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14. ABSTRACT
Purpose: This research proposal was to investigate specific compounds, tetrahydropalmatine (THP) and L-Theanine (L-Th), on neurobehavioral effects and specific gene expression in a

PTSD rodent model. The aims were to determine the effects of THP and L-Th on anxiety, locomotion, memory, hyperarousal, and gene expression in the brain in the rodent PTSD model. Design: A prospective experimental between groups design was used. Methods: Eighty rats were equally divided into two groups, non-stressed and PTSD-stressed. They were then subdivided into four groups: control, THP or L-Th, midazolam, or THP or L-Th and midazolam. The behavioral component was evaluated using the elevated plus-maze (EPM), acoustic startle reflex (ASR), or Morris water maze (MWM), in a restraint/shock stress model. Sample: Eighty rats were used for each herbal supplement (THP or L-Th) studied. Analysis: Data analysis was performed using two-tailed Multivariate Analysis of Variance (MANOVA) and LSD post-hoc tests. Findings: These studies establish a solid framework for future investigation of PTSD treatments. Data showed that there were significant differences in anxiety between groups in both the THP and L-Th studies ($p<0.05$). Significant transcriptional fold changes were found in important genes involved in dopamine, serotonin, acetylcholine, and GABA neurotransmitter systems with both herbal compounds. These results provide quantifiable data demonstrating gene expression changes in PTSD-stressed and non-stressed rats receiving various treatments. Additionally, these findings contribute important data to the limited molecular details pertaining to the understanding of the genetic mechanisms involved in the neurobiology of PTSD. Implications for Military Nursing: This proposal assists military nurses and other health care personnel to expand their understanding of the neurobehavioral and basic physiologic and cellular mechanisms responsible for PTSD. It is imperative that treatment of PTSD be investigated and possible therapies employed to sustain Force Health Protection and a Fit and Ready Force

15. SUBJECT TERMS

PTSD, specific gene expression, neurobehavioral effects, Force Health Protection, Fit and Ready Force

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USU Grant Number	HU0001-10-1-TS08
USU Project Number	N10-P12
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Table of Contents

Abstract

Purpose: This research proposal was to investigate specific compounds, tetrahydropalmatine (THP) and L-Theanine (L-Th), on neurobehavioral effects and specific gene expression in a PTSD rodent model. The aims were to determine the effects of THP and L-Th on anxiety, locomotion, memory, hyperarousal, and gene expression in the brain in the rodent PTSD model.

Design: A prospective experimental between groups design was used.

Methods: Eighty rats were equally divided into two groups, non-stressed and PTSD-stressed. They were then subdivided into four groups: control, THP or L-Th, midazolam, or THP or L-Th and midazolam. The behavioral component was evaluated using the elevated plus-maze (EPM), acoustic startle reflex (ASR), or Morris water maze (MWM), in a restraint/shock stress model.

Sample: Eighty rats were used for each herbal supplement (THP or L-Th) studied.

Analysis: Data analysis was performed using two-tailed Multivariate Analysis of Variance (MANOVA) and LSD post-hoc tests.

Findings: These studies establish a solid framework for future investigation of PTSD treatments. Data showed that there were significant differences in anxiety between groups in both the THP and L-Th studies ($p<0.05$). Significant transcriptional fold changes were found in important genes involved in dopamine, serotonin, acetylcholine, and GABA neurotransmitter systems with both herbal compounds. These results provide quantifiable data demonstrating gene expression changes in PTSD-stressed and non-stressed rats receiving various treatments. Additionally, these findings contribute important data to the limited molecular details pertaining to the understanding of the genetic mechanisms involved in the neurobiology of PTSD.

Implications for Military Nursing: This proposal assists military nurses and other health care personnel to expand their understanding of the neurobehavioral and basic physiologic and cellular mechanisms responsible for PTSD. It is imperative that treatment of PTSD be investigated and possible therapies employed to sustain Force Health Protection and a Fit and Ready Force

TSNRP Research Priorities that Study or Project Addresses

Primary Priority

Force Health Protection:	☒ Fit and ready force ☐ Deploy with and care for the warrior ☒ Care for all entrusted to our care
Nursing Competencies and Practice:	☐ Patient outcomes ☐ Quality and safety ☐ Translate research into practice/evidence-based practice ☐ Clinical excellence ☐ Knowledge management ☐ Education and training
Leadership, Ethics, and Mentoring:	☐ Health policy ☐ Recruitment and retention ☐ Preparing tomorrow's leaders ☐ Care of the caregiver
Other:	☒ Mentoring future military nursing research scientists

Progress Towards Achievement of Specific Aims of the Study or Project

SPECIFIC AIMS AND RESERCH QUESTIONS FROM THE GRANT

The aims of this study were to determine the effects of tetrahydropalmatine (THP) and L-Theanine (L-Th) in a PTSD rodent model. Specifically, the aims were as follows:

1. Determine the effects of THP and L-Th on anxiety.
2. Determine the effects of THP and L-Th on locomotion
3. Determine the effects of THP and L-Th on memory.
4. Determine the effects of THP and L-Th on hyperarousal or startle.
5. Determine the possible interaction effects of THP and L-Th with midazolam on anxiety, locomotion, memory, and hyperarousal or startle.
6. Determine the effects of THP and L-Th on gene expression in the brain.

Research Questions

This study consisted of eight groups of rats for each herbal supplement investigated (THP or L-Th), see table below. Rats were assigned to the nonstressed groups or the restraint shock PTSD rodent model groups. There were four groups within the nonstressed rats (control, herbal, midazolam, and herbal + midazolam) and four groups in the PTSD rats (control, herbal, midazolam, and herbal + midazolam) (Table 1).

Nonstressed	Control (saline)	Herbal	Midazolam	Herbal + Midazolam
PTSD	Control (saline)	Herbal	Midazolam	Herbal + Midazolam

Table 1 Research Groups

The aims of this research protocol were guided by the following questions:

1. Is there a significant difference in the anxiolytic effects between the groups?
2. Is there a significant difference in locomotion between the groups?
3. Is there a significant difference in memory between the groups?
4. Is there a significant difference in hyperarousal between the groups?
5. Are there significant differences or interaction effects between groups regarding the above neurobehavioral effects (anxiety, locomotion, memory, hyperarousal)?
6. Are there significant differences in gene expression and regulation in the hippocampus between the groups?
7. Are there significant differences in gene expression and regulation in the amygdala between the groups?

Findings related to each specific aim, research or study questions, and/or hypothesis:

***This section will be addressed separately for each herbal supplement investigated (THP and L-Theanine)**

SPECIFIC AIMS AND RESERCH QUESTIONS - THP

The aims of this study were to determine the effects of THP in a PTSD rodent model. Specifically, the aims and their corresponding research questions were as follows:

Aim #1: Determine the effects of tetrahydropalmatine (THP) on anxiety.
Question# 1: Is there a significant difference in the anxiolytic effects between the groups?

Aim# 5: Determine the possible interaction effects of THP with midazolam on <u>anxiety</u>, locomotion, memory, and hyperarousal or startle.
Question#5: Are there significant differences or interaction effects between groups regarding the above neurobehavioral effects (<u>anxiety</u>, locomotion, memory, hyperarousal)?

Anxiety was measured by the elevated plus maze (EPM), an instrument utilized to measure anxiety in the rodent model. This model has been validated in previous research studies. **Anxiety** is defined as the ratio of open arm time to total time on the elevated plus maze (EPM). A rat was considered to have entered an arm via the MotorMonitor

software. At the end of the test, the time spent on the open arms was expressed as a percentage of the time spent on both the open and the closed arms. An increase in the percentage of time spent in the open arms reflects decreased anxiety.

Data analyses were conducted using a 2-tailed multivariate analysis of variance and Least Significant Difference (LSD) post hoc test. Analysis of the ratio of open arm time versus total time spent in the elevated plus maze revealed statistically significant increases between the control midazolam and control vehicle group (P=.027); the control midazolam and control midazolam plus THP group (P=.017); the control midazolam and PTSD vehicle group (P=.001); the control midazolam and PTSD THP group (P=.006); and control midazolam and PTSD midazolam group (P=.014). However, there was no significance found between the control THP and PTSD vehicle group (P=.71) and the PTSD vehicle group compared to the PTSD THP group (p=.530) (see Table 3 and Figure 3).

Group	Sample Size	Mean Ratio Open Arm/Total Time ± SEM	Basic Motor Movement ± SEM	Fine Motor Movement ± SEM
Control Vehicle	10	43.7 ± 5.8*	1103.2 ± 52.7	794.3 ± 33.9
Control THP	9	53.6 ± 15.3	222.4 ± 53.5*	185.3 ± 40.0*
Control Midazolam	9	77.8 ± 7.6*	391 ± 109.4*	276 ± 73.6*
Control Midazolam + THP	9	39.9 ± 11.3*	478.1 ± 115.3*	366.3 ± 84.6*
PTSD Vehicle	9	25.2 ± 4.2*	1017.4 ± 73.3	717.9 ± 47.3
PTSD THP	10	34.7 ± 11*	264.5 ± 56.7*	208.4 ± 41.8*
PTSD Midazolam	10	39.7 ± 9.3*	731.5 ± 135.1*	499.2 ± 89*
PTSD Midazolam + THP	10	53.9 ± 14.8	130 ± 54.3*	102.8 ± 43.6*

Table 3. *P<0.05. Table showing treatment groups, sample size, mean ratio of open arm time to total maze time (in seconds), the number of basic motor movements and number of fine motor movements on elevated plus maze. Data are presented as mean ± standard error of the mean. SEM = Standard Error of the Mean, THP = tetrahydropalmatine, PTSD = Post Traumatic Stress Disorder.

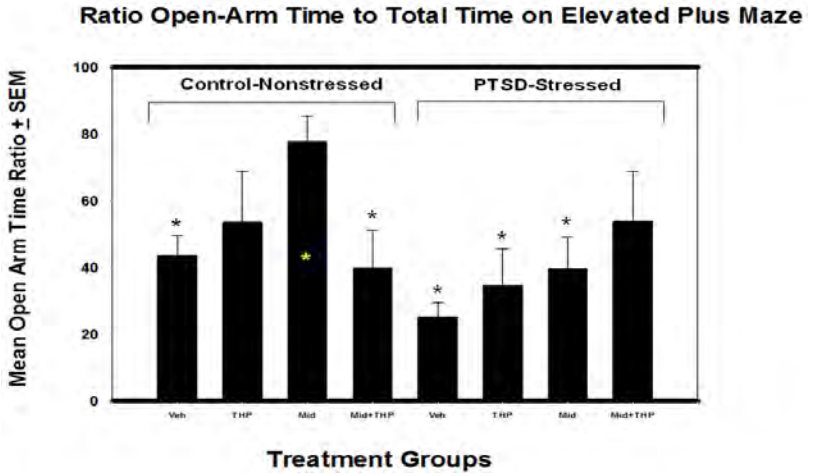

Figure 3. Bar graph representing the ratio of open-arm time to total time on Elevated Plus Maze. The X axis is the treatment groups and the Y axis shows the calculated ratio of the mean open-arm time to total time plus or minus the standard error of the mean in seconds. The asterisks show the groups that showed significance. Veh = Vehicle, THP = tetrahydropalmatine, Mid = Midazolam, Mid+THP = Midazolam and tetrahydropalmatine, SEM = Standard Error of the Mean

Aim# 2: Determine the effects of THP on locomotion.
Question#2: Is there a significant difference in locomotion between the groups?

