c-fos Expression in Rodent Model of Traumatic Brain Injury and Anxiety-Like Behavior

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Abstract

Traumatic brain injury (TBI) impacts over one million people in the United States every year and significantly increases an individual's risk of developing a psychiatric disorder. Previous research in our lab has highlighted the role of neuroinflammation in TBI and the development of post-injury anxiety in rodent models. The objective of this study is to characterize the brain regions involved in the anxiety-like behaviors observed in previous studies and in an immediate shock paradigm. The rats were randomly assigned to one of six groups: LFPI+shock, LFPI+no shock, naïve+shock, naïve+no shock, sham-operated, and LFPI+MN166. Lateral fluid percussion injury (LFPI) was used to model TBI in the rodents and shock refers to animals that were shocked in the immediate shock paradigm. The expression of c-fos was measured and compared between groups across multiple brain regions including the hippocampus, insula, amygdala, paraventricular nucleus, central gray, bed nucleus of the stria terminalis, and regions of the prefrontal cortex. The LFPI+shock rats displayed significantly higher freezing behavior in the immediate shock paradigm than all other treatment groups. The results of the c-fos expression measurements partially support previous findings on brain regions involved in anxiety, but are not consistent with the expected pattern of activation based on the behavioral results of the immediate shock paradigm.

Keywords: c-fos expression; traumatic brain injury; anxiety-like behavior

Introduction

Annually, over 1.7 million people in the United States suffer a traumatic brain injury (TBI) (Faul, Xu, Wald, & Coronado, 2010). One of the most common long-term impacts of TBI is an increased risk for psychiatric disorders post-injury, particularly anxiety disorders (Vaishnavi, Rao, & Fann, 2009). Anxiety disorders are classified by a heightened sensitivity to perceived threats which is observed through behavioral, physiological and cognitive responses including increased avoidance, muscle tension, and worrying about future threat (Craske, Rauch, & Ursano, 2009). The prevalence rates for anxiety disorders post-traumatic brain injury are significantly higher than the rates within the general public (Deb, Lyons, Koutzoukis, Ali, & McCarthy, 1999). Despite the large impact, research and understanding of this interaction at a neurobiological level is still limited due to previous and current methodological barriers. By better understanding the neural mechanisms underlying the increased prevalence of anxiety disorders post-injury, more targeted interventions can be developed to address the additional psychiatric challenges faced when trying to reintegrate into society following a TBI.

Previous research on the link between TBI and the development of anxiety disorders has identified several biochemical factors that contribute to this relationship including excessive inflammation resulting from the neuroimmune response to TBI (Rodgers et al., 2012), excitoxicity due to increased glutamatergic action (Reger et al., 2012), and oxidative stress (Prasad & Bondy, 2015). Additionally, research on the underlying structural and functional abnormalities in anxiety disorders indicate hyperactivation in the amygdala and insular cortex (Etkin & Wager, 2007; Paulus & Stein, 2006; Rauch, Shin, & Phelps, 2006; Simmons, Strigo, Matthews, Paulus, & Stein, 2006), diminished hippocampal volume and activation (Bremner et al., 1995; Shin et al., 2004), decreased activation of the medial prefrontal cortex (mPFC),

resulting in reduced inhibition of the amygdala (Britton, Phan, Taylor, Fig, & Liberzon, 2005; Shin & Liberzon, 2009), and decreased gray matter densities and volume in the anterior cingulate cortex (ACC) (Shin & Liberzon, 2009; Woodward et al., 2006; Yamasue et al., 2011). However, in a meta-analysis of functional neuroimaging studies, hypoactivation of the mPFC and ACC was only seen in patients with post-traumatic stress disorder (PTSD) and not in other anxiety-related disorders (Etkin & Wager, 2007). Since PTSD could be a consequence of an event that resulted in the TBI, rat models allow the controlled study of TBI as a physical trauma without the potentially confounding factor of the conscious memory of an emotional trauma. This is conducive to being able to determine if the injury itself results in a brain state that is more likely to acquire fear and what neural factors contribute to this state. Through the analysis of c-fos expression, a proto-oncogene that serves as an indirect marker for neuronal activity (Dragunow & Faull, 1989), this study looks further into the hypothesis of the brain being primed post-injury for increased fear-learning and fear response through examining TBI-induced changes in neuronal activation, with and without exposure to the immediate shock paradigm, in brain regions associated with anxiety disorders. In a previous experiment in the lab, ibudilast (MN166) administration, a phosphodiesterase inhibitor, had neuroprotective effects on levels of inflammation from neuroimmune responses post-TBI (Rodgers et al., 2012; 2014). Following-up on these results, this study also assesses whether MN166 has an effect on c-fos expression post-injury.

Background

Traumatic Brain Injury and Anxiety Disorders: Current Understanding of Neurobiological Basis

Previous studies that have examined the link between TBI and an increased prevalence of anxiety-related disorders have proposed several different biological mechanisms that may underlie this relationship. The three most supported explanations are chronic inflammation, excitotoxicity, and oxidative stress.

Chronic inflammation.

There is prominent evidence for an increase in neuroinflammation due to an immune response following the TBI and its potential effects on the development of anxiety. Inflammation occurs following the injury when microglia and astrocytes are activated to protect the brain from damage (Farina, Aloisi, & Meinl, 2007). However, this response can become toxic if the levels of the pro-inflammatory cytokines remain elevated, causing damage to the tissue and neuronal degeneration (Lehnardt, 2009). Rodgers et al. (2012) found significantly higher levels of astrocyte activation in the insula, hippocampus, and the amygdala as well as increased microglia activation in the insula for brain-injured rats compared to all other groups. The injured group also had significantly higher freezing behavior, supporting a relationship between excessive inflammation and anxiety-like behavior. Additionally, administration of an anti-inflammatory drug (MN166) mitigated the level of inflammation and decreased anxiety-like behavior in the brain-injured rats (Rodgers et al., 2012). In a later study, treatment with MN166 was shown to reverse anxiety behavior through glial attenuation six months post-injury (Rodgers et al., 2014).

Chronic levels of inflammation have been shown repeatedly to exist in people with PTSD through findings of elevated levels of pro-inflammatory cytokines systemically, particularly IL-1beta and TNF-alpha (Hoge, Brandstetter, & Moshier, 2009; Känel et al., 2007; Spivak, Shohat, Mester, Avraham, & Gil-Ad, 1997). TBI can result in a similar pro-inflammatory state due to activating an immune response. In a study of patients undergoing surgery following brain trauma, immunohistochemistry was used to assess inflammatory responses at times varying from three hours to five days post-injury. The findings provide evidence for a delayed inflammatory response with greater levels of reactive microglia at five days out from the trauma (Holmin & Höjeberg, 2004). Similarly, time-dependent inflammatory responses were seen in individuals assessed over a 30-week period following a closed head injury. Granulocytes were detected early on in the response whereas leukocytes were not noticeable until a minimum of one day post-injury (Hausmann, Kaiser, Lang, & Bohnert, 1999). The consequence of a long-term pro-inflammatory state is the potential for neuronal degeneration that can cause dysfunction and contribute to the development of psychiatric disorders.

Excitotoxicity.

In addition to inflammation, hyperexcitation of the glutamatergic system has been implicated in the pathophysiology of both PTSD and TBI. Excitotoxic conditions can result from hyperactivation due to high levels of extracellular glutamate causing increased levels of intracellular Ca^{2+} ions. The hyperactivation causes a large influx of Na^{+} ions which results in dysregulation of ionic gradients and can lead to edema and cell death (Yi & Hazell, 2006). During excitation there is a decrease in activity of the inhibitory amino acid gamma aminobutyric acid (GABA) and an increase in N-methyl-D-aspartate (NMDA) receptor levels which bind glutamate, a neurotransmitter that has predominantly excitatory action. These changes can be induced by stress and have been shown to be mediated by a hormonal cascade in

PTSD (Nair & Ajit, 2008). Glutamate triggers the release of corticotropin-releasing factor (CRF) in the median eminence of the hypothalamus which activates the hypothalamic-pituitary-adrenal (HPA) axis, leading to an increase in adrenocorticotrophin hormone (ACTH) and cortisol (or corticosterone in rats). Because the neurons containing CRF are projecting from the paraventricular nucleus (PVN), activation of the PVN will activate the HPA axis. The dysregulation of the HPA axis has been linked to symptoms of hyperarousal, re-experiencing trauma, decreased fear extinction, and avoidance. When glutamate action is blocked, symptoms significantly decrease, providing further evidence for hyperactivation of the glutamatergic system in PTSD (Nair & Ajit, 2008). Additionally, lower levels of GABA have been shown in the insular, parieto-occipital, and temporal cortices in people with PTSD compared to people without PTSD (Meyerhoff, Mon, Metzler, & Neylan, 2014; Rosso, Weiner, & Crowley, 2014). Decreased GABA and increased glutamatergic action has also been linked to anxiety symptoms in social anxiety disorder and panic disorder (Goddard, Mason, & Almai, 2001; Pollack, Jensen, Simon, & Kaufman, 2008).

Increased levels of NMDA receptors and decreased GABA activity have also been observed in rodent models of TBI (Reger et al., 2012). Up-regulation of NMDA receptors in the amygdala and a decrease in inhibitory GABA activity have been associated with an increased risk of developing anxiety due to an enhanced fear response (Reger et al., 2012). From the excitotoxicity perspective, activation of NMDA receptors by excitatory neurotransmitters, including glutamate, regulates the cellular damage following a TBI and influences the severity of the symptoms experienced. In a mouse model of TBI, administration of NMDA prior to injury lessened both behavioral and motor symptoms (Costa, Constantino, & Mendonça, 2010). Heightened levels of excitatory amino acids, glutamate and aspartate, have also been observed in

patients who have suffered TBI and the levels are positively correlated with the severity of symptoms reported (Gopinath, Valadka, Goodman, & Robertson, 2000).

Oxidative Stress.

Oxidative stress refers to "a disturbance in the balance between the production of reactive oxygen species (free radicals) and antioxidant defenses" (Betteridge, 2000). As discussed above, stress activates glutamatergic action. Hyperactivation of the glutamatergic system can result in oxidative stress by stimulating nitric oxide synthase (NOS) and resulting in increased levels of nitric oxide (NO) which can be toxic to nerve cells at high concentrations (Harvey, Oosthuizen, Brand, & Wegener, 2004). In a rat model of PTSD, excessive levels of markers for oxidative stress and neuroinflammation (reactive oxidative species and pro-inflammatory cytokines, respectively) were found in areas associated with the progression of PTSD including the amygdala, hippocampus, and the prefrontal cortex (Wilson, McLaughlin, Nair, & Ebenezer, 2013). Stress-induced oxidation was also observed in a blast-induced rat model of TBI. Following the blast-injury, oxidative damage was identified in the blood-brain barrier. There were also increased levels of oxidative stress markers in hippocampal tissue and evidence of excess inflammation in the prefrontal cortex (Kochanek et al., 2013). Administration of methylene blue, a drug that has antioxidant properties, resulted in attenuation of neuronal degeneration and lessened behavioral symptoms in a rat model of TBI (Watts, Long, & Chemello, 2014). Methylene blue has also been shown to have positive effects in reducing neuroinflammation and decreasing depressive-like behavior in mice (Fenn et al., 2015).

The different findings support a multidimensional understanding of how TBI can prime the brain for an exaggerated stress response and increased fear learning. Across multiple studies there is significant overlap in brain regions that have been identified as essential components in

the neural networks involved in heightened anxiety as well as those impacted most frequently by TBI.

Functional Neuroanatomy of TBI and Anxiety: Relevance of Brain Regions Measured

In addition to biochemical changes seen in TBI and PTSD, functional and structural differences in neuroanatomy can be used to identify areas of potential dysfunction. Since neural networks influence behavior, rather than a specific region in isolation, it is important to look at multiple measures of different areas to accurately assess differences between treatment groups and examine the diffuse effect of TBI that can have implications on behavior. Regions associated with TBI and the development of PTSD include the hippocampus, the insula, the amygdala, the PVN, and the PFC. Models of PTSD emphasize an exaggerated amygdala response due to decreased inhibition from regions in the prefrontal cortex that contribute to a heightened fear response to perceived threat (Liberzon & Britton, 2003; Rauch et al., 2006). In a meta-analysis of functional neuroimaging studies of PTSD, social anxiety disorder, and specific phobia, increased activity was observed in the insula and amygdala across the three disorders, but only the PTSD group showed significantly decreased activity in the ACC and ventromedial prefrontal cortex (Etkin & Wager, 2007). Other imaging studies of PTSD have found consistent trends in the activation of the amygdala and the ACC, but have also observed hypoactivation in the hippocampus and orbital frontal cortex (Bremner, Staib, Kaloupek, & Southwick, 1999; Liberzon & Britton, 2003; Shin et al., 2004). Hippocampal atrophy has been observed in PTSD (Harvey et al., 2004) and in TBI (Lyeth, Jenkins, Hamm, Dixon, & Phillips, 1990), often corresponding with memory impairment.

