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| 13. SUPPLEMENTARY NOTES                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |                  |                          |                                      |                                                                    |                                            |
| 14. ABSTRACT<br>Sleep disturbances are an important pathway by which the negative effects of trauma exposure lead to PTSD and other psychological difficulties. Because it is difficult to test and control the effects of trauma exposure in humans, we have developed a novel mouse model of PTSD that is based on well-established paradigms of fear conditioning (FC). We specifically developed a conditioning stimulus of mild transient hypercapnia that we proposed could be used for re-exposure during periods of sleep. We have initially validated our model by showing that mice exhibit a marked bradycardia and changes in EMG activity with exposure to hypercapnia prior to foot shock (conditioned stimulus). We have gone on to show that the physiologic responses to the conditioning stimulus are dependent on genetic background. We have also been able to successfully re-expose conditioned animals to hypercapnia during sleep and again have shown genetic differences exist in the hyperarousal state that develops with re-exposure. These initial studies both validate our model and demonstrate that re-exposure of a conditioning stimulus during the unconscious state can produce or exacerbate and underlying state of hyperarousability. |                  |                          |                                      |                                                                    |                                            |
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# Sleep resilience, comorbid anxiety, and treatment in a murine model of PTSD

W81XWH-11-2-0060

## Annual Technical Progress Report

Progress Period: December 31, 2011 to December 31, 2012

### I. INTRODUCTION

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PTSD is a prevalent psychiatric disorder characterized by intrusive thoughts and images during wake and sleep, hyperarousal, and avoidance of trauma reminders persisting more than one month after trauma exposure. Recent estimates suggest that almost 20% of military personnel who serve in current conflicts meet PTSD diagnostic criteria. PTSD is associated with considerable health care utilization and costs, and psychiatric comorbidity is the norm rather than the exception in PTSD.

Sleep disturbances are an important pathway by which the negative effects of trauma exposure lead to PTSD and other psychological difficulties, and that protecting military personnel and civilians from the negative effects of trauma exposure may involve strategies to promote and protect consolidated sleep. Because it is difficult to test and control the effects of trauma exposure in humans, we will test our hypotheses by using a new mouse model of PTSD that is based on a well-established model of fear conditioning (FC).

Our overarching objective is to use our newly developed murine model of fear conditioning (FC) to (1) study physiological markers of sleep resilience to PTSD-like symptoms and (2) examine the role of anxiety and the serotonergic and sleep-related pathways that underly PTSD-like syndromes. The overarching hypothesis is that decreasing sleep resilience in susceptible individuals will accelerate and promote acquisition of FC, whereas strengthening serotonergic activity and state-dependent re-exposure to the conditioned stimulus will promote fear extinction (FE). We propose four specific aims that examine the role of sleep resilience and co-morbid anxiety on FC and FE (Aims 1 and 2). We will next examine the role of the serotonin 5-HT<sub>1A</sub> pathway in modulating FC and FE and the potential of pharmacologic and behavioral interventions to impede or accelerate FE (Aims 3 & 4). The stated specific aims are as follows:

### II. BODY

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**Research accomplishments associated with each task outlined in the approved Statement of Work.** The tasks and timeline initially proposed and approved in the approved Statement of Work are provided below. Progress and outcomes on each of the tasks listed are detailed for this review period.

**Task 1: Update all necessary approvals, order start-up supplies and materials, calibrate instrumentation and data acquisition and management, and update manuals of operations (Months 1-4)**

PROGRESS: All materials are in hand and all instrumentation and data acquisition equipment is in place and operational. Our initial IACUC protocol (#0806601) for work related to the murine model of PTSD underwent its three year renewal at the time of the last progress report (#1106265-1). The IACUC was subsequently re-approved on June 8, 2012 and is attached below as an appendix item.

## Task 2: Obtain USAMRMC ACURO approval

**PROGRESS:** The University of Pittsburgh IACUC protocol #1106265-1 was reviewed by the USAMRMC and approval notification received on November 18, 2011.

## Task 3: Hire and train new postdoctoral fellow.

**PROGRESS:** Dr. Angela McDowell, leading the data collection efforts has been with us for 20 months now and is performing exceptionally well. She is a dedicated and skilled scientist with a strong background in fear conditioning and sleep. Overall, the team that includes staff and Co-PI, Dr. Anne Germain, is working together in a highly productive manner and meeting regularly to track milestones and plan upcoming protocols. Dr. McDowell is committed to research in the area of PTSD and sleep and is working towards submitting a K-career development award to the NIH in the latter part of 2013.