Aim# 5: Determine the possible interaction effects of THP with midazolam on anxiety, locomotion, memory, and hyperarousal or startle.
Question#5: Are there significant differences or interaction effects between groups regarding the above neurobehavioral effects (anxiety, locomotion, memory, hyperarousal)?

Locomotion was defined as motor movements on the EPM. The EPM was networked with MotorMonitor software (Hamilton-Kinder, Poway, California) with laser sensors integrally attached to the EPM to track the number of entries into each arm, time spent in each arm, and total basic and fine motor movements. Basic motor movements were the simple count of beam breaks in the elevated plus maze. Each time a photobeam was interrupted, the basic movement count was increased. These movements reveal a gross measure of locomotion, but did not distinguish what type of activity is being performed. Fine motor movements were a compilation of small animal movements such as grooming, head weaves or bobs. When rats have increased anxiety or fear, they display freezing behaviors, or decreased movements.

Basic Motor Movements

Total number of basic (gross) and fine motor movements tracked during time in the EPM were analyzed. Analysis showed a significant increase in basic motor movement of rats in the control vehicle group compared to the control THP group (P<.000); the control midazolam group (P<.000); the control THP plus midazolam group (P<.000); the PTSD THP group (P<.000); the PTSD THP group (P<.000); the PTSD THP group (P<.000); the PTSD midazolam group (P=.003); and the PTSD midazolam plus THP group (P<.000). Significant increase in basic movement of rats was also noted in the PTSD vehicle group compared to the control THP group (P<.000); the control midazolam group (P<.000); the control midazolam plus THP group (P<.000); the PTSD THP group (P<.000); the PTSD midazolam group (P=.023); and the PTSD midazolam plus THP group (P<.000). Significant increases in basic motor movements of rats were also noted in the control midazolam plus THP compared to the control THP group (P=.046); the control midazolam plus THP group compared to the PTSD midazolam plus THP group (P=.006); the PTSD midazolam group compared to the control THP (P<.000); the PTSD midazolam compared to the control midazolam (P=.007); the PTSD midazolam group compared to the control midazolam plus THP group (P=.043); the PTSD midazolam group compared to the PTSD THP group (P<.000); and the PTSD midazolam group compared to the PTSD midazolam plus THP (P<.000) (see Table 3 and Figure 4).

Fine Motor Movements

Similarly, a significant increase in fine motor movement of rats was found in the PTSD vehicle group compared to the control THP group (P<.000); the control midazolam group (P<.000); the control midazolam plus THP group (P<.000); the PTSD THP group (P<.000); the PTSD midazolam group (P=.012); and the PTSD midazolam plus THP (P<.000). Other significant increases in fine motor movements of rats were found in the control vehicle group compared to the control THP group (P<.000); the control midazolam group (P<.000); the control midazolam plus THP group (P<.000); the PTSD THP group (P<.000); the PTSD midazolam group (P=.001); and the PTSD midazolam plus THP (P<.000). Further significant increases in fine motor movement of rats were found in the PTSD midazolam group compared to the control THP group (P<.000); the control midazolam group (P=.011); the PTSD vehicle group (P=.012); the PTSD THP group (P=.001); and the PTSD midazolam plus THP (P=.000). Significant increases in fine motor movement of rats were also found in the control THP group compared to the control midazolam plus THP group (P=.042); the control midazolam plus THP group compared to the PTSD midazolam plus THP group (P=.003); and the control midazolam group compared to the PTSD midazolam plus THP group (P=.045) (see Table 3 and Figure 5).

Group	Sample Size	Mean Ratio Open Arm/Total Time ± SEM	Basic Motor Movement ± SEM	Fine Motor Movement ± SEM
Control Vehicle	10	43.7 ± 5.8*	1103.2 ± 52.7	794.3 ± 33.9
Control THP	9	53.6 ± 15.3	222.4 ± 53.5*	185.3 ± 40.0*
Control Midazolam	9	77.8 ± 7.6*	391 ± 109.4*	276 ± 73.6*

Control Midazolam + THP	9	39.9 ± 11.3*	478.1 ± 115.3*	366.3 ± 84.6*
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PTSD THP	10	34.7 ± 11*	264.5 ± 56.7*	208.4 ± 41.8*
PTSD Midazolam	10	39.7 ± 9.3*	731.5 ± 135.1*	499.2 ± 89*
PTSD Midazolam + THP	10	53.9 ± 14.8	130 ± 54.3*	102.8 ± 43.6*

Table 3. *P<0.05. Table showing treatment groups, sample size, mean ratio of open arm time to total maze time (in seconds), the number of basic motor movements and number of fine motor movements on elevated plus maze. Data are presented as mean ± standard error of the mean. SEM = Standard Error of the Mean, THP = tetrahydropalmatine, PTSD = Post Traumatic Stress Disorder.

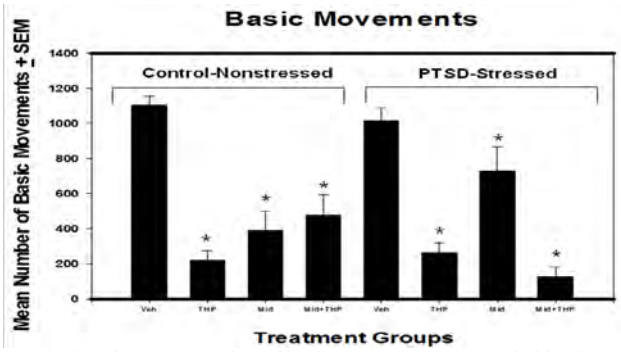

Figure 4. Bar graph representing the mean number of basic movements as recorded by the Motor monitor software on the Elevated Plus Maze. The X axis is the treatment groups and the Y axis shows the mean number of basic movements plus or minus the standard error of the mean. The asterisks show the groups that showed significance. Veh = Vehicle, THP = tetrahydropalmatine, Mid = Midazolam, Mid+THP = Midazolam and tetrahydropalmatine, SEM = Standard Error of the Mean

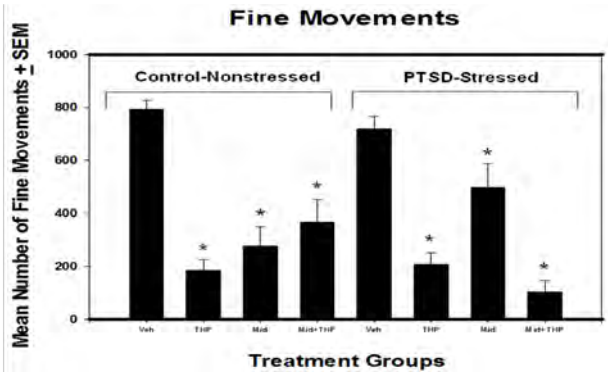

Figure 5. Bar graph representing the mean number of fine movements as recorded by the Motor monitor software on the Elevated Plus Maze. The X axis is the treatment groups and the Y axis shows the mean number of fine movements plus or minus the standard error of the mean. The asterisks show the groups that showed significance. Veh = Vehicle, THP = tetrahydropalmatine, Mid = Midazolam, Mid+THP = Midazolam and tetrahydropalmatine, SEM = Standard Error of the Mean

Aim# 3: Determine the effects of THP on memory.
Question#2: Is there a significant difference in memory between the groups?

Aim# 5: Determine the possible interaction effects of THP with midazolam on anxiety, locomotion, memory, and hyperarousal or startle.
Question#5: Are there significant differences or interaction effects between groups regarding the above neurobehavioral effects (anxiety, locomotion, memory, hyperarousal)?

Memory was defined as spatial memory as tested using the Morris water maze (MWM). This task is based upon the premise that animals have evolved an optimal strategy to explore their environment and escape from the water with a minimum amount of effort - i.e., swimming the shortest distance possible. The time it takes a rat to find a hidden platform in a water pool after previous exposure to the setup, using only available external cues, was determined as a measure of spatial memory.

In the MWM test, there were no statistically significant differences found between groups when looking at latency, time, and entries to the platform area or Zone 3 (see Figure 2). The nonstressed midazolam group had the overall highest mean time spent in Zone 3 (M=14.51; SEM = 0.680) and the control vehicle group had the lowest mean time spent in zone 3 (M=10.35; SEM=1.429) (see Table 4 and Figure 6).

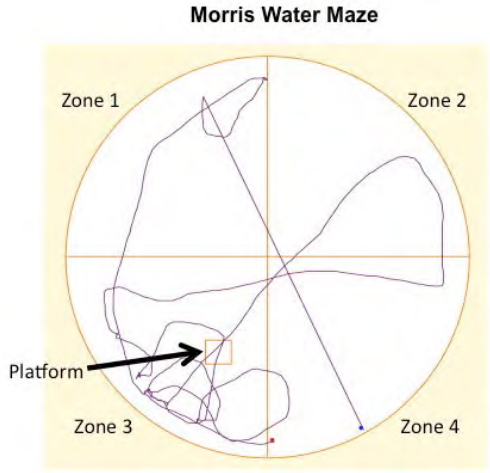

Figure 2. Map of the Morris water maze, as demonstrated from ANY-maze® software. Zone 3 is the area where platform was located, and the line illustrates the path of a rat searching for the platform.

Group	Sample Size	Mean Zone 3 Time	Standard Error of the Mean
Control Vehicle	10	10.35	1.429
Control THP	9	13.68	1.372
Control Midazolam	9	14.51	0.680
Control Midazolam + THP	9	13.41	0.869
PTSD Vehicle	9	13.29	1.546
PTSD THP	10	11.21	1.957
PTSD Midazolam	10	13.61	1.345
PTSD Midazolam + THP	10	13.89	0.922

Table 4. Table showing treatment groups, sample size, mean time spent in zone 3 and standard error of the mean. THP = tetrahydropalmatine, PTSD = Post Traumatic Stress Disorder.