TBI can cause structural damage due to the shearing forces experienced upon impact. Certain regions of the brain are more vulnerable to the shearing, as well as tensile effects, and are

more likely to be impacted as a result of a closed head injury (Bigler, 2008). The most frequently impacted region is the hippocampus and its connections to other regions of the brain due to axonal damage from the trauma (Povlishock, 1993). Regions in the frontal and temporal lobes are particularly vulnerable to injury due to the intersection of brain matter and skull with the presence of the anterior and middle cranial fossa (Bigler, 2007). During impact, the bony part of the skull can protrude into the brain matter being moved across it through rotation and deceleration, resulting in structural and functional damage.

Lateral Fluid Percussion Injury as a Model of TBI

Lateral fluid percussion injury (LFPI) is a well-accepted model of TBI in rodents and has been shown to produce reliable results. It has been recognized as clinically-relevant to understanding TBI in humans through translational behavioral changes as well as tissue damage (Thompson et al., 2005). The LFPI is done through rapid injection of fluid while the rodent is under anesthesia, resulting in an impact injury that mirrors a closed-head injury in humans (Dixon et al., 1987).

c-fos as a Marker of Neuronal Activation

c-fos is a well-studied immediate-early gene (IEG) that is used as a marker of neuronal activation in the brain (Herrera & Robertson, 1996). In previous studies of c-fos expression and TBI, increased levels were observed in the ACC and piriform cortex (Dragunow & Robertson, 1988) as well as the hippocampus and dentate gyrus (Dragunow, Faull, & Jansen, 1990; Yang, Mu, Xue, Whitson, & Salminen, 1994).

Methods

Animals

Thirty – six adult male Sprague-Dawley rats were housed in pairs with access to food and water *ad libitum*. A 12-hour light/dark cycle was maintained in the room and the temperature was controlled to a range of 20-26°C. All protocol was carried out in accordance with the University of Colorado Institutional Animal Care and Use Committee guidelines. Rats were randomly assigned to one of six treatment groups (n = 6/group). The six treatment groups are LFPI + MN166, naïve + no shock, naïve + shock, sham-operated, LFPI + no shock, and LFPI + shock.

Lateral fluid percussion injury (LFPI) was used to model the traumatic brain injury in the rats assigned to LFPI groups. In order to cause the closed injury, the LFPI apparatus delivers a quick impact force measuring approximately 2.0 atmospheres and lasting 10 milliseconds. Ibudilast (MN166), a phospodiesterase inhibitor, was administered to one treatment group before and after the LFPI. Naïve rats did not undergo surgery or receive the injection for the LFPI. Sham-operated rats underwent the surgery procedure, but did not receive the injection for the LFPI.

In order to provoke an excessive fear response that is a key aspect of PTSD, the immediate shock paradigm was used. As compared to contextual fear conditioning, the immediate shock paradigm does not allow a time delay between exposure to the environment and administration of the shock which prevents the development of a contextual memory (Fanselow, 1986; Landeira-Fernandez, DeCola, & Kim, 2006). Freezing is used as a measure of anxiety-like behavior because it is a behavior that is seen in response to perceived danger. Without a development of contextual cues to associate with the shock, no fear conditioning takes place and

freezing behavior in the context does not increase when the rats are later tested (Rudy & O'Reilly, 2001). However, despite this phenomenon of the immediate shock deficit, previous research in our lab found an increase in freezing behavior among brain-injured rats – suggesting that this unconditioned freezing could be due to pathological anxiety (Rodgers et al., 2012; 2014).

For the immediate shock paradigm, the rats in the shock condition were shocked in a randomized order at one-month post-injury date and tested twenty-four hours later. The rats were tested again at three months in the shock context, but did not receive a shock, and were immediately returned to their home changes for thirty minutes after to allow for maximum induction of c-fos. The tissue was then collected for *in situ* hybridization. Brain sections were sliced using a cryostat and assayed for c-fos mRNA expression. The slides were exposed to x-ray film for one to three weeks and were photographed individually to be analyzed.

Image Analysis

Using ImageJ software, the images were converted to gray scale and background areas were taken close to the region of interest. For each region, several measurements were taken for each rat and both mean gray and integrated density values were computed. These values were later combined for both measurement types to produce an average integrated density value and an average mean gray value for each region for each rat. The integrated density (area x mean gray value) for the different regions is reported for the results because it takes into account slight variation in brain size across the rats. All images were cross-checked with Paxinos and Watson's "The Rat Brain" atlas to confirm the presumed location in the brain vertically and horizontally before measurements were taken. Additionally, all measurements were taken blind to treatment groups.

The sub-regions measured can be categorized into four groups of images. The first image set includes the regions quantified within the dorsal hippocampus – CA1, CA2, CA3, and the dentate gyrus – as well as the barrel cortex (Appendix A). The second image set looks at regions of the insula and amygdala including the anterior insular point (AIP), dysgranular insular cortex (DI), granular insular cortex (GI), basolateral amygdala (BLA), and central nucleus of the amygdala (CE) (Appendix B). The third image set measurements were the paraventricular nucleus (PVN), the bed nucleus of the stria terminalis (BNST), and the central gray (CG) (Appendix C). And finally, the fourth image set was used to measure areas of the prefrontal cortex (PFC) – the infralimbic, prelimbic, and cingulate cortices (Appendix D). All regions were measured on both the right and left sides.

Statistical Analysis

In order to compare the different brain regions between groups, one-way analysis of variance (ANOVA) was used to assess the integrated density values and the mean gray values (conducted at one time point) with treatment as the independent variable. Due to overall significance in the ANOVA, *post hoc* testing was done using Fisher's least-significant difference (LSD) pairwise comparison. Homogeneity of variance was analyzed using Levene's test. Outlier exclusion (> 1.5 x IQR) was conducted, but had no significant effect on the results. Data were analyzed using SPSS Statistical software and statistical significance was set at $p < 0.05$. All results are expressed as mean ± standard error of the mean.

Results

Immediate Shock Paradigm

The LFPI+shock rats had significantly higher freezing behavior in the immediate shock paradigm than all other treatment groups (Figure 1).

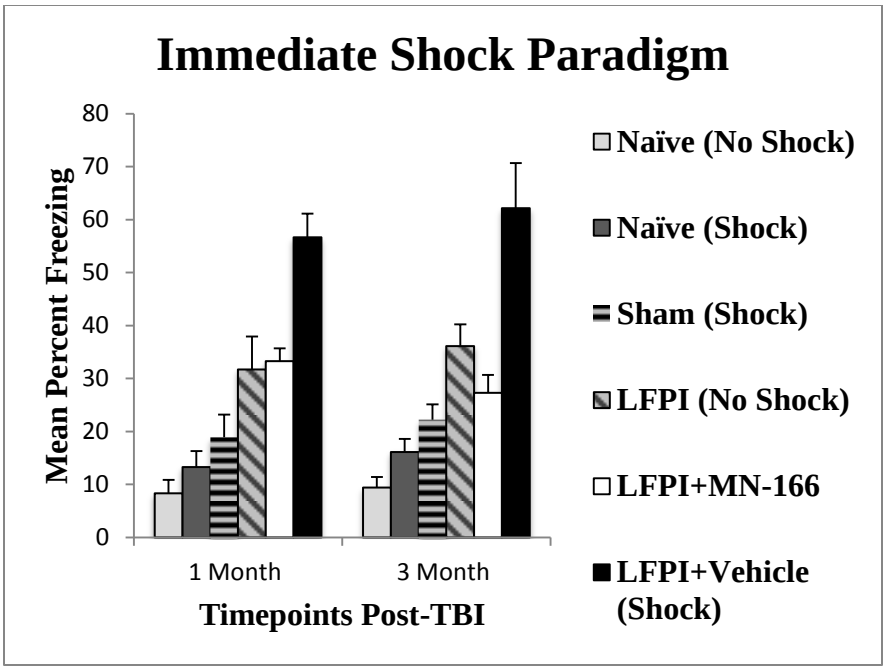

Figure 1: This graph shows the mean percent time spent freezing at one month and three months post-LFPI for all six groups.

c-fos Expression

For all of the following results, ipsilateral is the left hemisphere and refers to the side that was directly injured by the LFPI. Contralateral is the right hemisphere and is the side that was indirectly injured.

Image Set 1

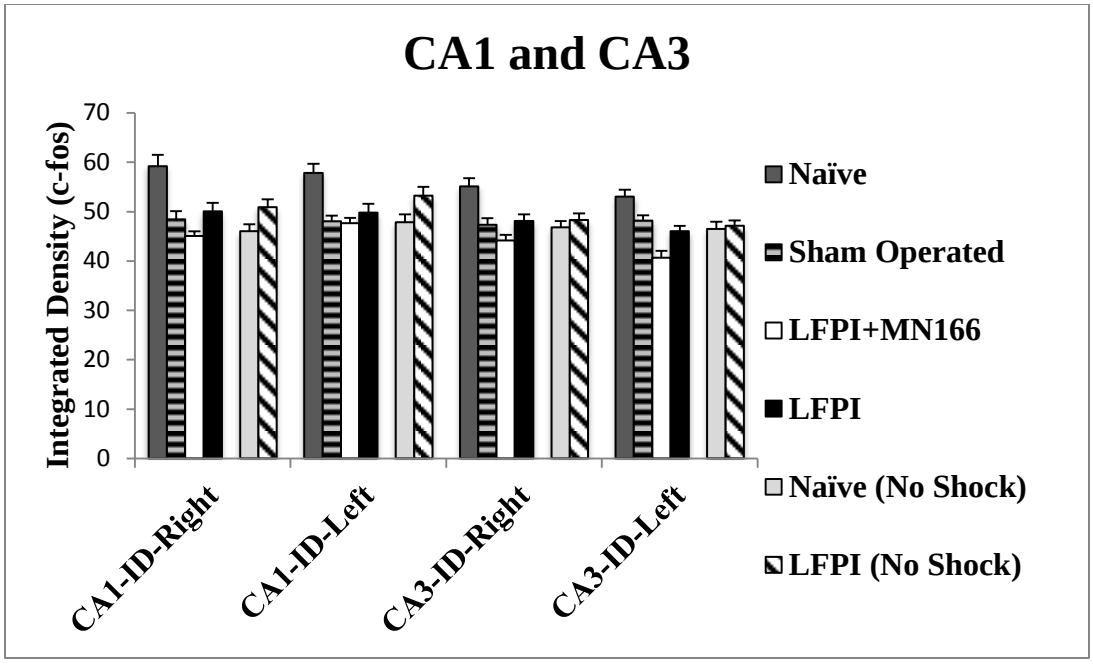

Figure 2: This graph shows the integrated density values for c-fos expression in the left and right CA1 and CA3 of the hippocampus for all six groups.

CA1.

There was a significant difference in both ipsilateral [$F(5,252)=6.287$, $p=0.000$] and contralateral [$F(5,253)=7.859$, $p=0.000$] CA1 between brain injured rats and controls. The c-fos expression was significantly higher in both the ipsilateral and contralateral CA1 of naïve+shock rats than the LFPI+shock rats ($p<0.001$) (Figure 2).

CA3.

There was a significant difference in both ipsilateral [$F(5,252)=8.999$, $p=0.000$] and contralateral [$F(5,251)=6.528$, $p=0.000$] CA3 between brain injured rats and controls. LFPI+shock rats had significantly higher c-fos expression than LFPI+MN166 in the ipsilateral CA3 ($p<0.01$). However, naïve+shock rats had significantly higher c-fos expression than the LFPI+shock rats in both the ipsilateral and contralateral CA3 ($p<0.001$) (Figure 2).

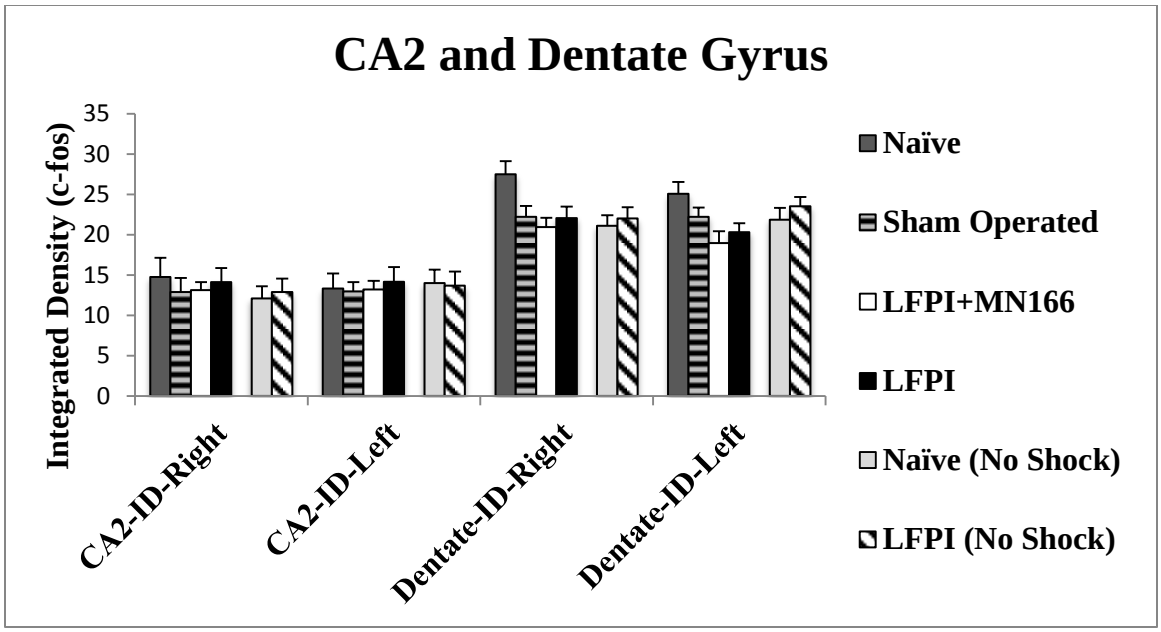

Figure 3: This graph shows the integrated density values for c-fos expression in the left and right CA2 of the hippocampus and the dentate gyrus for all six groups.