## Task 4 (Specific Aim 1): Examine the impact of sleep disruption on fear learning and extinction in a novel, physiologically-validated murine FC model of PTSD. (Months 5 to 18)

**PROGRESS:** We have submitted a manuscript for publication that outlines our novel physiologically validated model of FC that is currently under review. The manuscript is entitled 'Mild transient hypercapnia as a novel fear conditioning stimulus allowing re-exposure during sleep,' authored by Angela McDowell, Ashlee Filippone, Alex Balbir, Anne Germain and Chris O'Donnell.

The key findings of the manuscript are detailed briefly below.

**RATIONALE:** Fear-conditioning paradigms in rodents are used to investigate causal mechanisms of fear acquisition and the relationship between sleep and post-traumatic behaviors. We developed a novel conditioning stimulus (CS) that evoked fear and was subsequently used for re-exposure during sleep.

**METHODS:** Adult male FVB/nJ mice were implanted with EEG and EMG electrodes and femoral artery catheters. *Protocol A:* Physiological responses to the novel CS (mild transient hypercapnia, mthC; 3.0% CO<sub>2</sub>; n = 17) + footshock (FS) were compared to tone (T) + FS (n = 18); control groups of T alone (n = 17) and mthC alone (n=10) were included. *Protocol B:* In a second proof of principle study animals were fear-conditioned to mthC + FS and re-exposed during sleep to mthC or air (control).

## RESULTS:

### Protocol A

#### Heart rate (HR)

Figure 2 shows the changes in HR during the presentation of the CS, as well as the changes occurring post unconditioned stimulus (UCS) for all four groups. There was an overall significant effect of group during CS presentation ( $F = 25.36$ ,  $p < 0.01$ ) and UCS presentation ( $F = 18.75$ ,  $p < 0.001$ ). When mthC

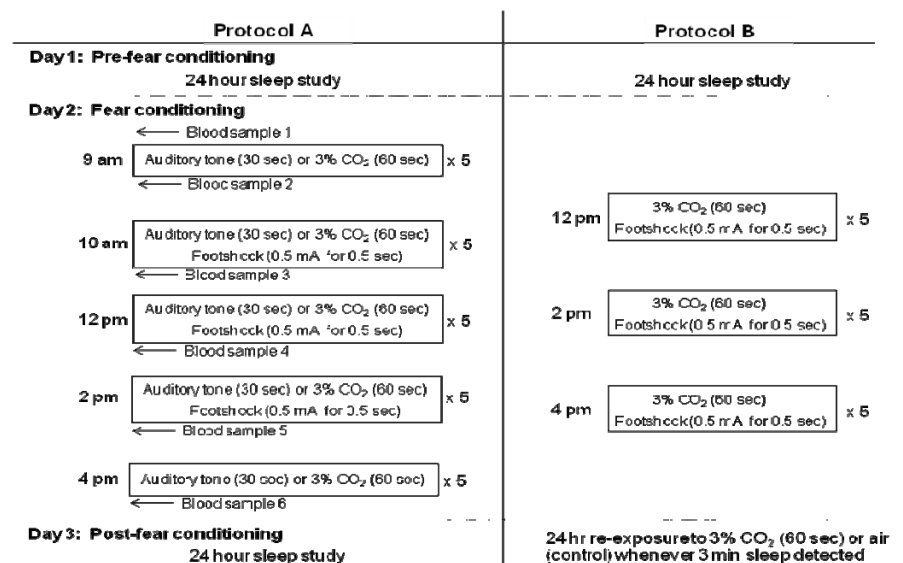


Figure 1: Shows the three day protocols for the two protocols.

was paired with footshock it induced a marked bradycardia that increased in magnitude across series, with a mean value of  $-31 \pm 4$  bpm and a maximum value above 40 bpm (Figure 2A, striped bars).

Footshock induced a tachycardic response with a mean response of  $44 \pm 7$  bpm (Figure 2A, black bars). Notably, the magnitude of the bradycardia was sustained ( $> 40$  bpm) in the final CS series of exposures in the absence of footshock (Figure 2A, far right striped bar), whereas the tachycardia response was not (Figure 2A, far right black bar).

A different heart rate response pattern was observed when mthC was presented alone. The initial mthC exposure in the unpaired (Figure 2B) group was similar to the paired group's initial mthC alone series with a response pattern of mild bradycardia to CO<sub>2</sub> and no tachycardia in the absence of footshock. For the unpaired group there was a relatively small and inconsistent mean bradycardic response of  $-13 \pm 4$  bpm (Figure 2B, three middle striped bars) that was found to be significantly different from the paired mthC group in the post hoc analysis ( $p < 0.01$ ). As expected there was no tachycardia generated in the absence of footshock which contrasted with the significant tachycardia exhibited in the paired mthC group ( $p < 0.01$ ) (Figure 2B vs. 2A, black bars). Thus, the bradycardia occurring during paired mthC and footshock represents a learned physiological response to a novel conditioned stimulus, which did not extinguish during the fifth exposure series of mthC alone.