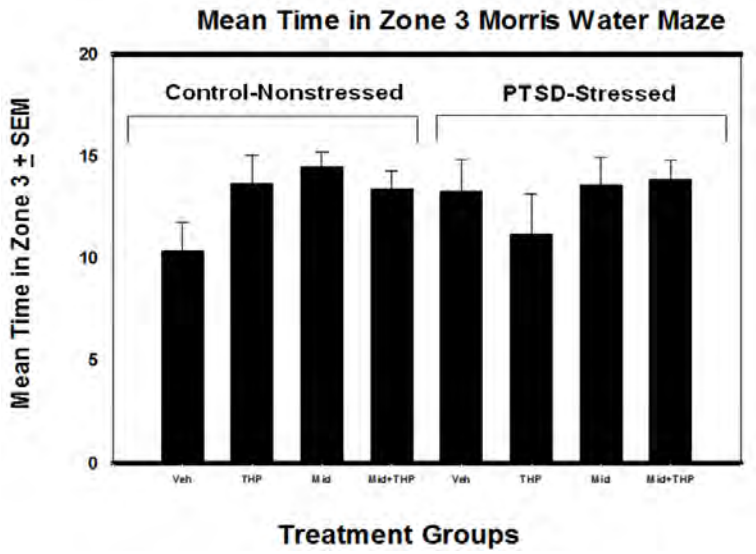

Figure 6. Bar graph representing the mean time spent in Zone 3 of Morris water maze plus or minus the standard error of the mean. The X axis is treatment groups and the Y axis is mean time spent in Zone 3 plus or minus the standard error of the mean. Veh = Vehicle, THP = tetrahydropalmatine, Mid = Midazolam, Mid+THP = Midazolam and tetrahydropalmatine, SEM = Standard Error of the Mean

Aim# 4: Determine the effects of THP on hyperarousal or startle.
Question#4: Is there a significant difference in hyperarousal between the groups?

Aim# 5: Determine the possible interaction effects of THP with midazolam on anxiety, locomotion, memory, and hyperarousal or startle.
Question#5: Are there significant differences or interaction effects between groups regarding the above neurobehavioral effects (anxiety, locomotion, memory, hyperarousal)?

Unable to evaluate because of the malfunction of the equipment.

Aim# 5: Determine the possible interaction effects of THP with midazolam on anxiety, locomotion, memory, and hyperarousal or startle.
Question#5: Are there significant differences or interaction effects between groups regarding the above neurobehavioral effects (anxiety, locomotion, memory, hyperarousal)?

See responses and data described under Aims 1-4

Aim# 6: Determine the effects of THP on gene expression in the brain.
Questions# 6: Are there significant differences in gene expression and regulation in the hippocampus between the groups?
Questions# 7: Are there significant differences in gene expression and regulation in the amygdala between the groups?

Gene Analysis

After completion of neurobehavioral tests, the rats were anesthetized using a bell jar filled with isoflurane. The calvarium was then opened exposing the whole brain, which was then removed intact and placed immediately on ice. Coronal slices (200-µm thickness) in the vicinity of interaural and bregma coordinates 6.96 mm and -2.04

respectively were dissected using a brain block (see red shaded region in Figure 1A). Investigators took samples (circular punches 80-100 µm in diameter) from the left and right amygdala (see lower left red circle in area 6 of Figure 1B). Following dissection of the amygdala, the investigators grossly dissected the bilateral hippocampi (see upper medial red outlined area in area 6 of Figure 1B). Tissue was snap frozen in liquid nitrogen, placed in pre-labeled eppendorf tubes, and packed in dry ice for shipping to the QIAGEN Service Core for Genomics and Gene Expression in Frederick, MD.

Following the manufacturer's protocol, the RNA was isolated using the QIAGEN RNEasy Mini Kit (Cat # 74104). The quality of RNA was determined using the Agilent Bioanalyzer (Agilent) with RNA 6000 Nano Kits (Agilent, Cat #5067-1511). Total RNA yield, 260/280, and 260/230 ratios were measured using a NanoDrop spectrophotometer (Thermo). QIAGEN completed a reverse transcription reaction using 500 ng of total RNA using the QIAGEN RT² First Strand Kit (QIAGEN, Cat # 330401). In accordance with the manufacturer's instructions, cDNA samples were assayed using a modified QIAGEN RT² PCR Arrays (Cat # PARN-60). This array, containing 84 assays related to rat neurotransmitter receptors and regulators, was modified to include four additional genes. These genes are related to neurotransmission pathways that have been identified in previous PTSD studies: p11 (*S100a10*) (Cat # PPR06766); 5HT2A receptor (*Htr2a*) (Cat # PPR06850); alpha-1 adrenergic receptor (*Adra1a*) (Cat # PPR43329); and *Egr1* (Zif/268) (Cat #PPR44272).

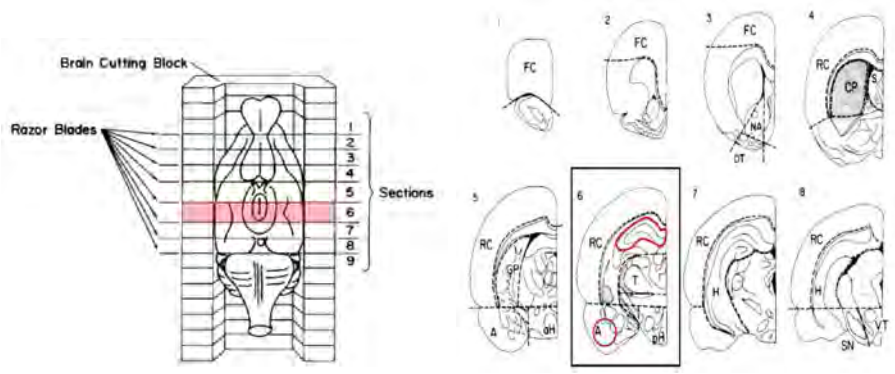

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Figure 1 – (A) Orientation of brain within brain cutting block; (B) Coronal brain sections from dissected regions
(A) Illustration of rat brain in cutting block depicting specific areas where coronal slices were performed; (B) Numbers correspond to dissected sections from Figure 1B. FC, frontal cortex; NA, nucleus accumbens; OT, olfactory tubercle; S, septum; CP, caudate putamen; RC, remaining cortex; GP, globus pallidus; aH, anterior hypothalamus; pH, posterior hypothalamus; A, amygdala; T, thalamus; SN, substantia nigra; VT, ventral tegmentum; H, hippocampus.

Rat Neurotransmitter Receptors and Regulators PCR Array

The Rat Neurotransmitter Receptors and Regulators RT² Profiler™ PCR Array profiles the expression of 84 genes involved in modulating the biological processes of neurotransmitter biosynthesis, uptake, transport, and signaling. This array includes receptors for acetylcholine, dopamine, gamma-aminobutyric acid (GABA), glutamate, serotonin, somatostatin and neuropeptides. Genes involved in the regulation of neurotransmitter levels were included as well. Analysis of the expression of a focused panel of genes related to neuronal systems with this array was performed using real-time PCR.

Statistical Analyses

A one-way ANOVA was performed for this cross-sectional, randomized, prospective study in order to compare the eight groups. Investigators ensured all assumptions were examined (e.g., homogeneity of variance,

univariate normality, etc.) and in the event that the assumptions were not tenable, remedial (e.g., transformation) or alternative (e.g., nonparametric tests, such as Kruskal-Wallis) strategies were considered. The investigators also performed multiple comparison procedures (MCP) in the event that significance ($\alpha = .05$) was obtained, with the Tukey post hoc test. A variance explained statistic eta-squared ($\eta 2$) was used as the reported effect size of small (0.01), medium (0.59) or large (0.138) [23]. The data was then analyzed based on gene cycle thresholds normalized to five housekeeping genes per QIAGEN procedures.

Results

Eighty-eight genes were investigated in this study. Data analysis showed a number of significant differences in gene expression in both the hippocampus and amygdala. Volcano plots graphically display the relationship of –log10 p-value and the log2 fold change for the combined PTSD treatment groups and nonstressed treatment groups for all of the genes (Figure 2). For the 8-group one-way ANOVA, a large effect size ($\eta^2 > .138$) was chosen to signify between-group difference in gene expression. A Tukey's post hoc test was performed to further delineate significance between groups.

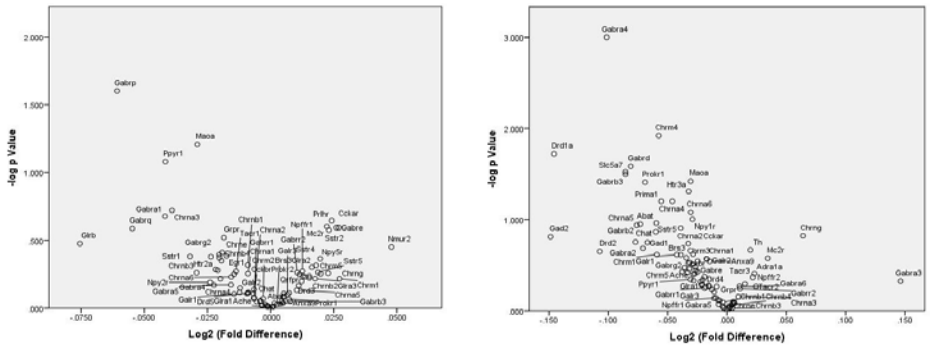

Figure 2 – (A) Differential expression of hippocampal and (B) amygdalar mRNA between groups
Volcano plot between Log2 fold change on the x-axis (for neurotransmitter receptors and regulators in Tetrahydropalmatine/midazolam treated groups 40 PTSD-stressed vs. 40 nonstressed (control) groups) vs. –log of p-value on the y-axis. Eighty male Sprague-Dawley rats were injected subcutaneously 30 minutes prior to evaluation of their performance on neurobehavioral tests. The rats were then euthanized and cDNA prepared from the hippocampus (A) and amygdala (B) were subjected to RT^{2}profiler PCR array for rat neurotransmitter receptor and regulator analysis, as described in Materials and Methods. PCR Array profiles were performed for the expression of 88 genes potentially involved in PTSD and/or rat neurotransmitter receptors and neurotransmitter regulation.