CA2.

There was only significant difference in the contralateral [F(5,250)=5.025, p=0.000] CA2 between brain injured rats and controls and no significant difference in the ipsilateral [F(5,250)=1.219, p=0.301] CA2. LFPI+shock rats had significantly higher c-fos expression in the contralateral CA2 when compared to sham-operated ($p<0.05$), naïve+no shock ($p<0.01$), and LFPI+no shock ($p<0.05$) (Figure 3).

Dentate gyrus.

There was a significant difference in both the ipsilateral [F(5,250)=6.932, p=0.000] and contralateral [F(5,255)=7.544, p=0.000] dentate gyrus (DG) between brain injured rats and controls. Naïve+shock rats had significantly higher c-fos expression than LFPI+shock rats in the ipsilateral DG ($p<0.001$) as did LFPI+no shock rats ($p<0.01$). Only naïve+shock rats had significantly higher c-fos expression than LFPI+shock rats in the contralateral DG ($p<0.001$) (Figure 3).

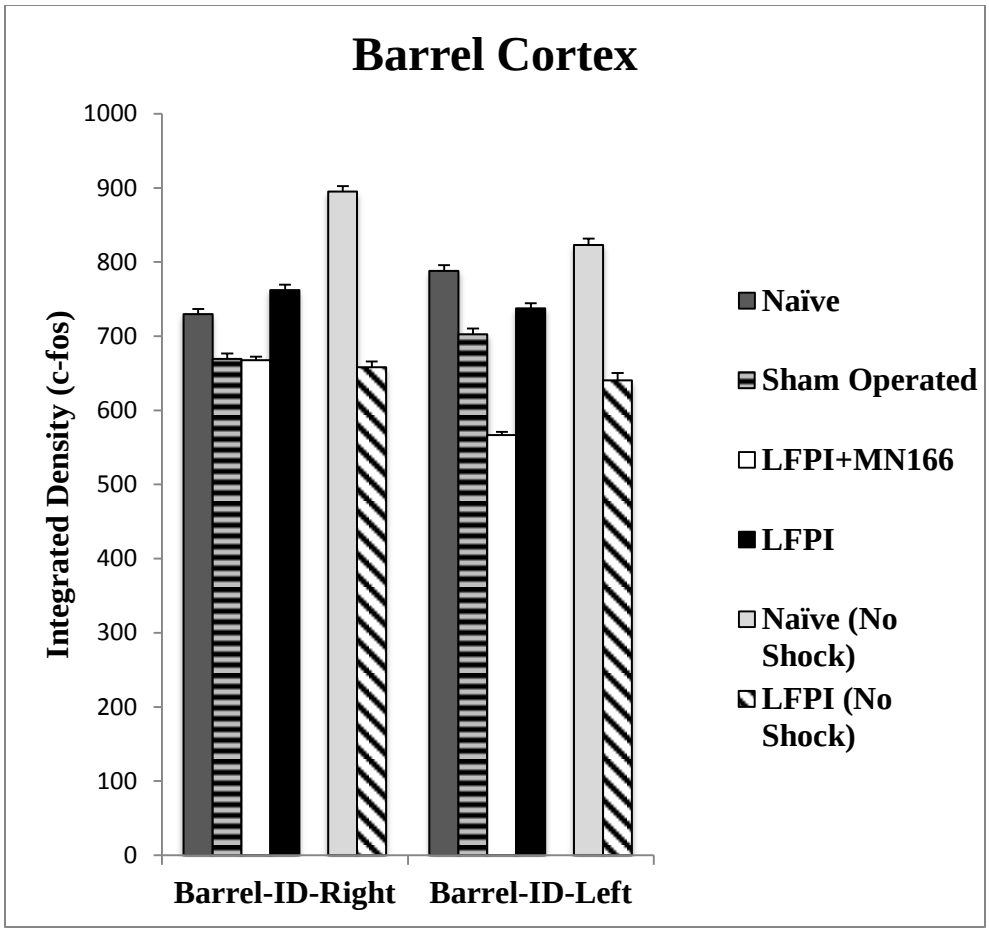

Figure 4: This graph shows the integrated density values for c-fos expression in the left and right barrel cortex for all six groups.

Barrel cortex.

There was a significant difference in both the ipsilateral [$F(5,252)=9.877$, $p=0.000$] and contralateral [$F(5,252)=11.326$, $p=0.000$] barrel cortex between brain injured rats and controls. The LFPI+shock rats had significantly higher c-fos expression when compared to the LFPI+MN166 rats ($p<0.001$) and the LFPI+no shock rats ($p<0.05$) in the ipsilateral barrel cortex. The LFPI+shock rats also had significantly higher c-fos expression when compared to the LFPI+MN166 rats ($p<0.05$), sham-operated ($p<0.05$) and the LFPI+no shock rats ($p<0.01$) in the contralateral barrel cortex. The naïve+no shock rats had significantly higher c-fos expression

than all other groups in the contralateral ($p<0.001$) barrel cortex and all groups except naïve+shock rats ($p=0.404$) in the ipsilateral ($p<0.05$) barrel cortex (Figure 4).

Image Set 2

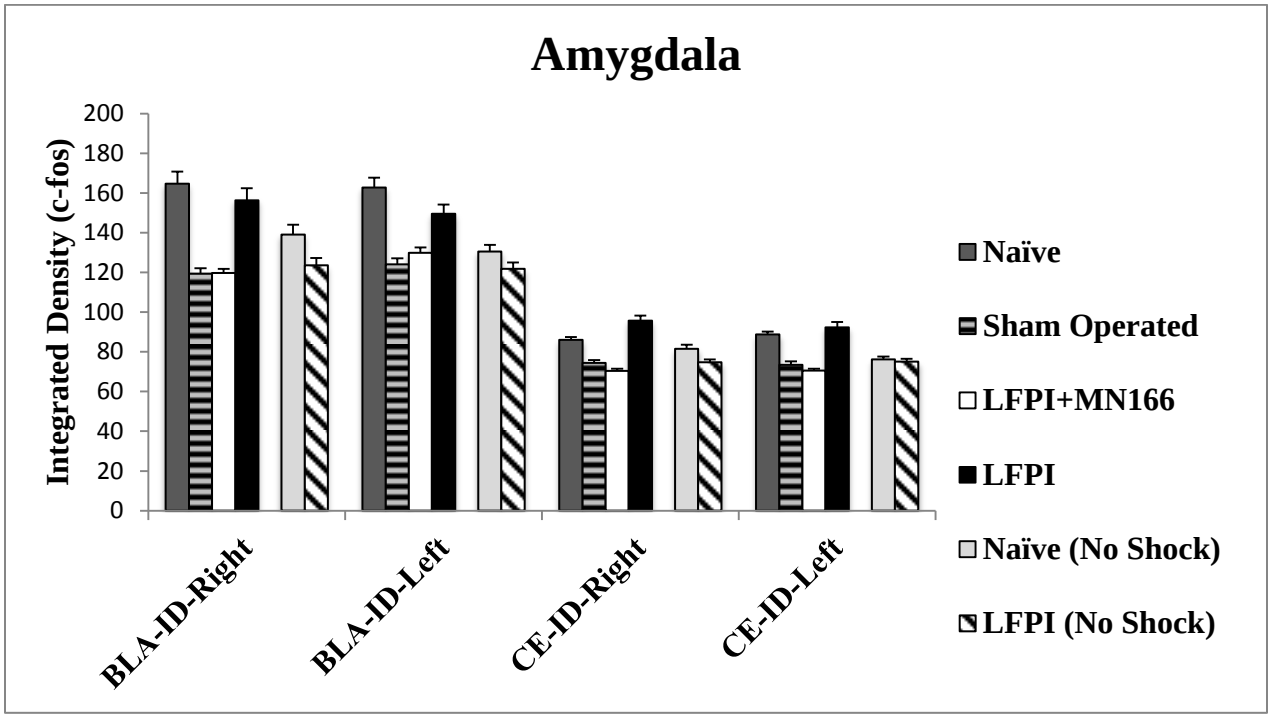

Figure 5: This graph shows the integrated density values for c-fos expression in the left and right basolateral and central nucleus of the amygdala for all six groups.

Basolateral amygdala.

There was a significant difference in both ipsilateral [$F(5,262)=18.174$, $p=0.000$] and contralateral [$F(5,270)=17.897$, $p=0.000$] basolateral amygdala (BLA) between brain injured rats and controls. LFPI+shock rats had significantly higher c-fos expression in the BLA compared to sham-operated, LFPI+MN166, and both no shock control groups ($p<0.001$) (Figure 5). Surprisingly, the naïve+shock rats had significantly higher c-fos expression than LFPI+shock rats in ipsilateral BLA ($p=0.014$), and did not statistically differ in the contralateral BLA ($p=0.200$) expression, in spite of demonstrating significantly less freezing behavior in the immediate shock paradigm (Figure 1).

Central nucleus of the amygdala.

There was a significant difference in both ipsilateral [F(5,261)=23.843, p=0.000] and contralateral [F(5,260)=26.021, p=0.000] central amygdala (CE) between brain injured rats and controls. The c-fos expression in the ipsilateral CE was significantly higher in the LFPI+shock rats as compared to all other treatment groups ($p<0.001$), except the naïve+shock rats ($p=0.160$). Expression of c-fos in the contralateral CE for the LFPI+shock rats was significantly higher than all other treatment groups ($p<0.001$) (Figure 5).

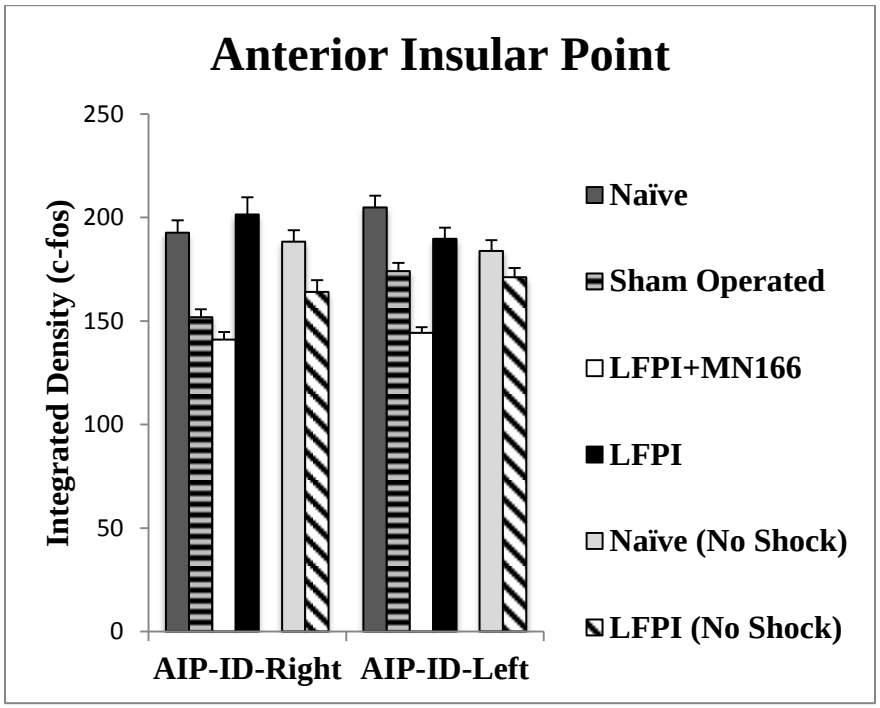

Figure 6: This graph shows the integrated density values for c-fos expression in the left and right anterior insular point for all six groups.

Anterior insular point.

There was a significant difference in both ipsilateral [F(5,260)=17.011, p=0.000] and contralateral [F(5,262)=17.292, p=0.000] anterior insular point (AIP) between brain injured rats and controls. LFPI+shock rats had significantly higher c-fos expression in the ipsilateral AIP when compared to LFPI+MN166 ($p<0.001$), sham-operated ($p<0.05$), and LFPI+no shock

($p<0.01$) rats. However, naïve+shock rats had significantly higher c-fos expression in the ipsilateral AIP than LFPI+shock rats ($p<0.05$). LFPI+shock rats also had significantly higher c-fos expression in the contralateral AIP then LFPI+MN166, sham-operated, and LFPI+no shock ($p<0.001$). There was no significant difference in contralateral AIP c-fos expression between LFPI+shock rats and either naïve+shock ($p=0.277$) or naïve+no shock ($p=0.100$) rats (Figure 6).