In contrast to what was seen with mthC, when tone was paired with footshock there was no learned heart rate response to tone alone ( $p > 0.05$ ) and a negligible mean change ( $-1 \pm 1$  bpm); however, there was the expected tachycardic response to footshock of  $31 \pm 3$  bpm (Figure 2C). For the tone alone group there were also negligible changes in mean heart rate across the repeated exposures of  $-4 \pm 1$  bpm and no tachycardic response in the absence of footshock (Figure 2D).

### Mean Arterial Blood Pressure

Exposure to mthC alone caused a small increase in blood pressure that was consistent across all exposures and was independent of whether it was paired with footshock (mean response of  $3.0 \pm 0.3$  mmHg) or unpaired (mean response of  $3.0 \pm 0.4$  mmHg;  $p > 0.05$ ; Figure 3A and 3B, striped bars). The effect of tone on blood pressure was small and relatively inconsistent (Figure 3C and 3D, striped bars) and unrelated to whether it was paired (mean response of  $0.8 \pm 0.2$  mmHg) or unpaired (mean response  $0.4 \pm 0.2$  mmHg) with footshock ( $p > 0.05$ ; Figure 3C and 3D). A small increase in blood pressure occurred across the three paired CS-UCS periods and was not different between the mthC and tone stimuli (Figure 3A and 3C). However, comparing all series between groups, the small but consistent hypertensive response to mthC seen in the presence or absence of footshock was statistically greater than for either of the two groups exposed to tone as a CS ( $F = 21.69$ ,  $p < 0.001$ ).

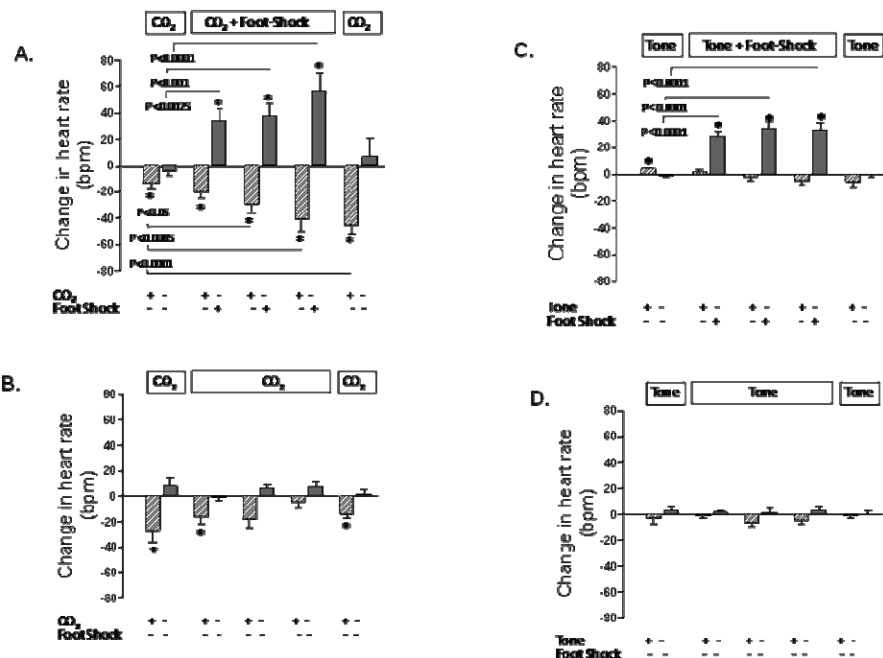


Figure 2: The mean  $\pm$  s.e.m change in heart rate in response to the CS (end-stimulus – pre-stimulus heart rate; striped bars) and the UCS (post-stimulus – end-stimulus heart rate; dark bars).

Considering the blood pressure and heart rate data together we show that tone alone has no effect on heart rate or blood pressure across repeated exposures, whereas mtHC induces a mild hypertensive response and is associated with a small bradycardia. The effect of footshock induced an acute tachycardic and mild hypertensive response. Only when mtHC predicted footshock did a learned FC response develop consisting of an increasing bradycardic response across exposures that occurred in the presence of a consistent, but mild, hypertensive response which did not change across series.