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Hippocampus

In the hippocampus, the genes with an effect size > 0.138 are displayed in Figure 3. In accordance with Figure 3, Table 1 summarizes between group changes in gene expression and fold changes in the hippocampus. The following genes in the hippocampus showed significant differences between groups: *Gabra2*, *Chrne*, *Chrna2*, and *Galr2*. The *Gabra2* gene was significant: $p = .006$, $\eta^2 = .238$. Post hoc tests revealed that the C-M+T group had a significantly lower mean ($M = 1.39$) than each of the following: (1) P-M ($M = 2.11$), (2) C-V ($M = 2.04$), and (3) P-V ($M = 2.04$). We found the *Chrne* gene to be significant: $p = .007$, $\eta^2 = .236$. Multiple comparison showed two significant tests: the P-M group had a higher mean ($M = 9.29$) than the (1) C-T ($M = 8.10$) and (2) C-M ($M = 8.28$) groups. The *Chrna2* gene was significant: $p = .031$, $\eta^2 = .189$. The post hoc test found two significant pairwise comparisons: The C-M+T group had a significantly lower mean ($M = 6.18$) than the (1) C-T ($M = 6.87$) and (2) P-V ($M = 6.96$) groups. The *Galr2* gene was found to be significant: $p = .042$, $\eta^2 = .18$. Though none of the multiple comparison tests were significant, the C-V group had the highest mean ($M = 11.04$) and the C-T group had the lowest ($M = 10.0$).

The following genes in the hippocampus did not show statistical significant differences between groups: *Chrna6*, *Galr1*, *Chrnd*, *Sstr1*, *Cckar*, *Slc5a7*, *Npffr1*, *Maoa*, *Gabra3*, *Qrfpr*, and *Htr2a*. However, secondary to a

large effect size, these genes are presented in Table 1. For the *Chrna6* gene (p = .213, η^2 = .545), the C-V group had the highest mean (M = 15.14) and the C-M+T group had the lowest (M = 6.91). Concerning the *Galr1* gene (p = .053, η^2 = .172), the P-M group had the highest mean (M = 11.94) and the P-V group had the lowest (M = 9.75). Though not significant, for the *Chrnd* gene (p = .131, η^2 = .159) the P-M+T group had the highest mean (M = 17.19) and the C-M+T group had the lowest (M = 14.68). After evaluating the *Sstr1* gene (p = .083, η^2 = .158), the P-M group had the highest mean (M = 7.29) and the C-M group had the lowest (M = 6.05). While not significant, for the *Cckar* gene (p = .113, η^2 = .155) the P-V group had the highest mean (M = 13.30) and the C-T group had the lowest (M = 11.27). Examining the *Slc5a7* gene (p = .102, η^2 = .152), the P-M+T group had the highest mean (M = 9.88) and the C-T group had the lowest (M = 8.79). For the *Npffr1* gene (p = .10, η^2 = .151), the P-M group had the highest mean (M = 11.56) and the C-M+T group had the lowest (M = 10.25). The *Maoa* gene did not show significance (p = .107, η^2 = .148), however the C-V group had the highest mean (M = 3.23) and the C-M+T group had the lowest (M = 2.83). Analysis of the *Gabra3* gene (p = .109, η^2 = .148) revealed the P-M group had the highest mean (M = 5.64) and the C-T group had the lowest (M = 4.38). For the *Qrfpr* (p = .136, η^2 = .14), the P-M group had the highest mean (M = 10.89) and the C-T group had the lowest (M = 9.35). Lastly, the *Htr2a* gene was not significant (p = .139, η^2 = .139), however the P-M group had the highest mean (M = 8.15) and the C-M+T group had the lowest (M = 6.91).

Gene	#Group Comparison	Fold Change	Effect Size	P-value	Description of Gene
Gabra2	C-M+T vs. P-M C-M+T vs. C-V C-M+T vs. P-V	.72 .68 .68	.238	.006	Gamma-Aminobutyric Acid (GABA) A Receptor, Alpha 2
Chrne	P-M vs. C-T P-M vs. C-M	1.19 .98	.236	.007	Cholinergic Receptor, Nicotinic, Epsilon (Muscle)
Chrna2	C-M+T vs. C-T C-M+T vs. P-V	.69 .78	.189	.031	Cholinergic receptor, nicotinic, alpha 2
Galr2	C-V vs. C-T	1.04	.180	.042	Galanin receptor 2
Chrna6	C-V vs. C-M+T	8.23	.545	.213	Cholinergic Receptor, Nicotinic, Alpha 6 (Neuronal)
Galr1	P-M vs. P-V	2.19	.172	.053	Galanin receptor 1
Chrnd	P-M+T vs. C-M+T	2.51	.151	.131	Cholinergic receptor, nicotinic, delta (muscle)
Sstr1	P-M vs. C-M	1.24	.158	0.083	Somatostatin receptor 1
Cckar	P-V vs. C-T	2.03	.155	.113	Cholecystokinin A receptor
Slc5a7	P-M+T vs. C-T	1.09	.152	.102	Solute carrier family 5 (choline transporter), member 7
Npffr1	P-M vs. C-M+T	1.31	.151	.100	Neuropeptide FF receptor 1
Maoa	C-V vs. C-M+T	.40	.148	.107	Monoamine oxidase A
Gabra3	P-M vs. C-T	1.26	.148	.109	Gamma-aminobutyric acid (GABA) A receptor, alpha 3
Qrfpr	P-M vs. C-T	1.54	.140	.136	Pyroglutamylated RFamide peptide receptor
Htr2a	P-M vs. C-M+T	1.24	.139	.139	5-hydroxytryptamine (serotonin) receptor 2A, G protein-coupled

Table 1 – Between group changes in gene expression within the hippocampus
#Group comparison column presents the highest and lowest mean; *Significant p-value < 0.05. PTSD Vehicle (P-V); PTSD Midazolam (P-M); PTSD tetrahydropalmatine (P-T); PTSD tetrahydropalmatine + midazolam (P-M+T); Control Vehicle (C-V); Control Midazolam (C-M); Control Tetrahydropalmatine (C-T); Control tetrahydropalmatine + midazolam (C-M+T); Large effect size > 0.138; Significant p-value < 0.05; All genes were confirmed via the National Center for Biotechnology Information (NCBI) database.

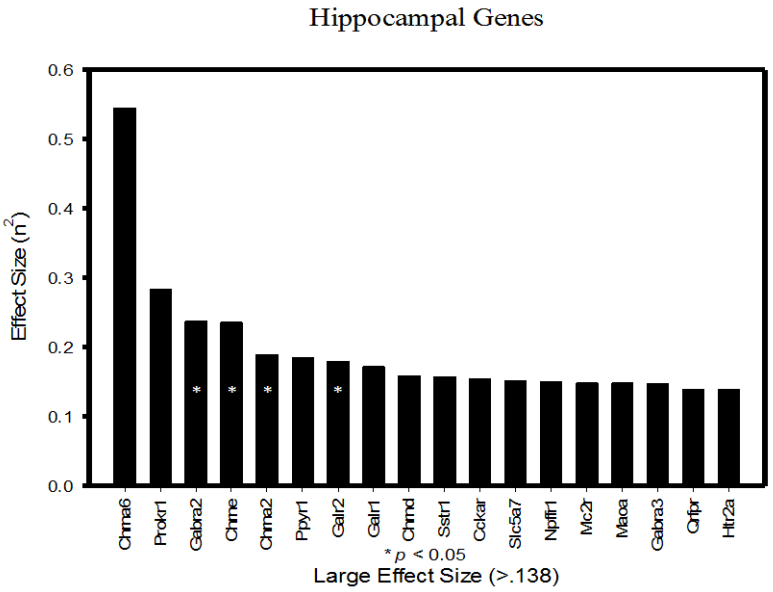


Figure 3 – Effect size of gene changes in the hippocampus
Genes demonstrating a large effect size (η^2 > .138) in the hippocampus as described in the Statistical Analysis; *Significant p-value < 0.05.

Amygdala

Genes in the amygdala with an effect size greater than 0.138 are displayed in Figure 4. In accordance with Figure 4, Table 2 summarizes between group differences in gene expression and fold changes within the amygdala. The *Gabra3* gene was found to be significant: p = .043, η^2 = .191. The P-V group had the highest mean (M = 4.05) and the P-M group had the lowest (M =2.66), however as per the post hoc test, this pairwise comparison was not significant.

The following genes were not found to be statistically significant: *Th*, *Chrna4*, *Sstr1*, *Chrnd*, *Tacr3*, *Prlhr*, *Adra1a*, *Chrnb3*, *Cckbr*, *Gabrd*, *Chrnb1*, and *Gabrg1*. However, based on the large effect size used in the study these genes are included in Table 2. For the *Th* gene (p = .077, η^2 = .188), we found the C-M group had the highest mean (M = 11.34) and the P-T group had the lowest (M = 9.88). Examining the *Chrna4* gene (p = .057, η^2 = .182), the P-V group had the highest mean (M =6.19) and the P-T group had the lowest (M = 4.71). For the *Sstr1* gene (p = .065, η^2 = .177), the P-V group had the highest mean (M = 5.77) and the C-M+T group had the lowest (M = 4.49). Though not significant, for the *Chrnd* gene (p = .126, η^2 = .176) the P-V group had the highest mean (M = 16.97) and the C-M group had the lowest (M = 13.73). Analysis of the *Tacr3* gene (p = .115, η^2 = .16) showed the C-M group had the highest mean (M = 7.59) and the P-T group had the lowest (M = 6.17). Evaluating the *Prlhr* gene (p = .15, η^2 = .156), the C-M group had the highest mean (M = 11.33) and the P-T group had the lowest (M = 9.6). For the *Adra1a* gene (p = .131, η^2 = .155), the P-M+T group had the highest mean (M = 6.39) and the P-M group had the lowest (M = 5.61). Concerning the *Chrnb3* gene (p = .294, η^2 = .149), the P-M+T group had the highest mean (M = 13.02) and the C-M+T group had the lowest (M = 10.55). Evaluation of the *Cckbr* gene (p = .145, η^2 = .149) showed the P-V group had the highest mean (M = 6.51) and the P-T group had the lowest (M = 5.16). For the *Gabrd* gene (p = .148, η^2 = .146), the P-V group had the highest mean (M = 5.03) and the C-M+T group had the lowest (M = 4.14). Analysis of the *Chrnb1* gene (p = .426, η^2 = .100) showed the C-T group had the highest mean (M = 8.63) and the P-V group had the lowest (M = 7.82). Lastly, for the *Gabrg1* gene (p = .196, η^2 = .14), we found the C-M group had the highest mean (M = 3.01) and the P-T group had the lowest (M = 2.24).