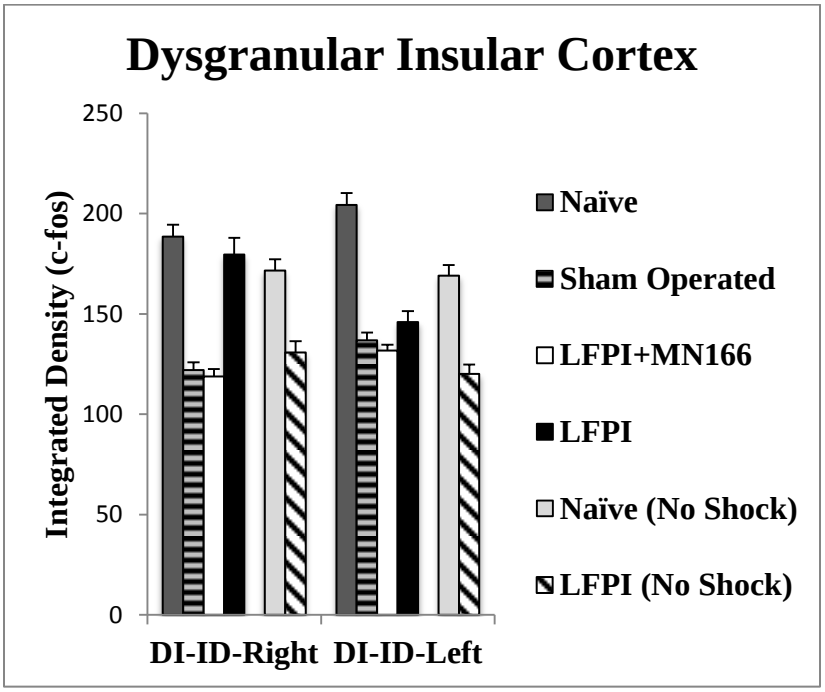

Figure 7: This graph shows the integrated density values for c-fos expression in the left and right dysgranular insular cortex for all six groups.

Dysgranular insular cortex.

There was a significant difference in both ipsilateral [$F(5,264)=20.176$, $p=0.000$] and contralateral [$F(5,271)=15.776$, $p=0.000$] dysgranular insular cortex (DI) between brain injured rats and controls. There was significantly higher c-fos expression in the ipsilateral DI of naïve+shock rats when compared to all other treatment groups ($p<0.001$). The naïve+no shock rats also had significantly higher ipsilateral DI c-fos expression than the LFPI+shock rats

($p<0.05$). The LFPI+shock rats only had significantly higher c-fos expression in the ipsilateral DI than LFPI+no shock ($p<0.01$). In contrast, the LFPI+shock rats had significantly higher c-fos in the contralateral DI when compared to LFPI+MN166, sham-operated, and LFPI+no shock rats ($p<0.001$). There was no significant difference in the contralateral DI c-fos expression levels between LFPI+shock rats and either naïve+shock ($p=0.415$) or naïve+no shock ($p=0.469$) rats (Figure 7).

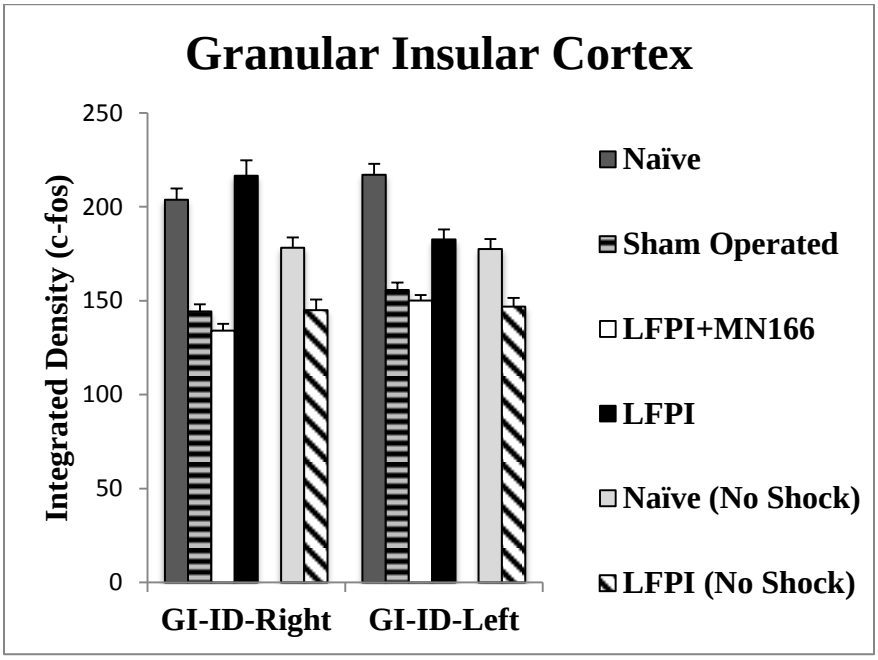

Figure 8: This graph shows the integrated density values for c-fos expression in the left and right granular insular cortex for all six groups.

Granular insular cortex.

There was a significant difference in both ipsilateral [$F(5,266)=15.345$, $p=0.000$] and contralateral [$F(5,269)=20.910$, $p=0.000$] granular insular cortex (GI) between brain injured rats and controls. LFPI+shock rats had significantly higher c-fos expression in the ipsilateral GI than LFPI+MN166 ($p<0.01$), sham-operated ($p<0.01$), and LFPI+no shock ($p<0.001$) rats. However, naïve+shock rats had significantly higher c-fos expression than LFPI+shock ($p<0.001$) rats in the

ipsilateral GI. In the contralateral GI, LFPI+shock rats had significantly higher c-fos expression when compared to all other treatment groups ($p<0.001$), except naïve+shock rats ($p=0.227$) (Figure 8).

Image Set 3

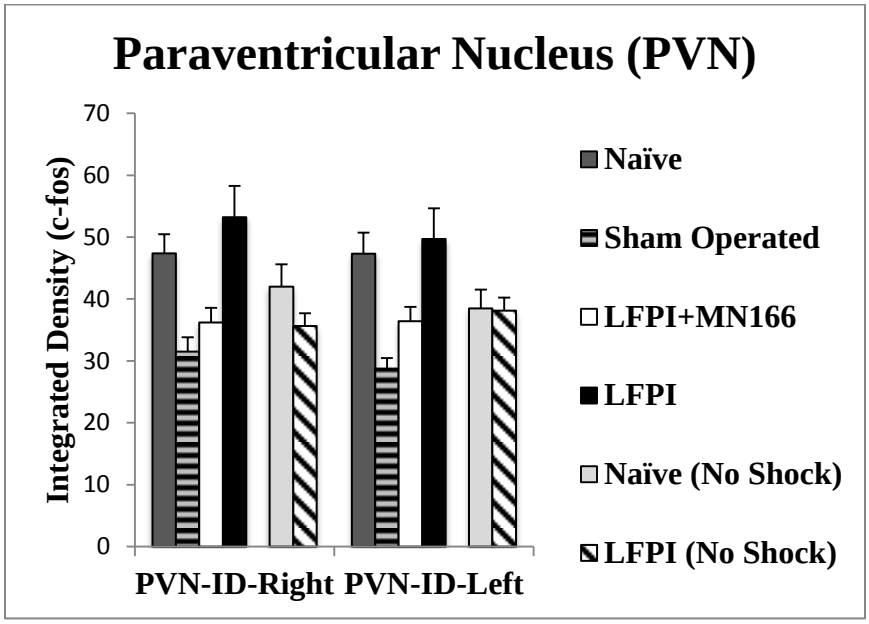

Figure 9: This graph shows the integrated density values for c-fos expression in the left and right paraventricular nucleus of the hypothalamus for all six groups.

Paraventricular nucleus.

There was a significant difference in both ipsilateral [$F(5,133)=5.805$, $p=0.000$] and contralateral [$F(5,133)=6.085$, $p=0.000$] paraventricular nucleus (PVN) between brain injured rats and controls. The c-fos expression in the ipsilateral PVN was significantly higher for the LFPI+shock rats than sham-operated ($p<0.001$), LFPI+MN166 ($p<0.01$), and both no shock groups ($p<0.05$). In the contralateral PVN, LFPI+shock rats had significantly higher c-fos expression as compared to LFPI+MN166 ($p<0.001$), naïve+no shock ($p<0.05$), sham-operated ($p<0.001$) and LFPI+no shock ($p<0.001$). There was no significant difference in c-fos expression

between the LFPI+shock rats and the naïve+shock rats in the either the ipsilateral (p=0.592) or contralateral (p=0.209) PVN (Figure 9).

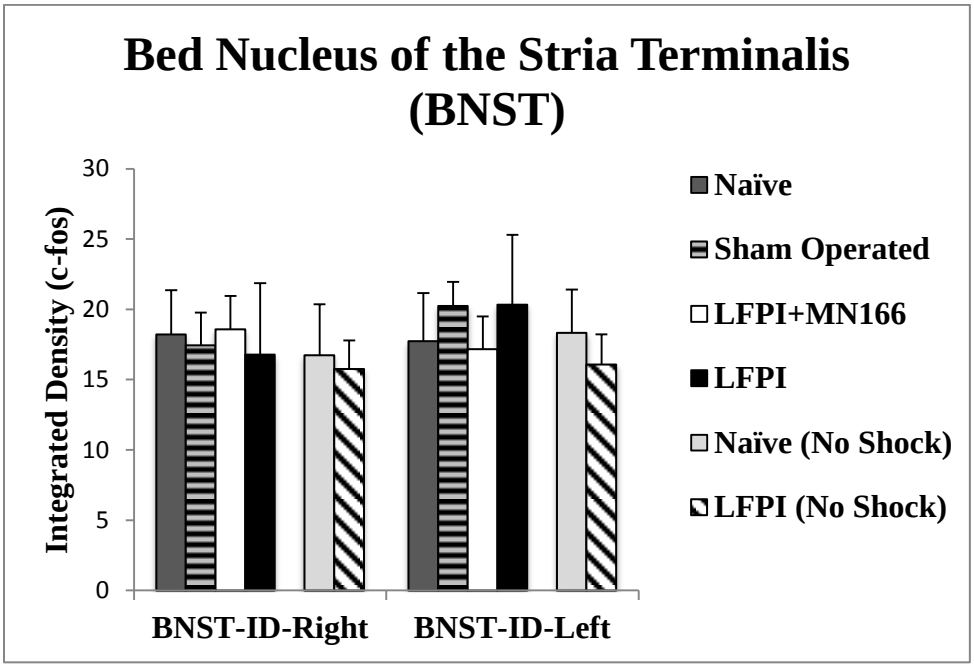

Figure 10: This graph shows the integrated density values for c-fos expression in the left and right bed nucleus of the stria terminalis for all six groups.

Bed nucleus of the stria terminalis.

There was a significant difference in the ipsilateral [F(5,128)=3.374, p=0.007] bed nucleus of the stria terminalis (BNST) between brain injured rats and controls, but no significant difference in the contralateral [F(5,127)=1.269, p=0.281] BNST. LFPI+shock rats had significantly higher c-fos expression in the ipsilateral BNST compared to LFPI+MN166 ($p<0.05$) and LFPI+no shock ($p<0.01$). However, there was no significant difference between the LFPI+shock rats and the naïve+no shock (p=0.131), naïve+ shock (p=0.052), or the sham-operated (p=0.944) in the ipsilateral BNST (Figure 10).

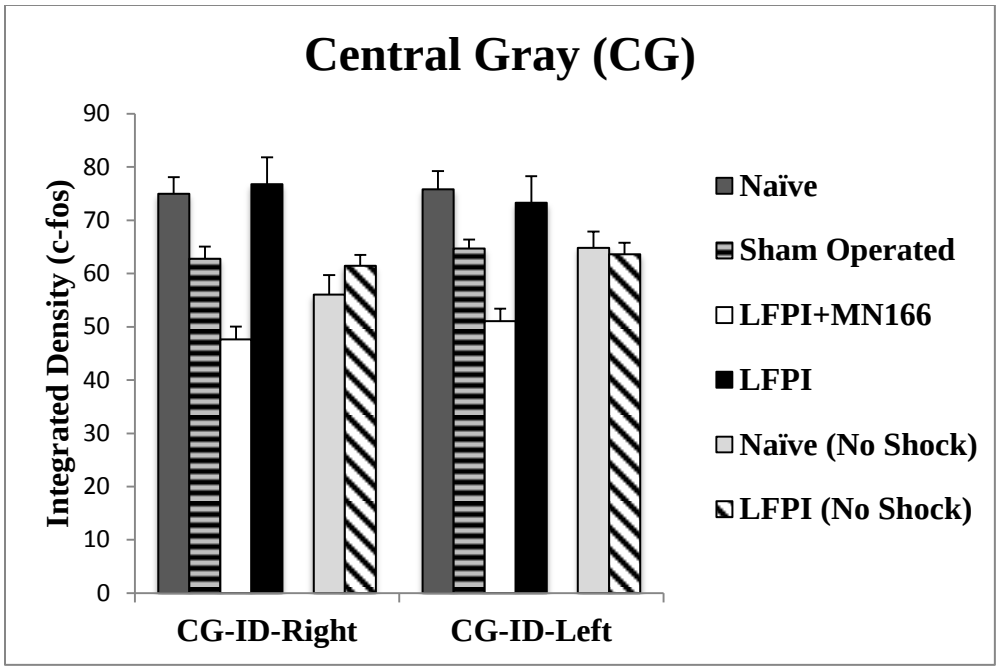

Figure 11: This graph shows the integrated density values for c-fos expression in the left and right central gray (also known as the periaqueductal gray (PAG)) for all six groups.