### Catecholamines

There was a small, but statistically significant increase in plasma epinephrine during the last two presentations of the paired CS-UCS for the mtHC + FS exposure (Figure 4A, two right black bars). There was a return of plasma epinephrine to baseline levels for the re-exposure to mtHC alone series at 3 pm (Figure 4A, far right dark gray bar). We did not see the same response pattern for the mtHC alone (control) group, with plasma epinephrine remaining at basal levels across all six samples taken (Figure 4B). The T + FS group exhibited a three to four-fold increase in plasma epinephrine and similar to the paired mtHC group plasma epinephrine returned to baseline levels after the final series of the tone alone (Figure 4C, right dark gray bar). Consistent with the mtHC alone group, there was no change in the response pattern across all six series for the tone alone group (Figure 4D). When comparing between groups, there was an overall significant effect of group ( $F = 41.74$ ,  $p < 0.001$ ) and post hoc tests revealed that plasma epinephrine in response to the three pairings of CS-UCS was significantly higher in the tone + FS group than the mtHC + FS group ( $p < 0.01$ ), and both were significantly higher than their non-shocked counterparts ( $p < 0.05$ ).

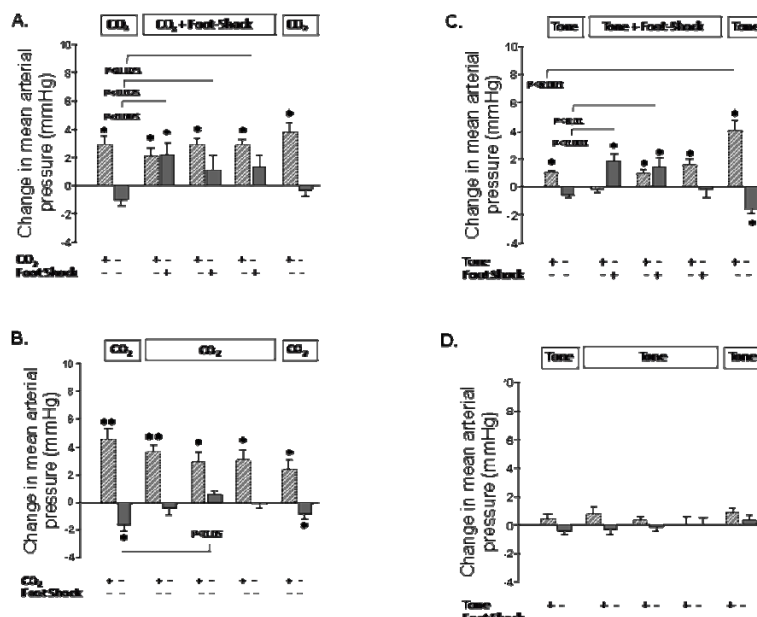


Figure 3: The mean  $\pm$  s.e.m change in mean arterial blood pressure in response to the CS (end-stimulus – pre-stimulus heart rate; striped bars) and the UCS (post-stimulus – end-stimulus heart rate; dark bars).

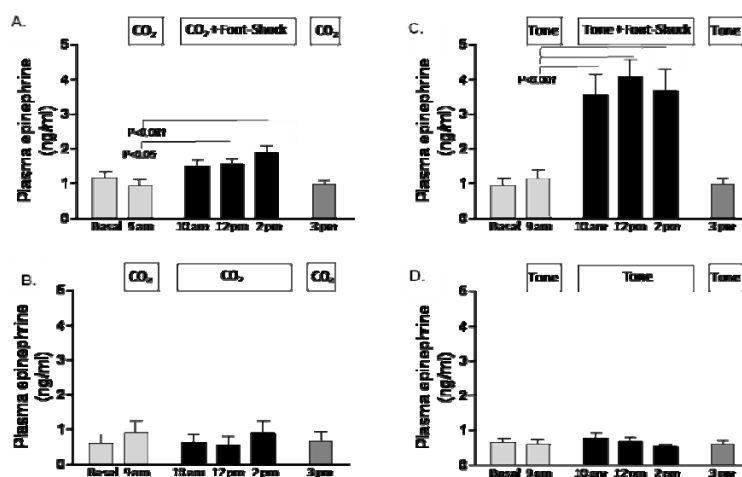


Figure 4: Mean  $\pm$  s.e.m plasma epinephrine under basal conditions and after each series of exposures to the CS or the paired CS-UCS at 9 am, 10 am, 12 noon, 2 pm, and 3 pm.

## Sleep

There were no differences in total time spent in any of the three sleep/wake states within or between groups on either the pre-FC day or the post-FC day.