Gene	#Group Comparison	Fold Change	Effect Size	P-value	Description of Gene
Gabra3	P-V vs. P-M	1.39	.191	.043	Gamma-aminobutyric acid (GABA) A receptor, alpha 3
Th	C-M vs. P-T	1.46	.188	.077	Tyrosine hydroxylase
Chrna4	P-V vs. P-T	1.48	.182	.057	Cholinergic receptor, nicotinic, alpha 4 (neuronal)
Sstr1	P-V vs. C-M+T	1.28	.177	.065	Somatostatin receptor 1
Chrnd	P-V vs. C-M	3.24	.176	.126	Cholinergic receptor, nicotinic, delta (muscle)
Tacr3	C-M vs. P-T	1.42	.160	.115	Tachykinin receptor 3
Prlhr	C-M vs. P-T	1.73	.156	.150	Prolactin releasing hormone receptor
Adra1a	P-M+T vs. P-M	.78	.155	.131	Adrenoceptor alpha 1A
Chrnb3	P-M+T vs. C-M+T	2.47	.149	.294	Cholinergic receptor, nicotinic, beta 3 (neuronal)
Cckbr	P-V vs. P-T	1.35	.149	.145	Cholecystokinin B receptor
Gabrd	P-V vs. C-M+T	.89	.146	.148	Gamma-aminobutyric acid (GABA) A receptor, delta
Chrnb1	C-T vs. P-V	.81	.143	.182	Cholinergic receptor, nicotinic, beta 1 (muscle)
Gabrg1	C-M vs. P-T	.77	.140	.196	Gamma-aminobutyric acid (GABA) A receptor, gamma 1

Table 2 – Between group changes in gene expression within the amygdala
#Group comparison column presents the highest and lowest mean; *Significant p-value < 0.05. PTSD Vehicle (P-V); PTSD Midazolam (P-M); PTSD Tetrahydropalmatine (P-T); PTSD Midazolam+ Tetrahydropalmatine (P-M+T); Control Vehicle (C-V); Control Midazolam (C-M); Control Tetrahydropalmatine (C-T); Control Midazolam+ Tetrahydropalmatine (C-M+T); Large effect size > 0.138; Significant p-value < 0.05; All genes were confirmed via the National Center for Biotechnology Information (NCBI) database.

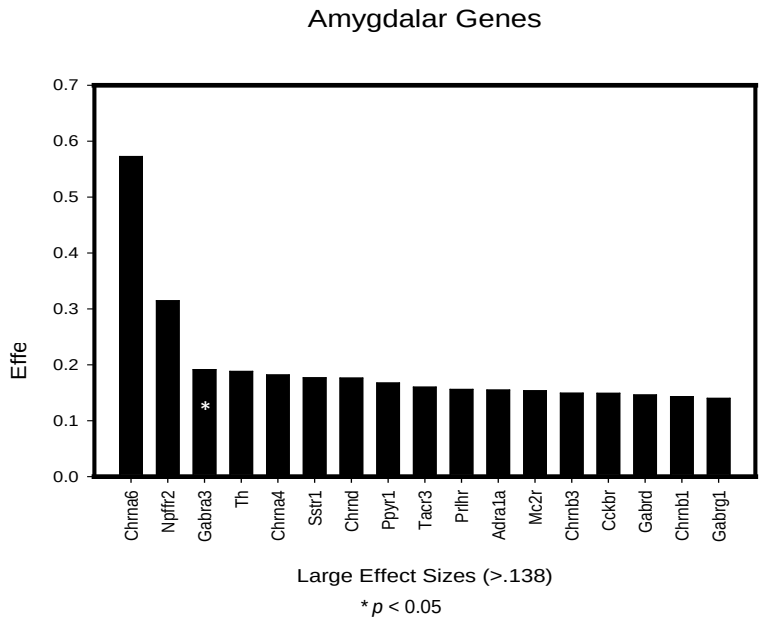


Figure 4 - Effect size of gene changes in the amygdala
Genes demonstrating a large effect size ($\eta^2 > .138$) in the amygdala as described in the Statistical Analysis; *Significant p-value < 0.05.

SPECIFIC AIMS AND RESERCH QUESTIONS – L-Th
The aims of this study were to determine the effects of L-Th in a PTSD rodent model. Specifically, the aims and their corresponding research questions were as follows:

Aim #1: Determine the effects of L-Theanine (L-Th) on anxiety.
Question# 1: Is there a significant difference in the anxiolytic effects between the groups?

Aim# 5: Determine the possible interaction effects of L-Th with midazolam on <u>anxiety</u>, locomotion, memory, and hyperarousal or startle.
Question#5: Are there significant differences or interaction effects between groups regarding the above neurobehavioral effects (<u>anxiety</u>, locomotion, memory, hyperarousal)?

Data regarding weight gain between the 40 control (non-stressed) and 40 PTSD (stressed) rats were significantly different ($p < .001$), where the control rats gained an average of 55.4 grams compared to 37.4 grams for the PTSD rats over the 10 post stress days. All neurobehavior data were analyzed using a two-tailed multivariate analysis of variance (MANOVA). A determination of statistical significance in neurobehavior using L-Theanine in a rodent model was made. If significance was found, a LSD post hoc was used.

Elevated Plus Maze:

Analysis of the ratio open arm time to total time on EPM revealed a statistically significant difference between the control midazolam and PTSD control group ($\rho = .004$). For the one-way ANOVA, significant between group differences were obtained: $F(7,72) = 2.62$, $p = .018$, $\eta^2 = .203$ (20.3% of the variability in the outcome was

attributable to between-group differences). The C-M group had the highest mean (M = 36.04) and the P-V group had the lowest (M=10.52). Given the homogeneity of variance assumption was not met (p=.004), the Games-Howell MCP was performed and no pairwise comparisons were significant. Given outliers and/or slight non-normality of the distribution, the investigators used the Kruskal-Wallis test and, as with the ANOVA, significance was obtained: $\chi^2(7) = 22.65$, $p = .002$. Moreover, a square root transformation did improve the distribution of this outcome, and significance was still obtained per the ANOVA: $F(7,72) = 3.32$, $p = .004$, $\eta^2 = .244$ and per the Tukey post hoc test, there is one pairwise significant comparison, that being CM (higher mean) vs. PV (See Figure 3, 4 and Table 4).

Group	Sample Size	Mean Open Arm Entries	Open Arm Entries SEM	Mean Ratio Open Arm /Total Time	Ratio Open Arm /Total Time SEM
Control Vehicle	10	8.00	1.23	12.83	2.39
Control L-Theanine	10	10.00	1.63	12.61	2.36
Control Midazolam	10	4.40	0.96	36.04	9.76
Control Midazolam + L-Theanine	10	7.60	1.90	15.42	6.85
PTSD Vehicle	10	6.70	1.25	10.52	1.64
PTSD L-Theanine	10	16.10	1.85	17.53	2.41
PTSD Midazolam	10	6.70	1.23	28.84	8.75
PTSD Midazolam + L-Theanine	10	10.60	2.12	27.84	5.66

Table 4. Open Arm Entries & Ratio of Open Arm Time in groups of rats in the Elevated Plus Maze, SEM=Standard Error of the Mean

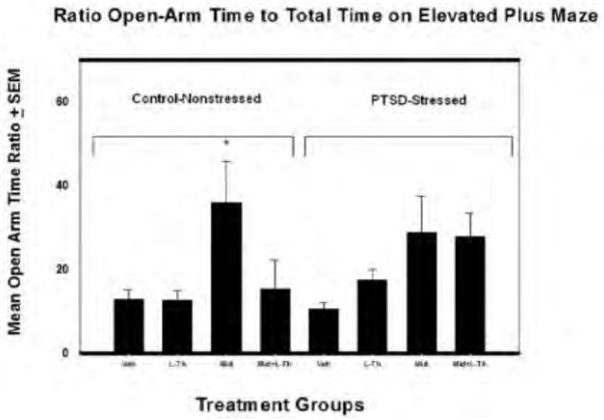


Figure 3. Ratio Open-Arm Time to Total Time on Elevated Plus Maze shows the calculated ratio of time that the rat spent on the open arm compared to the total time on the maze. X axis is treatment groups and Y axis is mean open arm time ratio in seconds. Veh=Vehicle, L-Th= L-Theanine, Mid=Midazolam, Mid+L- Th=Midazolam and L-Theanine, SEM=Standard Error of the Mean

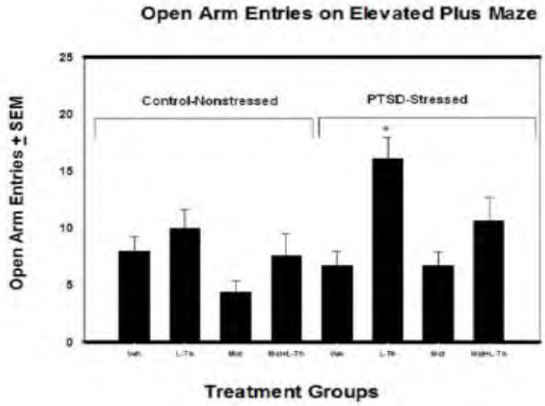


Figure 4. Open-Arm Entries on Elevated Plus Maze shows the number of times the rat ventured onto the open arm on the elevated plus maze. X axis is treatment groups and Y axis is mean open arm time in seconds. Veh=Vehicle, L-Th= L-Theanine, Mid=Midazolam, Mid+L-Th=Midazolam and L-Theanine, SEM=Standard Error of the Mean

Aim# 2: Determine the effects of L-Th on locomotion.
Question#2: Is there a significant difference in locomotion between the groups?

Aim# 5: Determine the possible interaction effects of L-Th with midazolam on anxiety, locomotion, memory, and hyperarousal or startle.
Question#5: Are there significant differences or interaction effects between groups regarding the above neurobehavioral effects (anxiety, locomotion, memory, hyperarousal)?

No data collected regarding locomotion.
Aim# 3: Determine the effects of L-Th on memory.
Question#2: Is there a significant difference in memory between the groups?

Aim# 5: Determine the possible interaction effects of L-Th with midazolam on anxiety, locomotion, memory, and hyperarousal or startle.
Question#5: Are there significant differences or interaction effects between groups regarding the above neurobehavioral effects (anxiety, locomotion, memory, hyperarousal)?

Morris Water Maze

In the MWM test, there were no statistically significant differences found between groups when looking at distance, mean speed, and entries to the platform area (Zone 3). For the one-way ANOVA, significance between group differences were obtained: F (7,72) =5.12, $p < .05$, $\eta^2 = .332$ (33.2% of the variability in the outcome was attributable to between-group differences). The P-M+L group had the highest mean ($M = 10.6$) and the C-M group had the lowest (M=4.4). Per the post hoc tests (i.e., Tukey HSD) the following pairwise comparisons were significant. P-L had a higher mean than C-M, C-V, C-M+L, P-V, and P-M (See Table 5 and Figure 5).