Central gray.

There was a significant difference in both ipsilateral [$F(5,132)=3.743$, $p=0.003$] and contralateral [$F(5,130)=6.637$, $p=0.000$] central gray (CG) between brain injured rats and controls. LFPI+shock rats had significantly higher c-fos expression in the contralateral CG compared to LFPI+MN166 ($p<0.001$), naïve+no shock ($p<0.01$), sham-operated ($p<0.05$), and LFPI+no shock ($p<0.05$). However, there was no significant difference in the contralateral CG of the LFPI+shock rats and the naïve+shock rats ($p=0.759$). The c-fos expression in the ipsilateral CG of LFPI+shock rats was only significantly different from that of LFPI+MN166 rats ($p<0.01$) (Figure 11).

Image Set 4

Prelimbic cortex.

There was no significant difference in either the ipsilateral [F(5,203)=1.928, p=0.091] or contralateral [F(5,202)=1.325, p=0.255] prelimbic cortex between brain injured rats and controls.

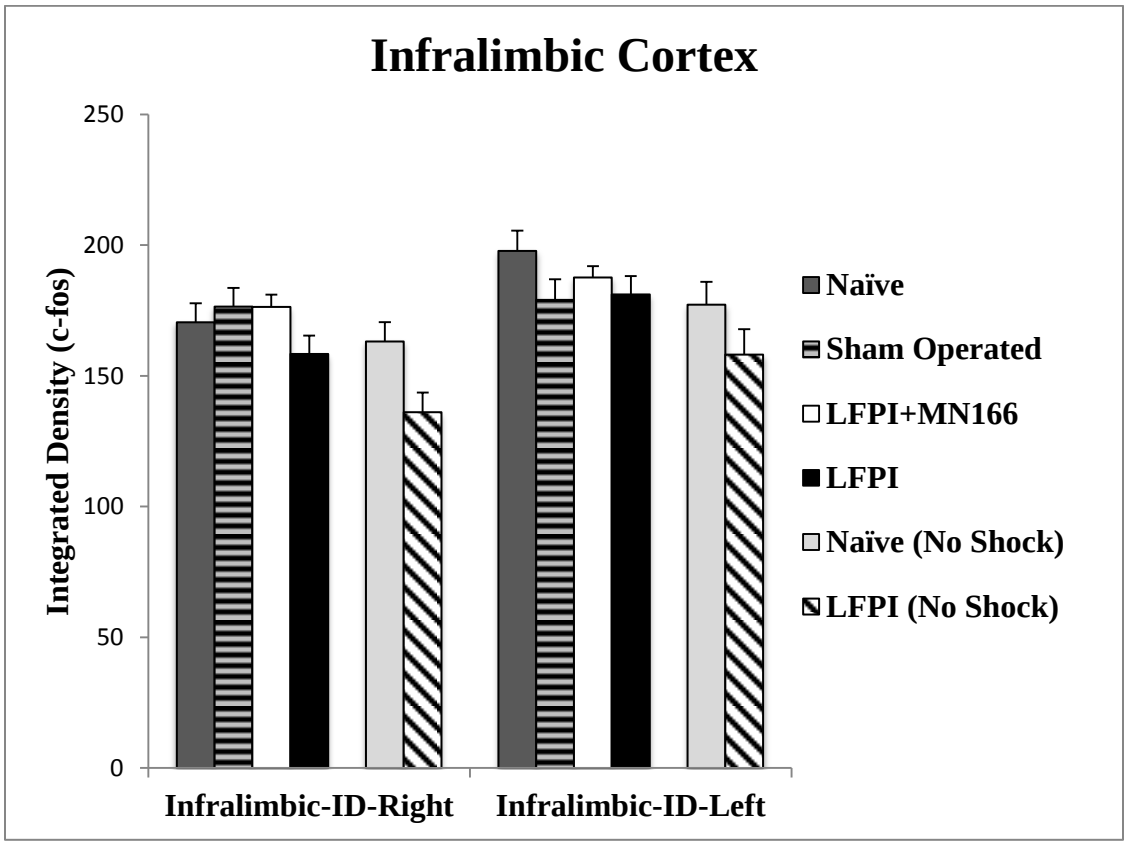

Figure 12: This graph shows the integrated density values for c-fos expression in the left and right infralimbic cortex for all six groups.

Infralimbic cortex.

There was a significant difference in the ipsilateral [F(5,196)=2.722, p=0.021] and contralateral [F(5,197)=4.698, p=0.000] infralimbic cortex between brain injured rats and controls. LFPI+shock rats had significantly higher c-fos expression in the both the ipsilateral and contralateral infralimbic cortex than the LFPI+no shock rats ($p<0.05$) (Figure 12).

Cingulate cortex.

There was no significant difference in either the ipsilateral [$F(5,201)=1.326$, $p=0.255$] or contralateral [$F(5,202)=1.493$, $p=0.194$] cingulate cortex between brain injured rats and controls.

Discussion

Several of our findings support previous functional neuroanatomical research on anxiety-related disorders. Based on the unconditioned freezing behavior, a potential indication of pathological anxiety, we would expect to see higher c-fos expression in the LFPI+shock group in regions associated with increased activation in anxiety disorders including the amygdala and insula (Carlson, Greenberg, & Rubin, 2011; Shin & Liberzon, 2009; Stein, Simmons, & Feinstein, 2007). The most consistent finding was significantly higher c-fos expression in the central amygdala (CE) in the LFPI+shock group as compared to all other groups, except with the ipsilateral CE in the naïve+shock animals where there was no significant difference. The CE is critical for fear learning and is considered an important region in the exaggerated fear response seen in chronic anxiety (Kalin, 2004; Rosen & Schulkin, 1998). In contrast, the basolateral amygdala (BLA), a region associated with developing and storing fear-based memories, had significantly higher c-fos expression for the naïve+shock rats than the LFPI+shock rats (Gale, 2004). In the insula, the LFPI+shock rats had higher expression of c-fos across the different regions as compared to LFPI+no shock, sham-operated, and LFPI+MN166. As an essential area for interoception and the ability to sense physiological information in the body, the insula has been identified as a critical part of the affective processes involved in anxiety, including worrying and avoidance behaviors (Etkin & Wager, 2007; Paulus & Stein, 2006; Simmons, Strigo, Matthews, Paulus, & Stein, 2006; Stein et al., 2007). We also found evidence for increased activation in the PVN for the LFPI+shock rats as compared to all other groups, except

naïve+shock rats. Since increased activation in the PVN is associated with heightened HPA activity, hyperactivation contributes to exaggerated stress responses in anxiety disorders (Claes, 2004; Liberzon, Krstov, & Young, 1997; Nair & Ajit, 2008). The significant differences in c-fos expression for the LFPI+shock rats in the BNST and CG were not consistent enough to provide any supportive or contradictive evidence for previous research.

Additionally, there was some evidence for less recent neuronal activation in the LFPI+shock group in areas associated with inhibition of anxiety compared to other groups. In the dorsal hippocampus, which mediates the fear circuit by contextualizing affective information from the amygdala (Sanders, Wiltgen, & Fanselow, 2003), there was significantly higher c-fos expression for the naïve+shock rats than the LFPI+shock rats in the CA1, CA3, and dentate gyrus (DG). These results could suggest greater involvement of the hippocampus in mediating the fear response in the naïve+shock group. The CA2 results were not consistent enough to support or contradict any trends. Interestingly, the LFPI+shock rats showed higher c-fos expression in the infralimbic cortex than LFPI+no shock rats. However, the c-fos expression is lower in both LFPI groups than the rest of the treatment groups in the infralimbic cortex, but not significantly. As a region involved in inhibition of behavioral and emotional responses to potentially aversive outcomes, impairments in the infralimbic cortex following injury could contribute to the increased freezing behavior (Vidal-Gonzalez, Vidal-Gonzalez, Rauch, & Quirk, 2006).

Since decreased inhibition of the prefrontal cortex and the anterior cingulate cortex has also been seen in imaging studies of people with PTSD (Davidson, 2002; Shin, Rauch, & Pitman, 2006), the LFPI+shock group would be expected to have lower levels of c-fos expression in these areas compared to controls. However, there were no significant differences between groups in either the prelimbic or cingulate cortices. In contrast, in the barrel cortex the naïve+no

shock rats had significantly greater c-fos expression than LFPI+shock rats, indicating increased whisker-activity and sensory processing (Petersen, 2007) which is likely inversely-correlated with the anxiety-like freezing behavior observed, but was not quantified.

The LFPI+MN166 group had lower c-fos expression than LFPI+shock group across all regions and significantly lower expression than LFPI+no shock group in most regions. Since c-fos can be a marker of both neuronal and glial activation (Dragunow & Robertson, 1988), these results support two previous studies in our lab that showed MN166 to have attenuating effects on neuroinflammation and development of anxiety-like behavior through suppression of glial cell activation (Rodgers et al., 2012; 2014).

Despite behavioral results that support LFPI-induced anxiety behaviors in the immediate shock paradigm, there were inconsistencies in the c-fos expression than would be expected from previous research on brain regions involved in anxiety disorders. The major inconsistency was that the naïve+shock rats had either significantly higher levels of c-fos expression or no significant difference in expression when compared to LFPI+shock rats in several regions associated with hyperactivation in anxiety disorders, even though the naïve+shock rats had significantly less anxiety-like freezing behavior in the immediate shock paradigm. These regions included the BLA, the ipsilateral AIP, and the ipsilateral GI. The increased expression could be due to edge effect when the c-fos was developed since both naïve groups were developed on the outer edge of the x-ray film which might have caused falsely elevated levels of expression.

Another possible explanation is that there was too long of a delay between being placed in the context and receiving the shock during the immediate shock paradigm and a contextualized fear response was developed. The box in which the rats were shocked could also have been an

aversive stimuli rather than a neutral stimuli, provoking a fear response. However, the behavioral results do not support these explanations and if this were the case, the fear response should have been seen in all groups that went through the immediate shock paradigm, not just the LFPI+shock and naïve+shock groups. Additionally, the rats were randomly placed in the shock paradigm, but the naïve+shock rats might have more frequently followed an LFPI+shock rat and could have responded to leftover odors from the previous rat which could be controlled for with a larger sample size. Therefore, to control for variability in c-fos expression for different brain regions, future studies should include more rats per treatment group. With women developing PTSD at twice the rate of men ("Women, Trauma and PTSD," 2014), it would also be valuable to repeat this study using both male and female rats to investigate whether there are sex differences in the brain regions involved in TBI-induced anxiety.

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References

Betteridge, D. J. (2000). What is oxidative stress?. *Metabolism*, 49, 3-8.

Bigler, E. D. (2007). Anterior and middle cranial fossa in traumatic brain injury: relevant neuroanatomy and neuropathology in the study of neuropsychological outcome *Neuropsychology*, 21, 515-531.

Bigler, E. D. (2008). Neuropsychology and clinical neuroscience of persistent post-concussive syndrome. *Journal Of The International Neuropsychological Society, 14*(01). doi:10.1017/S135561770808017X

Bremner, J. D., Randall, P., Scott, T. M., Bronen, R. A., Seibyl, J. P., Southwick, S. M., … Innis, R. B. (1995). MRI-based measurement of hippocampal volume in patients with combat related posttraumatic stress disorder. *The American Journal of Psychiatry*, 152, 973-981.

Bremner, J., Staib, L., Kaloupek, D., & Southwick, S. (1999). Neural correlates of exposure to traumatic pictures and sound in Vietnam combat veterans with and without posttraumatic stress disorder: a positron emission tomography study. *Biological Psychiatry*, 45, 806-816.

Britton, J., Phan, K., Taylor, S., Fig, L., & Liberzon, I. (2005). Corticolimbic blood flow in posttraumatic stress disorder during script-driven imagery. *Biological Psychiatry*, 57(8), 832-840.

Carlson, J., Greenberg, T., & Rubin, D. (2011). Feeling anxious: anticipatory amygdalo-insular response predicts the feeling of anxious anticipation. *Social Cognitive and Affective Neuroscience*, 6(1), 74-81.

Claes, S. (2004). Corticotropin-releasing hormone (CRH) in psychiatry: from stress to psychopathology. *Annals of Medicine*, *36*(1), 50-61. doi:10.1080/07853890310017044

Costa, T., Constantino, L., & Mendonça, B. (2010). N-methyl-D-aspartate preconditioning improves short-term motor deficits outcome after mild traumatic brain injury in mice. *Journal of Neuroscience, 88, 1329-1337.*

Craske, M., Rauch, S., & Ursano, R. (2009). What is an anxiety disorder?. *Depression and Anxiety,* 26, 1066-1085.

