fear extinction in control rats, and this effect will be attenuated in SPS exposed rats. Methods used – cannula infusion, single prolonged stress, and fear conditioning	75	6/1/11 – 10/30/11
Hypothesis #1b: SPS exposure induces extinction deficits by altering neural activity in the IL. Methods used - Single unit electrophysiology, single prolonged stress and fear conditioning	45	1/11/11 – 5/31/11
Hypothesis 1c: SPS exposure induces extinction deficits by altering brain glucocorticoid receptor expression. Methods used - Western Blotting, in situ hybridization, reverse transcriptase polymerase chain reaction, and single prolonged stress.	90	8/1/10 – 5/31/11

Specific Aim 2): ***Examine the roles of altered mPFC/amygdala function in social interactions (as a model of PTSD social avoidance cluster), determine if social interactions can modulate SPS-induced changes in fear behaviors and HPA axis responses, and determine the importance of mPFC/amygdala activity and HPA/glucocorticoid receptor function in these SPS effects.***	**Number of Animals**	**dates**

Hypothesis #2a: Temporary inactivation of the IL will lead to avoidance of social interactions. Methods used – cannula infusion, in situ hybridization, and social interaction test.	75	6/15/11 – 8/31/11
Hypothesis #2b: Temporary inactivation of the BLA will increase social interactions. Methods used – cannula infusion, in situ hybridization, and social interaction test.	75	6/15/11 – 9/30/11
Hypothesis #2c: Social buffering will not attenuate SPS-induced changes fear and stress reactivity. Methods used - Single prolonged stress, brief maternal separation, western blot electrophoresis, startle reactivity, fear conditioning	45	12/1/09 – 11/30/11 (partially completed)
Hypothesis #2d: Resistance to the beneficial effects of social buffering in SPS rats are due to aberrant neural activity in mPFC/amygdala circuits, and SPS-induced changes in the HPA axis. Methods used - Single prolonged stress, western blot electrophoresis, fear conditioning, in situ hybridization	90	10/1/10 – 8/31/11 (partially completed)

Specific Aim 3): ***Examine the role of altered mPFC/amygdala function and of altered HPA/glucocorticoid function in TMT-induced freezing, and determine if SPS disrupts social buffering of TMT-induced responses (as a model of emotional dysfunction in PTSD).***	**Number of Animals**	**dates**
Hypothesis #3a: Temporary inactivation of the IL will lead to deficits in defense behavior regulation similar to that observed in SPS animals. Methods used – cannula infusion, single prolonged stress, and predator induced freezing	75	8/1/10 – 10/31/10 (completed)
Hypothesis #3b: Temporary inactivation of the BLA will attenuate the defense behavior regulation deficit induced by SPS. Methods used – cannula infusion, single prolonged stress, and predator induced freezing	75	11/1/10 – 01/31/11
Hypothesis #3c: SPS exposure induces deficits in regulation of defensive behavior by altering neural activity in the IL/BLA. C-fos in situ hybridization, single prolonged stress and predator induced freezing	45	9/1/11- 12/15/11 (completed)
Hypothesis #3d: SPS induced changes in defense behavior regulation are mediated, in part, by altered HPA axis/glucocorticoid function. Methods used - Western Blotting, in situ hybridization, reverse transcriptase polymerase chain reaction, and single prolonged stress.	90	5/1/11- 7/31/11

Specific Aim 4): *Examine the ability of SSRI and*	**Number of**	**dates**

antikindling drug administration to alleviate SPS induced extinction deficit and social buffering deficit; and the role of mPFC/amygdala activity, and HPA/glucocorticoid function in these processes.	**Animals**	
Hypothesis #4a. SSRI administration will attenuate SPS-induced extinction deficits and social buffering deficits by altering IL/BLA electrophysiological activity in SPS animals and by reversing changes in glucocorticoid receptor and mRNA expression in the prefrontal cortex, hippocampus, and hypothalamus. Methods used - Single unit electrophysiology, single prolonged stress, fear conditioning, social interaction, predator induced freezing, western blotting, in situ hybridization, and reverse transcriptase polymerase chain reaction.	359	8/1/11 – 1/31/12
Hypothesis# 4b. Antikindling/mood stabilizer administration will attenuate SPS induced defensive behavior regulation deficits by modulating neural activity in the IL/BLA. (two different drugs will be tested). Methods used - Single unit electrophysiology, single prolonged stress, and predator induced freezing.	327	2/1/12 – 5/31/12 (partially completed)