Group	Sample Size	Mean Time in Zone 3	SEM
Control Vehicle	10	16.370	1.08
Control L-Theanine	10	18.830	1.23
Control Midazolam	10	17.230	1.89
Control Midazolam + L-Theanine	10	16.320	1.89
PTSD Vehicle	10	16.290	1.99
PTSD L-Theanine	10	16.680	1.26
PTSD Midazolam	10	17.970	2.39
PTSD Midazolam + L-Theanine	10	15.670	1.09

Table 5. Morris Water Maze time spent by each group of rats in Zone 3 (platform location), SEM=Standard Error of the Mean

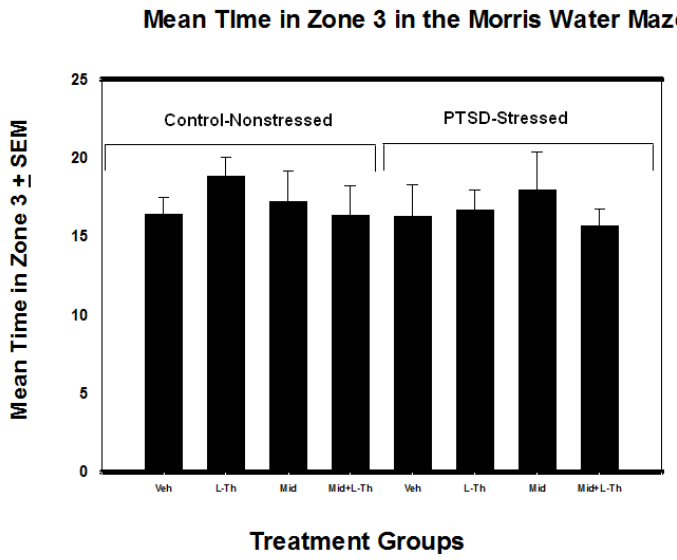


Figure 5. Mean time in Zone 3 in the Morris Water Maze. X axis is treatment groups and Y axis is mean open arm time in seconds. Veh=Vehicle, L-Th= L-Theanine, Mid=Midazolam, Mid+L-Th=Midazolam and L-Theanine, SEM=Standard Error of the Mean

Aim# 4: Determine the effects of L-Th on hyperarousal or startle.
Question#4: Is there a significant difference in hyperarousal between the groups?

Aim# 5: Determine the possible interaction effects of L-Th with midazolam on anxiety, locomotion, memory, and hyperarousal or startle.
Question#5: Are there significant differences or interaction effects between groups regarding the above neurobehavioral effects (anxiety, locomotion, memory, hyperarousal)?

Unable to evaluate because of the malfunction of the equipment.

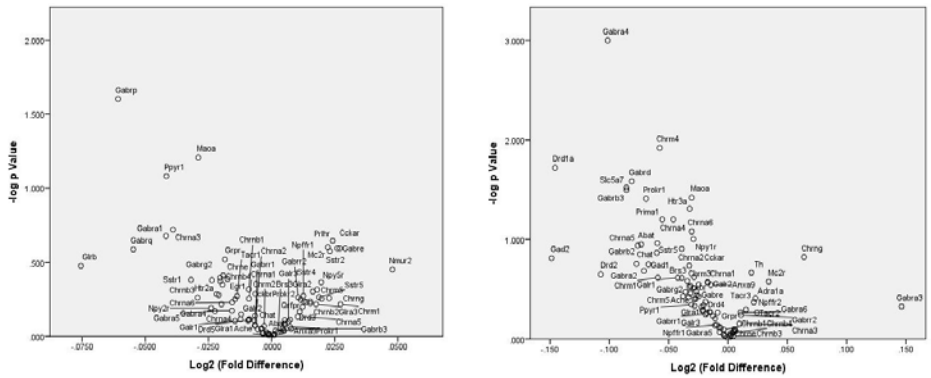
Aim# 5: Determine the possible interaction effects of L-Th with midazolam on anxiety, locomotion, memory, and hyperarousal or startle.
Question#5: Are there significant differences or interaction effects between groups regarding the above neurobehavioral effects (anxiety, locomotion, memory, hyperarousal)?

See data described under Aims 1-4

Aim# 6: Determine the effects of L-Th on gene expression in the brain.
Questions# 6: Are there significant differences in gene expression and regulation in the hippocampus between the groups?
Questions# 7: Are there significant differences in gene expression and regulation in the amygdala between the groups?

Results

Eighty-eight genes were investigated in this study. Data analysis showed a number of significant differences in gene expression in both the hippocampus and amygdala. Volcano plots graphically display the relationship of –log10 p-value and the log2 fold change for the combined PTSD treatment groups and nonstressed treatment groups for all of the genes (Figure 2). For the 8-group one-way ANOVA, a large effect size ($\eta^2 > .138$) was chosen to signify between-group difference in gene expression. A Tukey's post hoc test was performed to further delineate significance between groups.



2A **2B**

Figure 2 – (A) Differential expression of hippocampal and (B) amygdalar mRNA between groups
Volcano plot between Log2 fold change on the x-axis (for neurotransmitter receptors and regulators in Tetrahydropalmatine/midazolam treated groups 40 PTSD-stressed vs. 40 nonstressed (control) groups) vs. –log of p-value on the y-axis. Eighty male Sprague-Dawley rats were injected subcutaneously 30 minutes prior to evaluation of their performance on neurobehavioral tests. The rats were then euthanized and cDNA prepared from the hippocampus (A) and amygdala (B) were subjected to RT^2 profiler PCR array for rat neurotransmitter receptor and

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regulator analysis, as described in Materials and Methods. PCR Array profiles were performed for the expression of 88 genes potentially involved in PTSD and/or rat neurotransmitter receptors and neurotransmitter regulation.

Hippocampus

In the hippocampus, the genes with an effect size > 0.138 are displayed in Figure 3. In accordance with Figure 3, Table 1 summarizes between group changes in gene expression and fold changes in the hippocampus. The following genes in the hippocampus showed significant differences between groups: *Gabra2*, *Chrne*, *Chrna2*, and *Galr2*. The *Gabra2* gene was significant: $p = .006$, $\eta^2 = .238$. Post hoc tests revealed that the C-M+T group had a significantly lower mean ($M = 1.39$) than each of the following: (1) P-M ($M = 2.11$), (2) C-V ($M = 2.04$), and (3) P-V ($M = 2.04$). We found the *Chrne* gene to be significant: $p = .007$, $\eta^2 = .236$. Multiple comparisons showed two significant tests: the P-M group had a higher mean ($M = 9.29$) than the (1) C-T ($M = 8.10$) and (2) C-M ($M = 8.28$) groups. The *Chrna2* gene was significant: $p = .031$, $\eta^2 = .189$. The post hoc test found two significant pairwise comparisons: The C-M+T group had a significantly lower mean ($M = 6.18$) than the (1) C-T ($M = 6.87$) and (2) P-V ($M = 6.96$) groups. The *Galr2* gene was found to be significant: $p = .042$, $\eta^2 = .18$. Though none of the multiple comparison tests were significant, the C-V group had the highest mean ($M = 11.04$) and the C-T group had the lowest ($M = 10.0$).

The following genes in the hippocampus did not show statistical significant differences between groups: *Chrna6*, *Galr1*, *Chrnd*, *Sstr1*, *Cckar*, *Slc5a7*, *Npffr1*, *Maoa*, *Gabra3*, *Qrfpr*, and *Htr2a*. However, secondary to a large effect size, these genes are presented in Table 1. For the *Chrna6* gene ($p = .213$, $\eta^2 = .545$), the C-V group had the highest mean ($M = 15.14$) and the C-M+T group had the lowest ($M = 6.91$). Concerning the *Galr1* gene ($p = .053$, $\eta^2 = .172$), the P-M group had the highest mean ($M = 11.94$) and the P-V group had the lowest ($M = 9.75$). Though not significant, for the *Chrnd* gene ($p = .131$, $\eta^2 = .159$) the P-M+T group had the highest mean ($M = 17.19$) and the C-M+T group had the lowest ($M = 14.68$). After evaluating the *Sstr1* gene ($p = .083$, $\eta^2 = .158$), the P-M group had the highest mean ($M = 7.29$) and the C-M group had the lowest ($M = 6.05$). While not significant, for the *Cckar* gene ($p = .113$, $\eta^2 = .155$) the P-V group had the highest mean ($M = 13.30$) and the C-T group had the lowest ($M = 11.27$). Examining the *Slc5a7* gene ($p = .102$, $\eta^2 = .152$), the P-M+T group had the highest mean ($M = 9.88$) and the C-T group had the lowest ($M = 8.79$). For the *Npffr1* gene ($p = .10$, $\eta^2 = .151$), the P-M group had the highest mean ($M = 11.56$) and the C-M+T group had the lowest ($M = 10.25$). The *Maoa* gene did not show significance ($p = .107$, $\eta^2 = .148$), however the C-V group had the highest mean ($M = 3.23$) and the C-M+T group had the lowest ($M = 2.83$). Analysis of the *Gabra3* gene ($p = .109$, $\eta^2 = .148$) revealed the P-M group had the highest mean ($M = 5.64$) and the C-T group had the lowest ($M = 4.38$). For the *Qrfpr* ($p = .136$, $\eta^2 = .14$), the P-M group had the highest mean ($M = 10.89$) and the C-T group had the lowest ($M = 9.35$). Lastly, the *Htr2a* gene was not significant ($p = .139$, $\eta^2 = .139$), however the P-M group had the highest mean ($M = 8.15$) and the C-M+T group had the lowest ($M = 6.91$).