Davidson, R. J. (2002). Anxiety and affective style: role of prefrontal cortex and amygdala. *Biological Psychiatry*, 51, 68-80.

Deb, S., Lyons, I., Koutzoukis, C., Ali, I., & McCarthy, G. (1999). Rate of psychiatric illness 1 year after traumatic brain injury. *American Journal of Psychiarty,* 156 (3), 374-378.

Dixon, C. E., Lyeth, B. G., Povlishock, J. T., Findling, R. L., Hamm, R. J., Marmarou, A., et al. (1987). A fluid percussion model of experimental brain injury in the rat. *Journal of Neurosurgery*, 67, 110-119. doi:10.3171/jns.1987.67.1.0110

Dragunow, M, & Robertson, H. (1988). Brain injury induces c-fos protein (s) in nerve and glial like cells in adult mammalian brain. *Brain Research*, 455, 295-299.

Dragunow, M, Faull, R., & Jansen, K. (1990). MK-801, an antagonist of NMDA receptors, inhibits injury-induced c-fos protein accumulation in rat brain. *Neuroscience Letters*. 109, 128-133.

Dragunow, M., & Faull, R. (1989). The use of c-fos as a metabolic marker in neuronal pathway tracing. *Journal of Neuroscience Methods*, *29*(3), 261-265. doi:10.1016/0165 0270(89)90150-7

Etkin, A. & Wager, T. D. (2007). Functional neuroimaging of anxiety: a meta-analysis of emotional processing in PTSD, social anxiety disorder, and specific phobia. *American Journal of Psychiatry*, *164*(10), 1476-1488. doi:10.1176/appi.ajp.2007.07030504

Fanselow, M. (1986). Associative vs. topographical accounts of the immediate shock-freezing deficit in rats: implications for the response selection rules governing species-specific defensive reactions. *Learning and Motivation,* 17, 16-39.

Farina, C., Aloisi, F., & Meinl, E. (2007). Astrocytes are active players in cerebral innate immunity. *Trends in Immunology*, 28(3), 138-145.

Faul, M., Xu, L., Wald, M., & Coronado, V. (2010). Traumatic brain injury in the United States: emergency department visits, hospitalizations and deaths 2002–2006. Atlanta, Georgia, Centers for Disease Control and Prevention, National Center for Injury Prevention and Control.

Fenn, A. M., Skendelas, J. P., Moussa, D. N., Muccigrosso, M. M., Popovich, P. G., Lifshitz, J., et al. (2015). Methylene blue attenuates traumatic brain injury-associated neuroinflammation and acute depressive-like behavior in mice. *Journal of Neurotrauma, 32*(2), 127-138. doi:10.1089/neu.2014.3514

Gale, G. D. (2004). Role of the basolateral amygdala in the storage of fear memories across the adult lifetime of rats. *Journal of Neuroscience*, 24(15), 3810-3815. doi:10.1523/jneurosci.4100-03.2004

Goddard, A., Mason, G., & Almai, A. (2001). Reductions in occipital cortex GABA levels in panic disorder detected with 1h-magnetic resonance spectroscopy. *Archives of General Psychiatry,* 58, 556-561.

Gopinath, S., Valadka, A., Goodman, J., & Robertson, C. (2000). Extracellular glutamate and aspartate in head injured patients. *Acta Neurochirurgica Supplement*, 76, 437-438.

Harvey, B., Oosthuizen, F., Brand, L., & Wegener, G. (2004). Stress–restress evokes sustained iNOS activity and altered GABA levels and NMDA receptors in rat hippocampus. *Psychopharmacology,* 175(4), 494-502.

Hausmann, R., Kaiser, A., Lang, C., & Bohnert, M. (1999). A quantitative immunohistochemical study on the time-dependent course of acute inflammatory cellular response to human brain injury. *International Journal of Legal Medicine,* 112(4), 227-232.

Herrera, D., & Robertson, H. (1996). Activation of c-fos in the brain. *Progress in Neurobiology*, 50(2), 83-107.

Hoge, E., Brandstetter, K., & Moshier, S. (2009). Broad spectrum of cytokine abnormalities in panic disorder and posttraumatic stress disorder. *Depression and Anxiety*. 26(5), 447-455.

Holmin, S., & Höjeberg, B. (2004). In situ detection of intracerebral cytokine expression after human brain contusion. *Neuroscience Letters*, *369*(2), 108-114. doi:10.1016/j.neulet.2004.07.044

Kalin, N. H. (2004). The role of the central nucleus of the amygdala in mediating fear and anxiety in the primate. *Journal of Neuroscience*. doi:10.1523/jneurosci.0292 04.2004

Kochanek, P., Dixon, E., Shellington, D., Shin, S., Bayir, H., Jackson, E….Jenkins, L.W. (2013). Screening of biochemical and molecular mechanisms of secondary injury and repair in the brain after experimental blast-induced traumatic brain injury in rats. *Journal of Neurotrauma*, 30(11), 920-937.

Känel, von, R., Hepp, U., Kraemer, B., Traber, R., Keel, M., Mica, L., & Schnyder, U (2007). Evidence for low-grade systemic proinflammatory activity in patients with posttraumatic

stress disorder. *Journal of Psychiatric Research, 41*(9), 744-752. doi:10.1016/j.jpsychires.2006.06.009

Landeira-Fernandez, J., DeCola, J., & Kim, J. (2006). Immediate shock deficit in fear conditioning: effects of shock manipulations. *Behavioral Neuroscience,* 120(4), 873-879.

Lehnardt, S. (2009). Innate immunity and neuroinflammation in the CNS: The role of microglia in toll-like receptor mediated neuronal injury. *Glia*, *58*(3), 253-263. doi:10.1002/glia.20928

Liberzon, I., & Britton, J. (2003). Neural correlates of traumatic recall in posttraumatic stress disorder. *Stress,* 6, 151-156.

Liberzon, I., Krstov, M., & Young, E. (1997). Stress-restress: effects on ACTH and fast feedback. *Psychoneuroendocrinology*, 22, 443-453.

Lyeth, B. G., Jenkins, L., Hamm, R., Dixon, C. E., & Phillips, L. (1990). Prolonged memory impairment in the absence of hippocampal cell death following traumatic brain injury in the rat. *Brain Research*, 526, 249-258.

Meyerhoff, D., Mon, A., Metzler, T., & Neylan, T (2014). Cortical gamma-aminobutyric acid and glutamate in posttraumatic stress disorder and their relationships to self-reported sleep quality. *Sleep,* 37(5), 893-900.

Nair, J., & Ajit, S. S. (2008). The role of the glutamatergic system in posttraumatic stress disorder. *CNS Spectrums*, *13*(07), 585-591. doi:10.1017/s1092852900016862

Paulus, M. P., & Stein, M. B. (2006). An Insular View of Anxiety. *Biological Psychiatry,* 60, 383-387. doi:10.1016/j.biopsych.2006.03.042

Petersen, C. (2007). The functional organization of the barrel cortex. *Neuron*, 56, 339-355.

Pollack, M., Jensen, J., Simon, N., & Kaufman, R. (2008). High-field MRS study of GABA, glutamate and glutamine in social anxiety disorder: response to treatment with levetiracetam. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, 32(3), 739-743.

Povlishock, J. T. (1993). Pathobiology of traumatically induced axonal injury in animals and man. *Annals of Emergency Medicine*, 22, 980-986.

Prasad, K. N., & Bondy, S. C. (2015). Common biochemical defects linkage between posttraumatic stress disorders, mild traumatic brain injury (TBI) and penetrating TBI. *Brain Research*, 1599C, 103-114. doi:10.1016/j.brainres.2014.12.038

Rudy, J. W., & O'Reilly, R. C. (2001). Conjunctive representations, the hippocampus, and contextual fear conditioning. *Cognitive, Affective, and Behavioral Neuroscience*, 1, 66-82.

Rauch, S. L., Shin, L. M., & Phelps, E. A. (2006). Neurocircuitry models of posttraumatic stress disorder and extinction: human neuroimaging research—past, present, and future. *Biological Psychiatry, 60*(4), 376-382. doi:10.1016/j.biopsych.2006.06.004

Reger, M. L., Poulos, A. M., Buen, F., Giza, C. C., Hovda, D. A., & Fanselow, M. S. (2012). Concussive brain injury enhances fear learning and excitatory processes in the amygdala. *Biological Psychiatry, 71*(4), 335-343. doi:10.1016/j.biopsych.2011.11.007

Rodgers, K. M., Bercum, F. M., McCallum, D. L., Rudy, J. W., Frey, L. C., Johnson, K. W., ... Barth, D. S. (2012). Acute neuroimmune modulation attenuates the development of anxietylike freezing behavior in an animal model of traumatic brain injury. *Journal of Neurotrauma,* 29(10), 1886-1897. doi:10.1089/neu.2011.2273

Rodgers, K.M., Deming, Y. K., Bercum, F. M., Chumachenko, S. Y., Wieseler, J. L., Johnson, K. W., … Barth, D. S. (2014). Reversal of established traumatic brain injury-induced, anxiety-like behavior in rats after delayed, post-injury neuroimmune suppression. *Journal of Neurotrauma*, *31*(5), 487-497. doi:10.1089/neu.2013.3090

Rosen, J. B., & Schulkin, J. (1998). From normal fear to pathological anxiety. *Psychological Review,* 105(2), 325-350. doi:10.1037/0033-295X.105.2.325

Rosso, I., Weiner, M., & Crowley, D. (2014). Insula and anterior cingulate GABA levels in posttraumatic stress disorder: preliminary findings using magnetic resonance spectroscopy. *Depression and Anxiety,* 31(2), 115-123.

Sanders, M., Wiltgen, B., & Fanselow, M. (2003). The place of the hippocampus in fear conditioning. *European Journal of Pharmacology,* 463, 217-223.

Shin, L. M., Rauch, S., & Pitman, R. (2006). Amygdala, medial prefrontal cortex, and hippocampal function in PTSD. *Annals of the New York Academy of Sciences,* 1071, 67-79.

Shin, L. M., & Liberzon, I. (2009). The neurocircuitry of fear, stress, and anxiety disorders. *Neuropsychopharmacology*, *35*(1), 169-191. doi:10.1038/npp.2009.83

Shin, L. M., Shin, P. S., Heckers, S., Krangel, T. S., Macklin, M. L., Orr, S. P., … Rauch, S. L. (2004). Hippocampal function in posttraumatic stress disorder. *Hippocampus,* 14(3), 292-300.

Simmons, A., Strigo, I., Matthews, S. C., Paulus, M. P., & Stein, M. B. (2006). Anticipation of aversive visual stimuli is associated with increased insula activation in anxiety prone subjects. *Biological Psychiatry*, *60*(4), 402-409. doi:10.1016/j.biopsych.2006.04.038

Spivak, B., Shohat, B., Mester, R., Avraham, S., & Gil-Ad, I. (1997). Elevated levels of serum interleukin-1β in combat-related posttraumatic stress disorder. *Biological Psychiatry*, 42(5), 345-348.

Stein, M., Simmons, A., & Feinstein, J. (2007). Increased amygdala and insula activation during emotion processing in anxiety-prone subjects. *The American Journal of Psychiatry*, 164(2), 318-327.

Thompson, H. J., Lifshitz, J., Marklund, N., Grady, M. S., Graham, D. I., Hovda, D. A., & McIntosh, T. K. (2005). Lateral fluid percussion brain injury: a 15-year review and evaluation. *Journal of Neurotrauma*, 22(1), 42-75. doi:10.1089/neu.2005.22.42

Vaishnavi, S., Rao, V., & Fann, J. R. (2009). Neuropsychiatric problems after traumatic brain injury: unraveling the silent epidemic. *Psychosomatics*, *50*(3), 198-205. doi:10.1176/appi.psy.50.3.198

Vidal-Gonzalez, I., Vidal-Gonzalez, B., Rauch, S. L., & Quirk, G. J. (2006). Microstimulation reveals opposing influences of prelimbic and infralimbic cortex on the expression of conditioned fear. *Learning and Memory*, 13(6), 728-733. doi:10.1101/lm.306106

Walker, D., Toufexis, D., & Davis, M. (2003). Role of the bed nucleus of the stria terminalis versus the amygdala in fear, stress, and anxiety. *European Journal Of Pharmacology*, 463, 199-216.

Watts, L. T., Long, J., & Chemello, J. (2014). Methylene blue is neuroprotective against mild traumatic brain injury. *Journal of Neurotrauma*, 31(11), 1063-1071.

Wilson, C., McLaughlin, L., Nair, A., & Ebenezer, P. (2013). Inflammation and oxidative stress are elevated in the brain, blood, and adrenal glands during the progression of posttraumatic stress disorder in a predator. *Plos One*, 8(10).