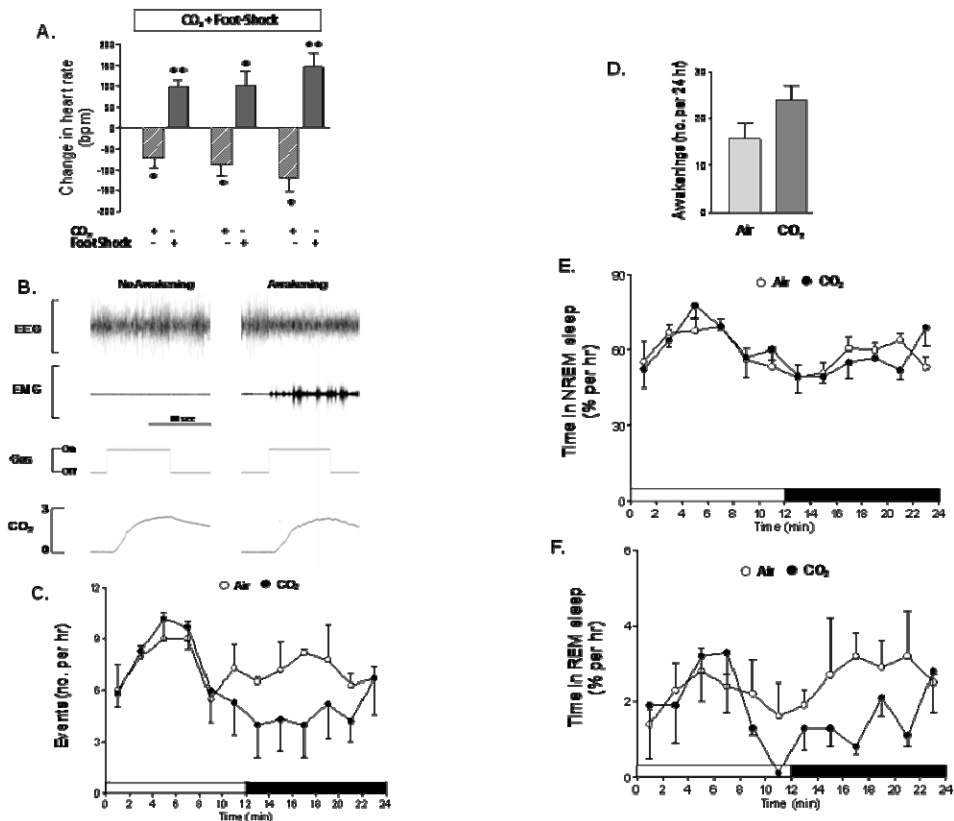
### Protocol B

#### Heart rate

We assessed change in heart rate in response to the three exposures of paired CS-UCS in the six mice that underwent FC in Protocol B (Figure 5A). We reproduced a similar pattern of marked bradycardia in response to mthC preceding footshock as seen in Protocol A (three middle striped bars in Figure 2A). However, the degree of bradycardia we saw in Protocol B (without prior exposure to mthC alone) reached a magnitude of  $-118 \pm 34$  bpm during the third exposure period (Figure 5A, far right striped bar). In contrast, in Protocol A when animals experienced prior exposure to mthC alone before the repeat CS-UCS pairings the comparable bradycardia was only  $-41 \pm 9$  bpm (Figure 2B, second striped bar from right).

#### Re-exposure to mthC during sleep

Sample tracings in Figure 5B show two separate one-minute periods of mthC exposure triggered automatically after three minutes of continuous sleep in one mouse. In the first sample trace mthC had no impact on sleep state whereas the second period of mthC induced an awakening. The number of mthC or air (control) events that occurred across the 24 hour period are shown in Figure 5C. Interestingly, in the dark period the patterns tended to diverge with the appearance of fewer events in animals experiencing mthC than those receiving the control air events (Figure 5C). The number of awakenings (Figure 5D) were more common with mthC (16% of events induced awakening) than air (9% of events induced awakening) and while there was no apparent difference during NREM sleep



**Figure 5:** Demonstration of the utility and impact of re-exposure to a CS of 3% CO<sub>2</sub> for 60 sec whenever three minutes of consolidated sleep occurred. (A) learned bradycardic response to the CS of 3% CO<sub>2</sub> (B) two sample tracings from a mouse showing a 60 sec exposure to 3% CO<sub>2</sub> during sleep with one event having no impact on sleep (left) and the other causing a distinct awakening (right). (C) the number of gas exposure events was averaged in two hour bins across the 24 hour re-exposure period for animals that were re-exposed to the CS+ (3% CO<sub>2</sub>) compared to those re-exposed to the CS- (air). (D) the total number of awakenings across the 24 hour period after fear conditioning for animals re-exposed to the CS+ compared to CS-. (E) time in REM sleep was averaged in two hour bins across the 24 hour re-exposure period for animals that were re-exposed to the CS+ compared to CS-. (F) time in NREM sleep was averaged in two hour bins across the 24 hour re-exposure period for animals that were re-exposed to the CS+ compared to CS-.