Gene	#Group Comparison	Fold Change	Effect Size	P-value	Description of Gene
Gabra2	C-M+T vs. P-M C-M+T vs. C-V C-M+T vs. P-V	.72 .68 .68	.238	.006	Gamma-Aminobutyric Acid (GABA) A Receptor, Alpha 2
Chrne	P-M vs. C-T P-M vs. C-M	1.19 .98	.236	.007	Cholinergic Receptor, Nicotinic, Epsilon (Muscle)
Chrna2	C-M+T vs. C-T C-M+T vs. P-V	.69 .78	.189	.031	Cholinergic receptor, nicotinic, alpha 2
Galr2	C-V vs. C-T	1.04	.180	.042	Galanin receptor 2
Chrna6	C-V vs. C-M+T	8.23	.545	.213	Cholinergic Receptor, Nicotinic, Alpha 6 (Neuronal)
Galr1	P-M vs. P-V	2.19	.172	.053	Galanin receptor 1
Chrnd	P-M+T vs. C-M+T	2.51	.151	.131	Cholinergic receptor, nicotinic, delta (muscle)
Sstr1	P-M vs. C-M	1.24	.158	0.083	Somatostatin receptor 1
Cckar	P-V vs. C-T	2.03	.155	.113	Cholecystokinin A receptor

Slc5a7	P-M+T vs. C-T	1.09	.152	.102	Solute carrier family 5 (choline transporter), member 7
Npffr1	P-M vs. C-M+T	1.31	.151	.100	Neuropeptide FF receptor 1
Maoa	C-V vs. C-M+T	.40	.148	.107	Monoamine oxidase A
Gabra3	P-M vs. C-T	1.26	.148	.109	Gamma-aminobutyric acid (GABA) A receptor, alpha 3
Qrfpr	P-M vs. C-T	1.54	.140	.136	Pyroglutamylated RFamide peptide receptor
Htr2a	P-M vs. C-M+T	1.24	.139	.139	5-hydroxytryptamine (serotonin) receptor 2A, G protein-coupled

Table 1 – Between group changes in gene expression within the hippocampus
#Group comparison column presents the highest and lowest mean; *Significant p-value < 0.05. PTSD Vehicle (P-V); PTSD Midazolam (P-M); PTSD tetrahydropalmatine (P-T); PTSD tetrahydropalmatine + midazolam (P-M+T); Control Vehicle (C-V); Control Midazolam (C-M); Control Tetrahydropalmatine (C-T); Control tetrahydropalmatine + midazolam (C-M+T); Large effect size > 0.138; Significant p-value < 0.05; All genes were confirmed via the National Center for Biotechnology Information (NCBI) database.

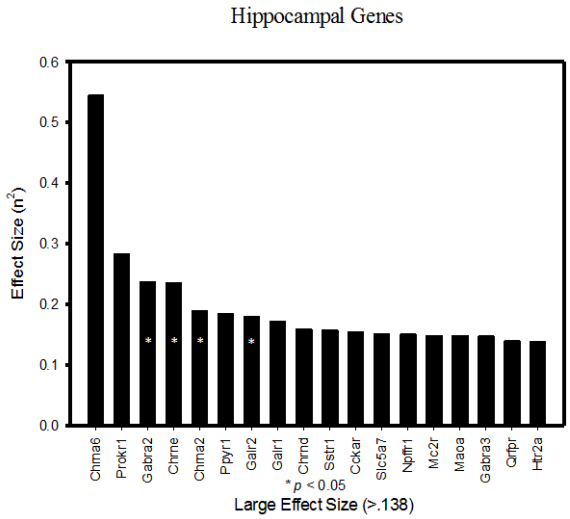

Figure 3 – Effect size of gene changes in the hippocampus
Genes demonstrating a large effect size ($\eta^2 > .138$) in the hippocampus as described in the Statistical Analysis; *Significant p-value < 0.05.

Amygdala

Genes in the amygdala with an effect size greater than 0.138 are displayed in Figure 4. In accordance with Figure 4, Table 2 summarizes between group differences in gene expression and fold changes within the amygdala. The *Gabra3* gene was found to be significant: $p = .043$, $\eta^2 = .191$. The P-V group had the highest mean ($M = 4.05$) and the P-M group had the lowest ($M = 2.66$), however as per the post hoc test, this pairwise comparison was not significant.

The following genes were not found to be statistically significant: *Th*, *Chrna4*, *Sstr1*, *Chrnd*, *Tacr3*, *Prlhr*, *Adra1a*, *Chrnb3*, *Cckbr*, *Gabrd*, *Chrnb1*, and *Gabrg1*. However, based on the large effect size used in the study these genes are included in Table 2. For the *Th* gene ($p = .077$, $\eta^2 = .188$), we found the C-M group had the highest mean ($M = 11.34$) and the P-T group had the lowest ($M = 9.88$). Examining the *Chrna4* gene ($p = .057$, $\eta^2 = .182$), the P-V group had the highest mean ($M = 6.19$) and the P-T group had the lowest ($M = 4.71$). For the *Sstr1* gene ($p = .065$, $\eta^2 = .177$), the P-V group had the highest mean ($M = 5.77$) and the C-M+T group had the lowest ($M = 4.49$). Though not significant, for the *Chrnd* gene ($p = .126$, $\eta^2 = .176$) the P-V group had the highest mean ($M = 16.97$)

and the C-M group had the lowest (M = 13.73). Analysis of the *Tacr3* gene (p = .115, η^2 = .16) showed the C-M group had the highest mean (M = 7.59) and the P-T group had the lowest (M = 6.17). Evaluating the *Prlhr* gene (p = .15, η^2 = .156), the C-M group had the highest mean (M = 11.33) and the P-T group had the lowest (M = 9.6). For the *Adra1a* gene (p = .131, η^2 = .155), the P-M+T group had the highest mean (M = 6.39) and the P-M group had the lowest (M = 5.61). Concerning the *Chrnb3* gene (p = .294, η^2 = .149), the P-M+T group had the highest mean (M = 13.02) and the C-M+T group had the lowest (M = 10.55). Evaluation of the *Cckbr* gene (p = .145, η^2 = .149) showed the P-V group had the highest mean (M = 6.51) and the P-T group had the lowest (M = 5.16). For the *Gabrd* gene (p = .148, η^2 = .146), the P-V group had the highest mean (M = 5.03) and the C-M+T group had the lowest (M = 4.14). Analysis of the *Chrnb1* gene (p = .426, η^2 = .100) showed the C-T group had the highest mean (M = 8.63) and the P-V group had the lowest (M = 7.82). Lastly, for the *Gabrg1* gene (p = .196, η^2 = .14), we found the C-M group had the highest mean (M = 3.01) and the P-T group had the lowest (M = 2.24).

Gene	**#Group Comparison**	**Fold Change**	**Effect Size**	**P-value**	**Description of Gene**
Gabra3	P-V vs. P-M	1.39	.191	.043	Gamma-aminobutyric acid (GABA) A receptor, alpha 3
Th	C-M vs. P-T	1.46	.188	.077	Tyrosine hydroxylase
Chrna4	P-V vs. P-T	1.48	.182	.057	Cholinergic receptor, nicotinic, alpha 4 (neuronal)
Sstr1	P-V vs. C-M+T	1.28	.177	.065	Somatostatin receptor 1
Chrnd	P-V vs. C-M	3.24	.176	.126	Cholinergic receptor, nicotinic, delta (muscle)
Tacr3	C-M vs. P-T	1.42	.160	.115	Tachykinin receptor 3
Prlhr	C-M vs. P-T	1.73	.156	.150	Prolactin releasing hormone receptor
Adra1a	P-M+T vs. P-M	.78	.155	.131	Adrenoceptor alpha 1A
Chrnb3	P-M+T vs. C-M+T	2.47	.149	.294	Cholinergic receptor, nicotinic, beta 3 (neuronal)
Cckbr	P-V vs. P-T	1.35	.149	.145	Cholecystokinin B receptor
Gabrd	P-V vs. C-M+T	.89	.146	.148	Gamma-aminobutyric acid (GABA) A receptor, delta
Chrnb1	C-T vs. P-V	.81	.143	.182	Cholinergic receptor, nicotinic, beta 1 (muscle)
Gabrg1	C-M vs. P-T	.77	.140	.196	Gamma-aminobutyric acid (GABA) A receptor, gamma 1

Table 2 – Between group changes in gene expression within the amygdala
#Group comparison column presents the highest and lowest mean; *Significant p-value < 0.05. PTSD Vehicle (P-V); PTSD Midazolam (P-M); PTSD Tetrahydropalmatine (P-T); PTSD Midazolam+ Tetrahydropalmatine (P-M+T); Control Vehicle (C-V); Control Midazolam (C-M); Control Tetrahydropalmatine (C-T); Control Midazolam+ Tetrahydropalmatine (C-M+T); Large effect size > 0.138; Significant p-value < 0.05; All genes were confirmed via the National Center for Biotechnology Information (NCBI) database.

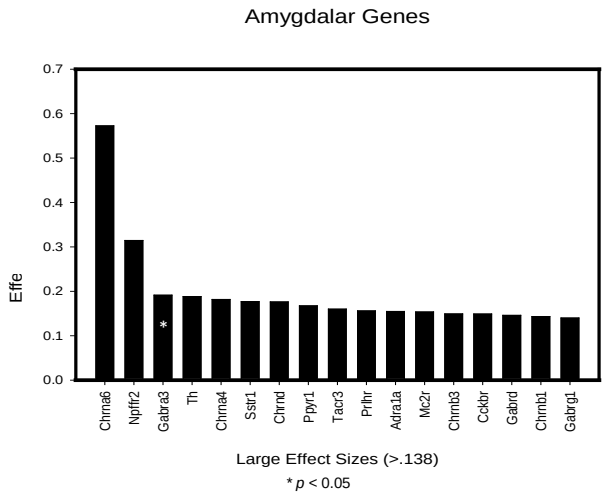

Figure 4 - Effect size of gene changes in the amygdala
Genes demonstrating a large effect size ($\eta^2 > .138$) in the amygdala as described in the Statistical Analysis; *Significant p-value < 0.05.

Relationship of current findings to previous findings:
All of the data and findings described above in the "**Progress Towards Achievement of Specific Aims of the Study or Project**" are new as these studies were the first of their kind. However, in our previous work, significant findings showed that THP and L-Th have been found to decrease anxiety and motor movements in the laboratory rat. There are no current published data directly related to altered gene expression with the use of THP or L-Th in PTSD.

Effect of problems or obstacles on the results:

1. The restrainers were too big to restrain the rats at the beginning, so we modified the restrainers and the shocking apparatus to adjust for the size of the rats.

2. The Acoustic Startle Reflex equipment was not operational during the experiments and the data obtained was not accurate. Therefore, we did not analyze these data or include them in our findings.

Limitations:

1. This was an animal study in the basic sciences and not generalizable to the human population, but these data can be used as part of the foundation of our sciences and building blocks of our practice.

2. The sample population for this study was male rats due to time and financial constraints. The use of male rats also eliminated the potential confounding variables arising from estrus cycles. A study that compares male and female rats would more than double the financial requirements and work load of the proposal, including the hiring of additional personnel.

3. A one-time dose for these herbal supplements was chosen for these experiments. The timing of administration, such as multidose or prophylaxis, may yield different results in preventing or aborting PTSD symptomology. It is known that some treatments of PTSD (i.e. antidepressants) may require an extended period of time to affect

neurobehavior. Future studies of THP and or L-Th may utilize our current validated model with an extended dosing period to obtain steady state for the period of time needed to alter neurobiology.