Women, Trauma and PTSD. (2014, January 3). Retrieved from http://www.ptsd.va.gov/public/PTSD-overview/women/women-trauma-and-ptsd.asp

Woodward, S. H., Kaloupek, D. G., Streeter, C. C., Martinez, C., Schaer, M., & Eliez, S. (2006). Decreased anterior cingulate volume in combat-related PTSD. *Biological Psychiatry*, 59, 582-587. doi:10.1016/j.biopsych.2005.07.033

Yamasue, H., Kasai, K., Iwanami, A., Ohtani, T., Yamada, H., Abe, O., et al. (2011). Voxel based analysis of MRI reveals anterior cingulate gray-matter volume reduction in posttraumatic stress disorder due to terrorism. *Proceedings of The National Academy of Sciences*, 100, 9039-9043. doi:10.1073/pnas.1530467100

Yang, K., Mu, X., Xue, J., Whitson, J., & Salminen, A (1994). Increased expression of c-fos mRNA and AP-1 transcription factors after cortical impact injury in rats. *Brain Research*, 664, 141-147.

Yi, J., & Hazell, A (2006). Excitotoxic mechanisms and the role of astrocytic glutamate transporters in traumatic brain injury. *Neurochemistry International*,48(5), 394-403.

Appendix A

Image Set 1

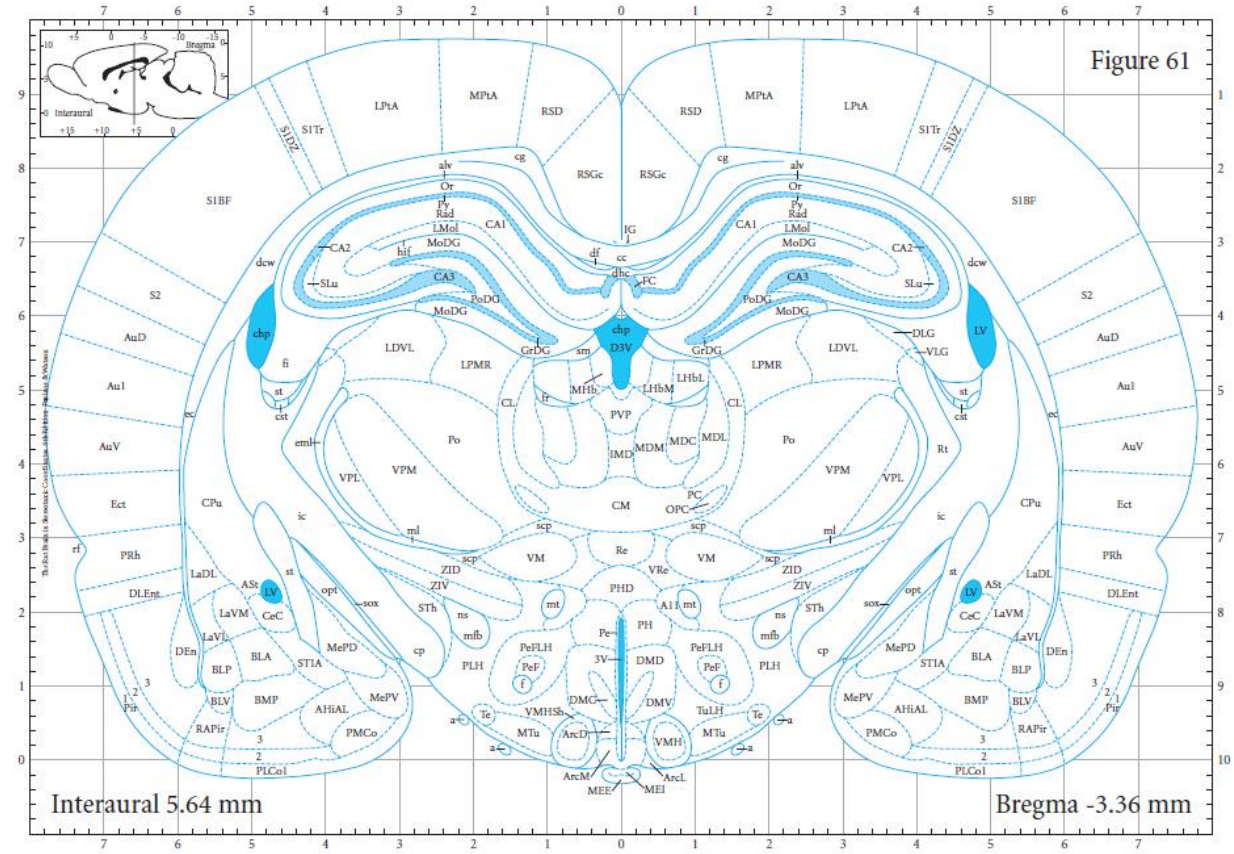
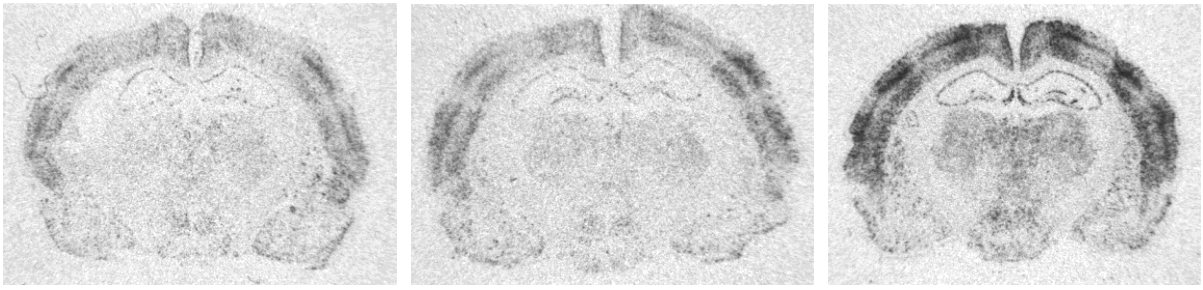


Figure 1: This diagram shows the general region where the measurements for Image Set 1 were taken. These measurements include CA1, CA2, CA3, dentate gyrus (DG), and barrel cortex (S1BF).



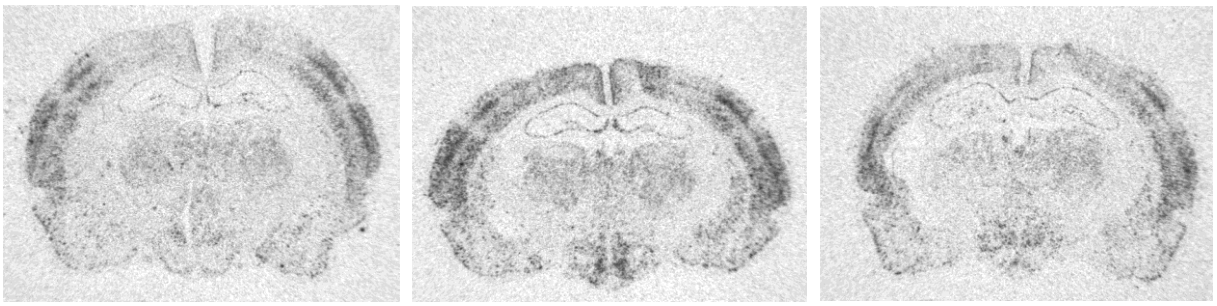


Figure 2: This figure shows example images from Image Set 1. From top left: LFPI+MN166, Naïve + No Shock, Naïve + Shock. From bottom left: Sham-operated, LFPI + No Shock, LFPI + Shock.

Appendix B

Image Set 2

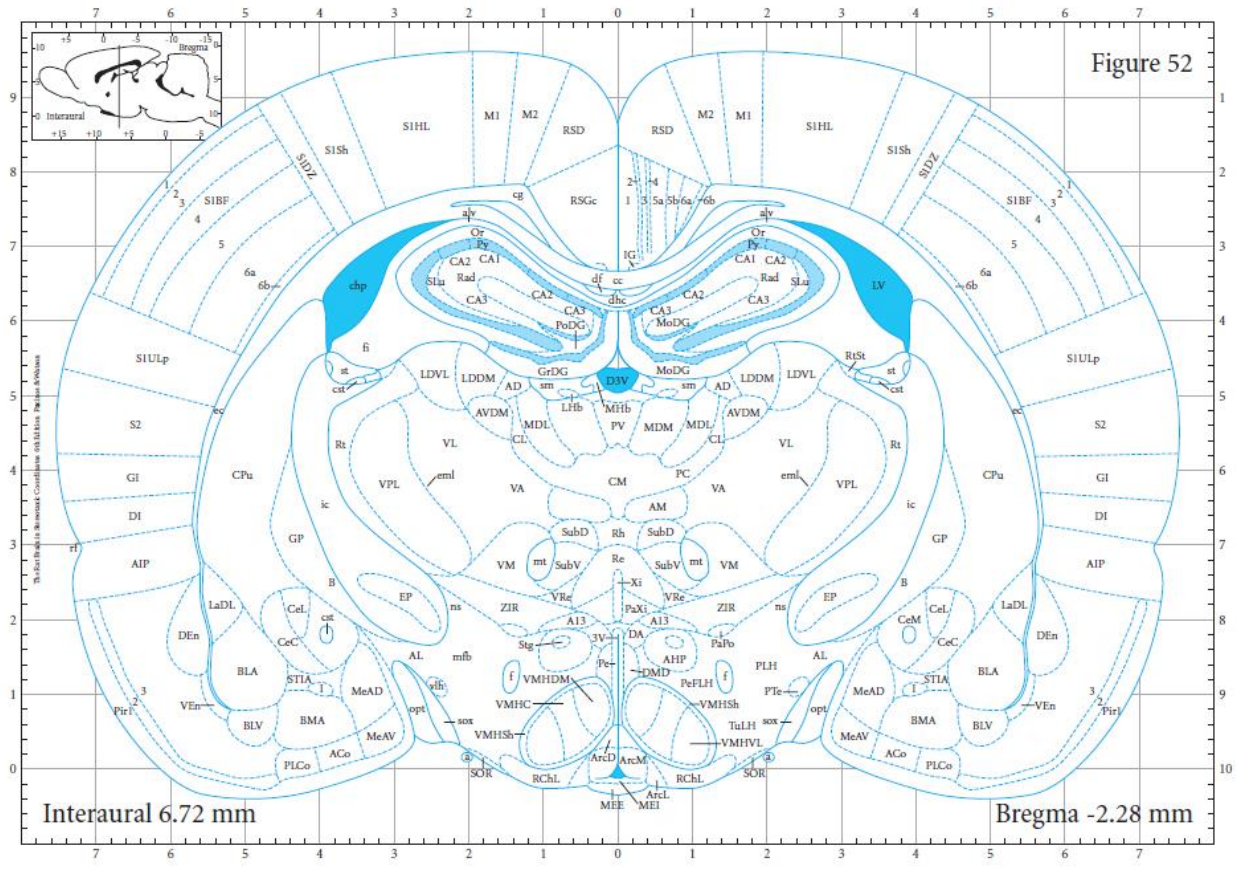


Figure 3: This diagram shows the general region where the measurements for Image Set 2 were taken. These measurements include basolateral amygdala (BLA), central nucleus of the amygdala (Ce), the anterior insular point (AIP), dysgranular insular cortex (DI), and granular insular cortex (GI).

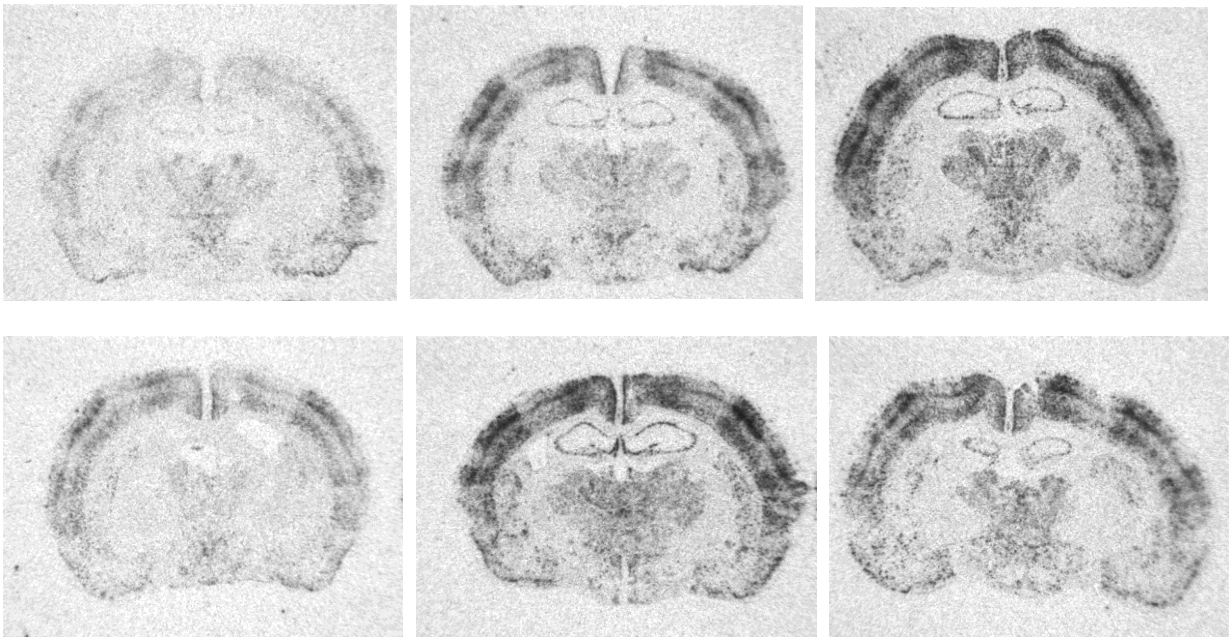


Figure 4: This figure shows example images from Image Set 2. From top left: LFPI+MN166, Naïve + No Shock, Naïve + Shock. From bottom left: Sham-operated, LFPI + No Shock, LFPI + Shock.