(Figure 5E), a pattern of decreased REM during the dark period in the mtHC group (Figure 5F) paralleled the pattern of number of events (Figure 5C).

**CONCLUSIONS:** The primary aim of the current study was to develop and test a novel conditioning stimulus for the purpose of re-exposing the animals during sleep as a proof of principle to study sleep-dependent fear conditioning processes. We determined that mtHC produced a robust, reproducible learned bradycardia response when paired with footshock that was not seen with mtHC alone or tone + footshock. Assessment of systemic stress through measurement of circulating epinephrine demonstrated an absence of response to mtHC alone and when mtHC was paired with footshock the increase in epinephrine was less than seen with the traditional pairing of tone + footshock. We subsequently demonstrate in a proof of principle study that mtHC can be reapplied during sleep in FC animals allowing a new experimental paradigm for future studies to examine unique relationships between learning, memory, and sleep with potential application to the field of PTSD.

These data were also presented in preliminary form at the 2011 APPS International meeting in Minneapolis, Minnesota. The title of the abstract was 'A novel murine fear conditioning model using mild hypercapnia as a conditioned stimulus to study sleep disturbances in PTSD,' authored by Ashlee Filippone, Angela McDowell, Lia Romano, Anne Germain, and Chris O'Donnell.

**Task 5 (Aim 2): To determine the impact of co-morbid conditions of genetic and environmental anxiousness on acquisition and extinction in a murine FC model of PTSD (Months 13 to 24).**

**PROGRESS:** The second aim of the study involved a comparison of fear-conditioning and sleep responses between two different genetic inbred strains: C57BL/6J and the Balb/C. We followed a similar FC protocol to that outlined above in Figure 1, with the exception that we did not expose the animals to the CS alone either before or after the three groupings of paired CS-UCS exposures. Our data detail that these two inbred strains exhibit a very different pattern of physiologic fear conditioning.

In the Balb/C mice, the conditioning stimulus (CS) of mild transient hypercapnia induces a marked bradycardic HR response during CS exposure that habituates and lessens across exposure periods (Figure 6A, green upward sloping line). In contrast, the C57BL/6J mice produce an almost opposite response with the degree of bradycardia sensitizing across sessions (Figure 6B, green downward sloping line). The conclusions from this study are that conditioned HR responses to our novel hypercapnic CS are robust and are either extinguished or heightened with re-exposure based on genetic background.

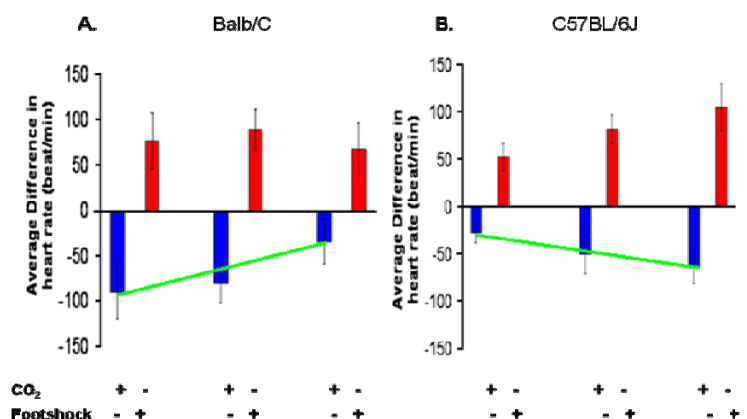


Figure 6: Mean  $\pm$  s.e.m heart rate changes during exposure to mild transient hypercapnia (conditioned stimulus) and footshock (unconditioned stimulus) in Balb/C and C57BL/6J mice.

These data are currently being combined from data from Aim 3 (Task 6) to produce a manuscript assessing genetic factors and the impact of re-exposure to the conditioning stimulus of mild hypercapnia during sleep.