Conclusion:

SPECIFIC AIMS

1. Determine the effects of tetrahydropalmatine and l-theanine on anxiety.
2. Determine the effects of tetrahydropalmatine and l-theanine on locomotion
3. Determine the effects of tetrahydropalmatine and l-theanine on memory.
4. Determine the effects of tetrahydropalmatine and l-theanine on hyperarousal or startle.
5. Determine the possible interaction effects of tetrahydropalmatine and l-theanine with midazolam on anxiety, locomotion, memory, and hyperarousal or startle.
6. Determine the effects of tetrahydropalmatine and l-theanine on gene expression in the brain.

The purpose of this study was to investigate THP and L-Th and their effects on PTSD induced neurobehavior in the rodent model. This was the first study evaluating THP in a PTSD model. The ability of THP administered to a rodent with induced PTSD to alleviate the symptoms of anxiety, locomotion, and memory was studied. Rats were separated into PTSD and non-stressed groups and administered various pharmacological agents. Through use of the Restraint Tail-Shock device, PTSD was induced in male Sprague-Dawley rats. After adequate time to develop symptomatology, depending upon group assignment, rats were given a pharmacological intervention including THP or L-Th. The rats were then subjected to a series of neurobehavioral testing in order to evaluate anxiety (EPM), spatial memory (MWM), and Acoustic Startle Reflex (ASR). Our theoretic framework and PTSD Disease Induction Model were validated based on this research. This model will be useful in future research applications. The PTSD induction model in which rats were exposed to a two-hour immobilization and tail-shock session over three days was validated in our study.

RT-PCR analysis revealed significant changes between groups in several genes implicated in a variety of disorders ranging from PTSD, anxiety, mood disorders, and substance dependence. These data further elucidate the transcriptomic footprint of PTSD in the rodent amygdala and hippocampus as well as transcriptome changes effected by THP, L-Th, and midazolam interventions. This understanding of the effect of PTSD at the level of mRNA transcription contributes to a more complete understanding of the overall metabonomics of PTSD and may allow for more targeted pharmacologic intervention in the future.

The next step to consider in clarifying the molecular mechanism of PTSD may be to characterize PTSD-induced changes to the rat proteome. Changes in mRNA expression are often linked to changes in protein expression, but not in a predictable manner. Small changes in mRNA expression can lead to variable changes in corresponding protein expression. We propose repeating the current experiment using protein assay methods to correlate changes in mRNA expression with changes in protein expression induced by PTSD and the effects of THP, L-Th, and midazolam on these changes. Multiple methods and computational approaches to protein assays are available.

We anticipate further investigation into the network of gene relationships between control and PTSD-induced rats using bioinformatic methods such as gene enrichment analysis and network enrichment analysis. Characterization of a comprehensive proteomic network in both control and PTSD-induced rats will allow for comparative proteomic analysis that may lead to the identification of biomarkers for PTSD susceptibility as well as potential targets for pharmacologic interventions. Additionally, we anticipate investigation into the chemical components of other herbals or pharmaceuticals that may have similar effects on gene expression at the level of mRNA transcription, with a particular emphasis on those with demonstrated efficacy in treating PTSD.

These descriptive gene expression findings provide insight into the possible genetic basis for PTSD in the rodent amygdala and hippocampus as well as gene changes resulting from THP, L-Th, and midazolam interventions. The results contribute to the body of knowledge in understanding the molecular pathology of PTSD, allowing for more specific focused investigations in the future.

Overall, evaluation of the data collected in this study did not support our hypothesis that THP or L-Th may ameliorate the symptoms of PTSD. However, our experimental model was validated through our implemented controls.

The next step to consider in clarifying the molecular mechanism of PTSD may be to characterize PTSD-induced changes to the rat proteome. Changes in mRNA expression are often linked to changes in protein expression, but not in a predictable manner. Small changes in mRNA expression can lead to variable changes in corresponding protein expression. We propose repeating the current experiment using protein assay methods to

correlate changes in mRNA expression with changes in protein expression induced by PTSD and the effects of THP, L-Th, and midazolam on these changes.

While a one-time dose was insufficient in providing a significant decrease in anxiety, a multi-dose regimen may yield more effective results. Future experiments should evaluate a multi-dose or prophylactic regimen. The timing of administration, such as multidose or prophylaxis, may yield different results. It is known that some treatments of PTSD (i.e. antidepressants) may require an extended period of time to affect neurobehavior. Future studies of THP and L-Th may utilize our current validated model with an extended dosing period, to obtain steady state for the period of time needed to alter neurobehavior.

Significance of Study or Project Results to Military Nursing

PTSD is a devastating, debilitating, and costly neuropathologic outcome of war. It is critical that nurses and other health care professionals investigate treatments to ameliorate the neurobehavioral sequelae from PTSD. While the results in this study showed no significant difference between rats, it validated the theoretic framework and PTSD Disease Induction Model. This model will be useful in future research applications. The neurobehavioral data gleaned from this study and future studies is critically important for the translation of bench research to clinical research in moving towards optimizing the treatment of patients with PTSD.

This proposal assists military nurses and other health care personnel in expanding their understanding of the neurobehavioral and basic physiologic and cellular mechanisms responsible for PTSD. Understanding new developments in behavioral and molecular neuroscience is not only fundamental, but relevant to the development of new and innovative treatments or therapies of PTSD in troops, veterans, and family members under the care of the Military Nurse Corps. PTSD and its treatment are unique to the military and critical to the health of military personnel.

Although this was an animal study in the basic sciences and not generalizable to the human population, these data can be used as part of the foundation of our sciences and building blocks of our practice. The next step to consider in clarifying the molecular mechanism of PTSD may be to characterize PTSD-induced changes to the rat proteome. Changes in mRNA expression are often linked to changes in protein expression, but not in a predictable manner. Small changes in mRNA expression can lead to variable changes in corresponding protein expression. We propose repeating the current experiment using protein assay methods to correlate changes in mRNA expression with changes in protein expression induced by PTSD and the effects of THP, L-Th, and midazolam on these changes.

Future studies of THP and L-Th may utilize our current validated model with an extended dosing period to obtain steady state for the period of time needed to alter neurobiology. The meticulous study of the neurobiological effects of THP and L-Th may further define the mechanism of action of these supplements, enabling future research to be more focused.

Changes in Clinical Practice, Leadership, Management, Education, Policy, and/or Military Doctrine that Resulted from Study or Project

None

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Summary of Dissemination

Type of Dissemination	Citation	Date and Source of Approval for Public Release
Published	Ceremuga T., Shellabarger P., Persson T., Fanning M., Galey P., Robinson D., Bertsch S., Ceremuga G., Bentley M. Effects of tetrahydropalmatine on post-traumatic stress disorder-induced changes in rat brain gene expression, *Journal of Integrative Neuroscience*, Vol. 12, No. 4 (December 2013) 1–16.	October 1, 2013 PAO approval
Published	Ceremuga, T., Bentley, M., Anderson, R., Frye, P., Duvall, C., Maan, J., Manjarres, C., Petsche, J., Ceremuga, G. Effects of Tetrahydropalmatine (THP) on PTSD-induced Changes in Rat Neurobehavior, *Plant Science Today*, accepted for publication – February 2014..	October 1, 2013 PAO approval
In Review	Ceremuga, T., Bentley, M., Wolfe, J., Baldwin, S., Onstott, T., Aytes, K., Ferrara, B., Alleyn, M., Fortner, C., Ceremuga, G., Padron, G. Effects of L-Theanine on PTSD-induced Changes in Rat Neurobehavior, *AMEDD Journal*, submitted - in review March 2014.	October 1, 2013 PAO approval
In Press	Ceremuga, T., Bentley, M., Martinson, S., Washington, J., Revels, R., Wojcicki, J., Crawford, D., Edwards, R., Kemper, J., Townsend, W., Ceremuga, G., Padron, G. Effects of L-theanine on Post Traumatic Stress Disorder Induced Changes in Rat Brain Gene Expression, *The Scientific World Journal - Neuroscience*, - In Press May 2014.	October 1, 2013 PAO approval
Published Abstracts	Ceremuga, T., Bentley, M., Shellabarger, P., Persson, T., Fanning, M., Robinson, D., Bertsch, S., Kunz, B., Ceremuga, G. Effects of Tetrahydropalmatine (THP) on PTSD-induced Changes in Rat Brain Gene Expression, *AANA J*, August 2013.	October 1, 2013 PAO approval
	Ceremuga, T., Bentley, M., Anderson, R., Frye, P., Duvall, C., Maan, J., Manjarres, C., Petsche, J., Ceremuga, G. Effects of Tetrahydropalmatine (THP) on PTSD-induced Changes in Rat Neurobehavior, *AANA J*, August 2013.	October 1, 2013 PAO approval
Podium Presentations	Ceremuga, T., Bentley, M., Shellabarger, P., Persson, T., Fanning, M., Robinson, D., Bertsch, S., Kunz, B., Ceremuga, G. Effects of Tetrahydropalmatine (THP) on PTSD-induced Changes in Rat Brain Gene Expression, American Association of Nurse Anesthetists (AANA) Conference, Las Vegas, August 2013.	June 3, 2013 PAO approval

	Ceremuga, T., Bentley, M., Anderson, R., Frye, P., Duvall, C., Maan, J., Manjarres, C., Petsche, J., Ceremuga, G. Effects of Tetrahydropalmatine (THP) on PTSD-induced Changes in Rat Neurobehavior, American Association of Nurse Anesthetists (AANA) Conference, Las Vegas, August 2013.	June 3, 2013 PAO approval
Poster Presentations	Ceremuga, T., Bentley, M., Shellabarger, P., Persson, T., Fanning, M., Robinson, D., Bertsch, S., Kunz, B., Ceremuga, G. Effects of Tetrahydropalmatine (THP) on PTSD-induced Changes in Rat Brain Gene Expression, American Association of Nurse Anesthetists (AANA) Conference, Las Vegas, August 2013.	June 3, 2013 PAO approval
	Ceremuga, T., Bentley, M., Anderson, R., Frye, P., Duvall, C., Maan, J., Manjarres, C., Petsche, J., Ceremuga, G. Effects of Tetrahydropalmatine (THP) on PTSD-induced Changes in Rat Neurobehavior, American Association of Nurse Anesthetists (AANA) Conference, Las Vegas, August 2013.	June 3, 2013 PAO approval

Reportable Outcomes

Reportable Outcome	Detailed Description
Applied for Patent	None
Issued a Patent	None
Developed a cell line	None
Developed a tissue or serum repository	None
Developed a data registry	None

Recruitment and Retention Table

Recruitment and Retention Aspect	Number	
Animals Projected in Grant Application	170	
Animals Purchased	170	
Model Development Animals	10	
Animals Intervention Group / Control or Sham Group	80	80
Intervention Group / Control or Sham Group Animals With Complete Data	80	80
Intervention Group / Control or Sham Group Animals With Incomplete Data	0	0