Appendix C

Image Set 3

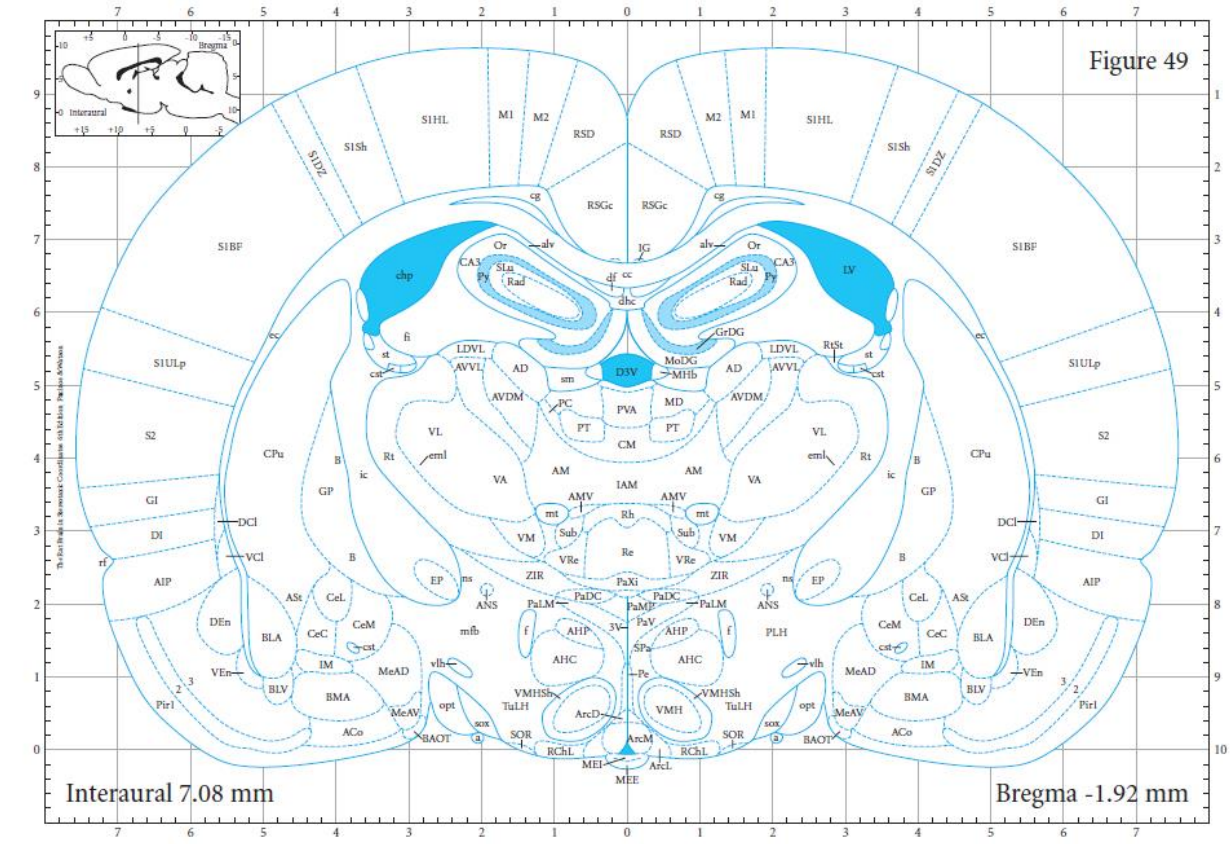
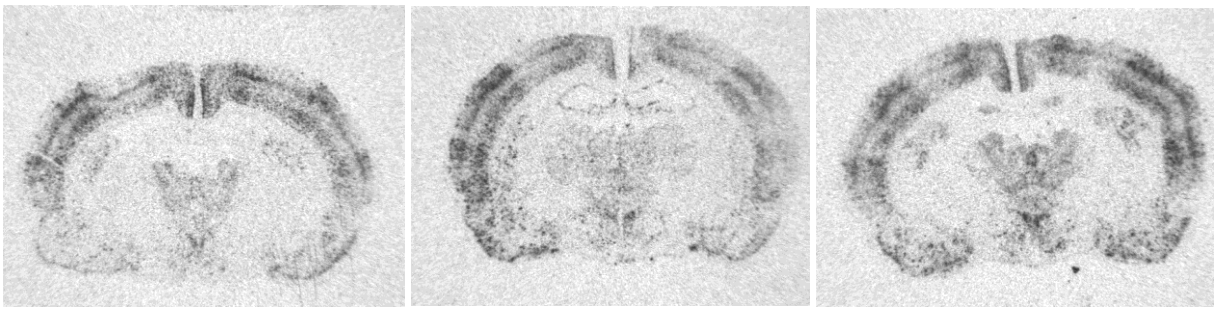


Figure 5: This diagram shows the general region where the measurements for Image Set 3 were taken. These measurements include the paraventricular nucleus (PVN) (PaV, PaM, PaDC, PaLM – paraventricular hypothalamic nucleus ventral part, medial parvicellular part, dorsal cap, lateral magnocellular part), the bed nucleus of the stria terminalis (BST), and the central gray (CG).



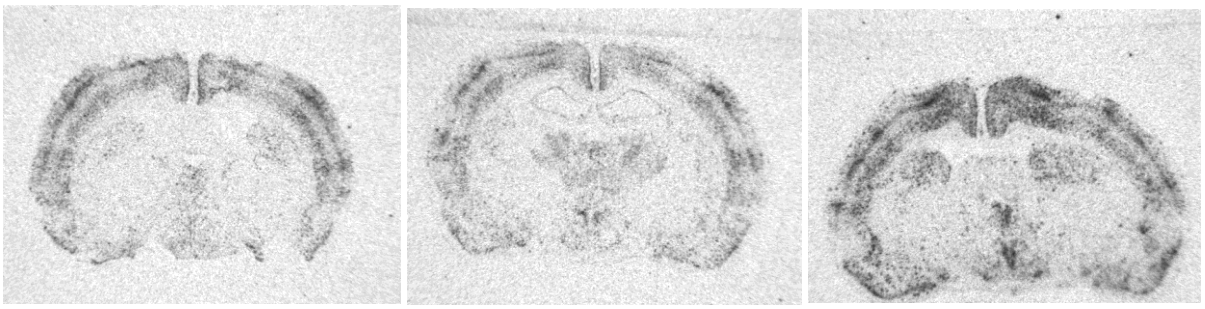


Figure 6: This figure shows example images from Image Set 3. From top left: LFPI+MN166, Naïve + No Shock, Naïve + Shock. From bottom left: Sham-operated, LFPI + No Shock, LFPI + Shock.

Appendix D

Image Set 4

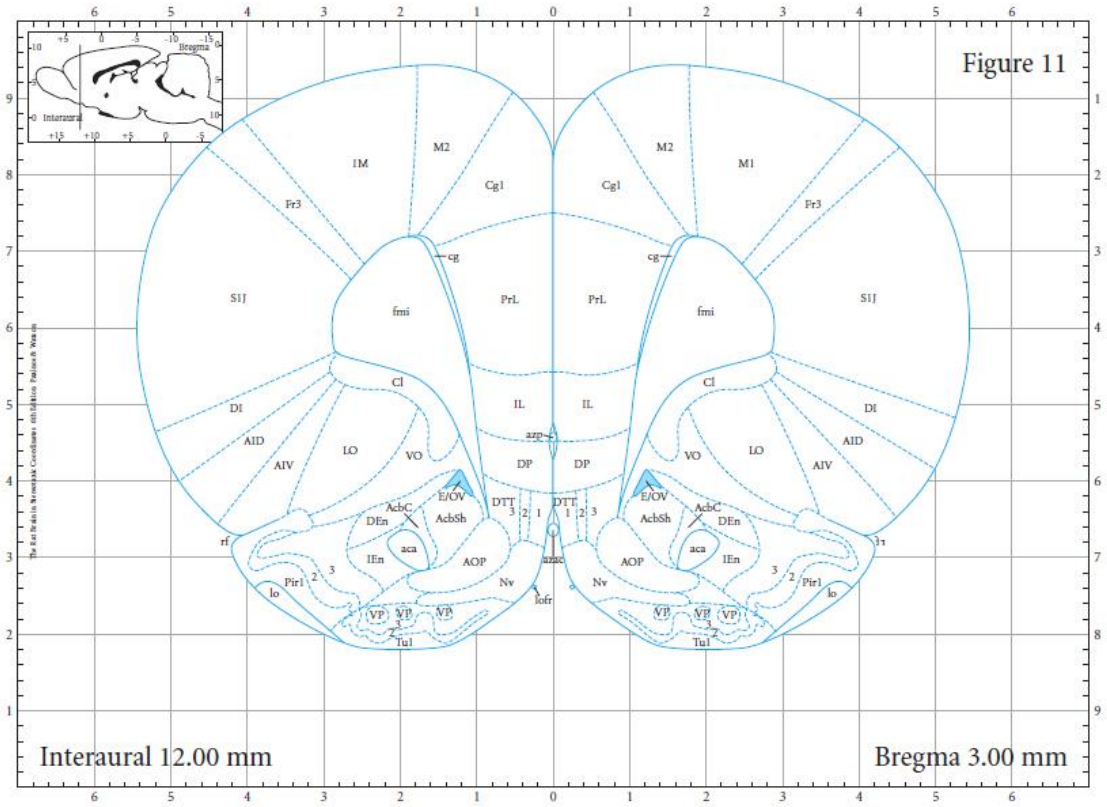


Figure 7: This diagram shows the general region where the measurements for Image Set 4 were taken. These measurements include the infralimbic cortex (IL), the prelimbic cortex (PrL), and the cingulate cortex (Cg1).

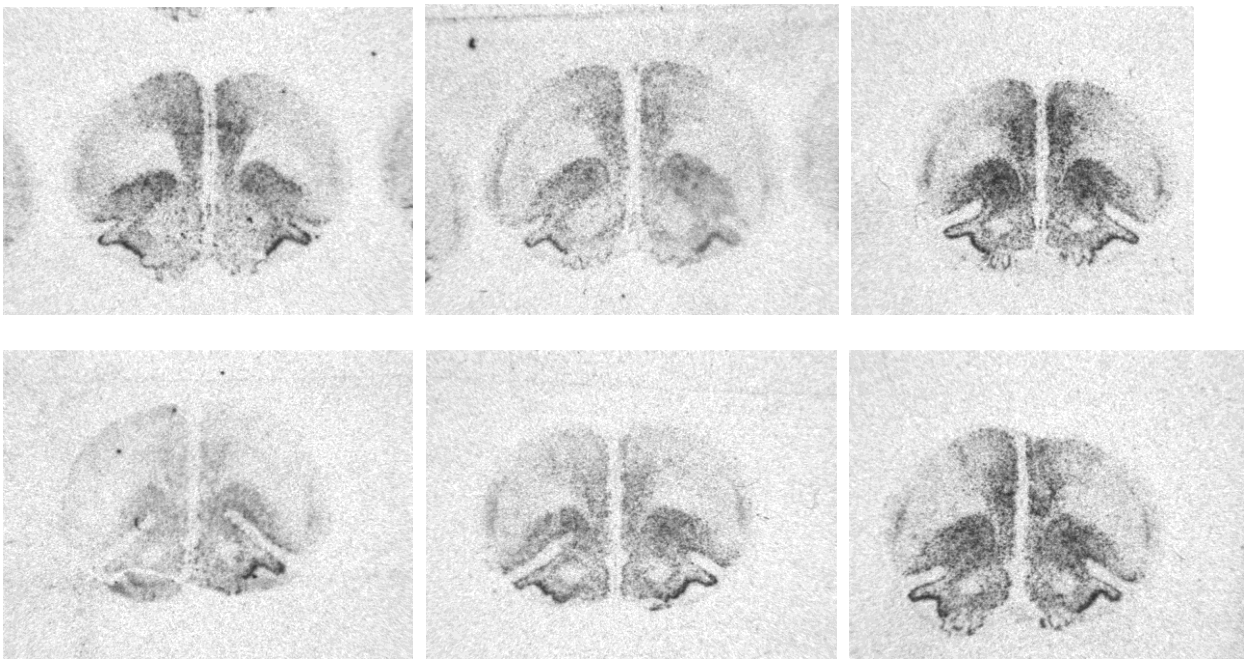


Figure 8: This figure shows example images from Image Set 4. From top left: LFPI+MN166, Naïve + No Shock, Naïve + Shock. From bottom left: Sham-operated, LFPI + No Shock, LFPI + Shock.