**Task 6 (Aim 3): To determine how re-exposure to a conditioned stimulus of mild hypercapnia across sleep-wake states promotes extinction in a murine FC model of PTSD.**

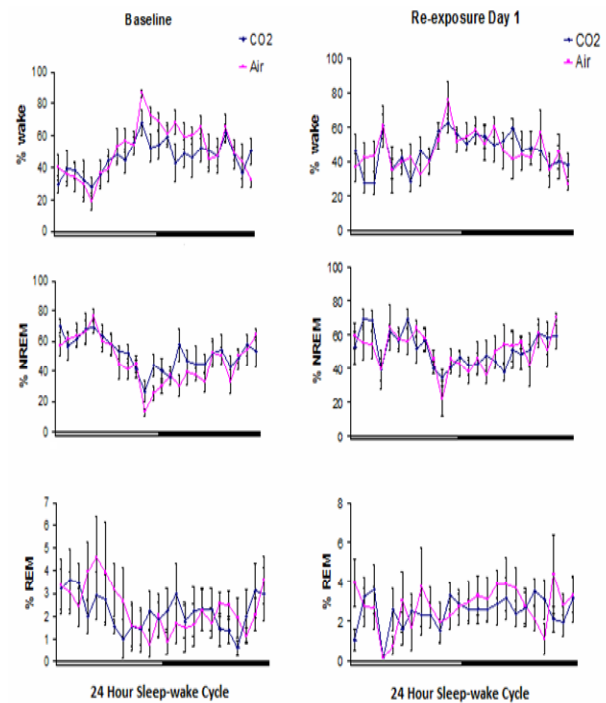
**PROGRESS:** We have now completed data collection on experiments that assess the impact of re-exposure to the conditioned hypercapnic stimulus specifically during NREM sleep in both Balb/C (fear susceptible strain) and C57BL/6J mice (neutral strain). We have adopted a protocol in which the mice are first fear-conditioned to a CS of mild hypercapnia and then re-exposed to the hypercapnia during sleep over a subsequent 24 hr period. Specifically, mice are re-exposed using our fully automated feedback system to a 60 sec period of mild hypercapnia only after 180 sec of consolidated sleep (NREM or REM) is observed. We have conducted studies in both the C57BL/6J and Balb/C strains and compared the impact of re-exposure of the CS on sleep architecture as well as arousals and sleep disruption.

Overall, we found no significant differences in sleep architecture in either strain of mice when comparing baseline (prior to fear-conditioning) to the re-exposure period over identical 24 hr periods. Data are shown in Figure 7 for the Balb/C strain which shows a clear circadian rhythm in NREM and REM sleep across the 24 hr time period, but comparable distributions of sleep between baseline and re-exposure conditions for animals re-exposed to mild hypercapnia or to air (controls).

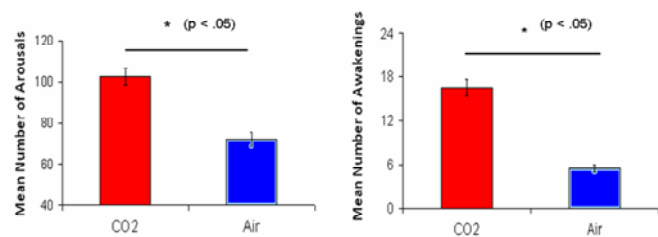
We also observed that the number of re-exposure events during the 24 hr re-exposure period exhibited a circadian rhythm with rates highest during the early part of the light or sleeping period and lowest at the beginning of the dark or active period. Again there were no differences between the strains with respect to the number of re-exposure events experienced. However, an interesting finding was that in the Balb/C strain, but not the C57BL/6J strain, animals re-exposed to hypercapnia during sleep exhibited a significantly greater number of arousals and awakenings when compared to mice that received the control air exposure (Figure 8; note: this difference in arousals and awakenings occurred despite an identical number of exposure events for CO<sub>2</sub> and air).

Our data demonstrate that re-exposure to a CS during sleep can impact arousability from sleep. Specifically, we show that the vulnerable Balb/C strain exhibits a state of heightened arousal after fear conditioning that remains present during sleep.

As noted above, the data from Aim 2 are currently being combined with these data from Aim 3 and prepared for submission of a publication.



**Figure 7:** Mean  $\pm$  s.e.m hourly sleep architecture (wake, NREM, and REM sleep) in Balb/C mice under baseline conditions and after fear conditioning (re-exposure day 1) in response to administration of hypercapnia (CO<sub>2</sub>) or air (control) for 1 min after at least 3 min of sleep.



**Figure 8:** Mean  $\pm$  s.e.m arousals and awakenings in Balb/C mice during the 24 period of re-exposure to either hypercapnia (CO<sub>2</sub>) or air (control) in fear conditioned animals.

The data from this study were presented in preliminary form as an abstract and subsequent presentation at the 2012 meeting of the APSS in Boston. **Re-exposure to a fear conditioned stimulus during sleep in a mouse model of PTSD.** Angela L. McDowell, Ashlee Fillipone, Lia Romano, Anne Germain and Chris O'Donnell. University of Pittsburgh, Pittsburgh, PA.

**Task 7 (Aim 4): To determine the mechanisms of acquisition and extinction in a murine FC model of PTSD and to evaluate potential therapeutic targets.**

PROGRESS: We have begun preliminary meetings with our Co-Investigator, Anne Germain, on refining the protocol for assessing the impact of the serotonin axis on fear conditioning learning and sleep. Dr. Germain is currently undertaking a DoD study in human subjects using a fear conditioning paradigm and examining the role of genetic variation in the promoter region of the serotonin transporter gene. We are excited about the paralleling the mouse and human studies as much as possible to bring a translational research slant to our ongoing work. The mouse studies will begin early in 2013.

**Task 8. Data review, quality control /insurance, processing, scoring, and storage for exploratory and confirmatory analyses.**

PROGRESS: Data review and quality control of scoring is underway for experimental data collection related to Tasks 4, 5 and 6 outlined above.

### **III. KEY RESEARCH ACCOMPLISHMENTS**

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- Development of a novel murine model of fear conditioning utilizing mild transient hypercapnia as the conditioning stimulus.
- Demonstration that a stimulus of mild transient hypercapnia can be used for re-exposure as a conditioning stimulus during the sleep state.
- Showing that re-exposure of a conditioning stimulus of mild transient hypercapnia during sleep can lead to increased arousals and awakenings despite normal amounts of NREM and REM sleep
- Demonstration that genetic background affects acquisition and habituation to a conditioning stimulus of mild transient hypercapnia and that a fear conditioning susceptible genetic mouse strain exhibits disturbed sleep when re-exposed to the conditioning stimulus.

### **IV. REPORTABLE OUTCOMES**

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The following abstract was accepted and presented at the APSS 2011 SLEEP meeting in Minneapolis. **A novel murine fear conditioning model using mild hypercapnia as a conditioned stimulus to study sleep disturbances in PTSD.** Ashlee Fillipone, Angela L. McDowell, , Lia Romano, Anne Germain and Chris O'Donnell. University of Pittsburgh, Pittsburgh, PA.

The following abstract was accepted and presented at the APSS 2012 SLEEP meeting in Boston. **Re-exposure to a fear conditioned stimulus during sleep in a mouse model of PTSD.** Angela L. McDowell, Ashlee Fillipone, Lia Romano, Anne Germain and Chris O'Donnell. University of Pittsburgh, Pittsburgh, PA.

In April 2012 Dr. Angela McDowell was invited to the NIH American Academy of Sleep Medicine Young Investigator Forum to give a presentation on **A novel model of fear-conditioning in mice and its impact on sleep.**

The following manuscript has been submitted for publication. **Mild transient hypercapnia as a novel fear conditioning stimulus allowing re-exposure during sleep.** Angela McDowell, Ashlee Filippone, Alex Balbir, Anne Germain and Chris O'Donnell.

The following abstract has been submitted for the APSS 2013 SLEEP meeting to be held in Baltimore. **The impact of exposure to adverse events occurring in early life and adolescence on sleep-wake patterns in adult mice.** Angela McDowell, Anne Germain and Chris O'Donnell.

## V. CONCLUSION

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The project continues to move forward in a timely manner. We have a well-trained and coordinated investigative team with data collection proceeding in a rigorous and reproducible fashion. The first manuscript, which was a major undertaking demonstrating and validating our novel murine model of fear conditioning with application during sleep, has now been submitted for publication. We have finished data collection and are currently preparing a draft of our second manuscript on the representation of the fear conditioning stimulus during sleep, and are well on the way to completing data collection for a third manuscript relating to induced sleep disruption on fear conditioning and learning. In addition, we have continued to present our data at National and International meetings related to sleep. We are in process of finalizing the study protocol for the initiation of studies related to Task 7 (Aim 4) which will begin early in 2013.

## VI. REFERENCES

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None applicable

## VII. APPENDIX

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- Draft of submitted manuscript.
- Most recent IACUC approval letter



# University of Pittsburgh

*Research Conduct and Compliance Office  
Institutional Animal Care and Use Committee*

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2500 Fifth Avenue  
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Fax: 412-383-2020

University of Pittsburgh Protocol Number: 1106265A-1

June 8, 2012

DOD (DM102174)

Assurance Number: A3187-01

To Whom It May Concern:

The Institutional Animal Care and Use Committee of the University of Pittsburgh has reviewed and approved on June 8, 2012 the research proposal submitted by Christopher O'Donnell.

Titled: A murine model for Post-traumatic Stress Disorder and its influence on sleep architecture

The committee finds that the protocol meets the standards for humane animal care and use as set by the Animal Welfare Act and the NIH Guide for the Care and Use of Laboratory Animals.

Sincerely,

Frank J. Jenkins, Ph.D., Chair  
Institutional Animal Care and Use Committee

**This letter is valid until June 30, 2013.**