EVALUATING THE IMPACT OF AGE AND TIME ON SEONSORY AND AFFECTIVE LEVELS OF PAIN PROCESSING

by

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Presented to the Faculty of the Graduate School of

The University of Texas at Arlington in Partial Fulfillment of the

Requirements for the Degree of

MASTER OF SCIENCE IN PSYCHOLOGY

THE UNIVERSITY OF TEXAS AT ARLINGTON

DECEMBER, 2018

ACKNOWLEDGMENTS

I would like to acknowledge and thank my supervising professor, Dr. Perry Fuchs, for his advice and guidance through this extensive project. In addition, I would like to thank my committee members for their support and encouragement. Finally, I would like to acknowledge the University of Texas at Arlington, Psychology department.

DEDICATION

I dedicate this work to my husband and parents, who have supported me throughout the length of my research. Their encouragement has kept me motivated and driven to push forward. I am grateful for their understanding of the long days, and the late nights. None of this would have been possible without them.

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ABSTRACT

EVALUATING THE IMPACT OF AGE AND TIME ON SEONSORY AND AFFECTIVE LEVELS OF PAIN PROCESSING

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The relationship between pain and age has been studied for decades. However, research has produced conflicting results for pain thresholds and pain affect in clinical assessments. Likewise, pre-clinical research is conflicting and sparse, limiting the extent of any overarching conclusions. Despite conflicting results on directionality (higher thresholds verses lower thresholds), pain sensitivity and pain affect seem to change with age. Two major arguments have surfaced to explain these differences. The first is, the aging process can lead to changes in peripheral pain signals of pain pathways. The second is, higher order processes that evaluate pain information change with age.

For the current project, it was hypothesized that older animals would have diminished peripheral pain pathways due to the natural aging process; leading to decreased nociceptive behaviors for acute pain in older animals compared to younger animals. However, chronic pain was predicted to strengthen the signals in these pathways for all age groups due to continued use of the pathway. Since older individuals have been shown to inhibit pain less effectively than younger individuals, it was also predicted that (contrary to acute pain) chronic pain should elicit greater nociceptive behaviors in older animals than in younger animals.

In this study, Sprague Dawley Rats were categorized in two groups: young and old. A limited arthritic model was used to induce pain, while the control group received saline

injections. Subjects were evaluated on mechanical and thermal thresholds to evaluate sensory processing of pain. Pain affect was evaluated using the Place Escape/Avoidance Paradigm (PEAP testing) and immunohistological assessments of c-fos expression in the anterior cingulate cortex. The current study verified that old rats have differing levels of pain sensitization and pain affect than young rats. It was determined that chronicity of pain could interact with age to alter pain processing, but did not have any main effect on threshold results or assessments of pain affect.

Keywords: age, nociception, affect, chronic pain

CHAPTER 1

1.1 INTRODUCTION

The pursuit to understand pain has become so pervasive in psychological research that pain research encompasses topics as divergent as art therapy to neuropharmacology. Over the past few decades of pain research, topics of interest have ebbed and flowed. One specific topic of interest that has surfaced is, the evaluation of pain in regards to age (Gagliese, & Melzack, 1997). Currently this research is particularly relevant; culture trends in population data have necessitated a greater understanding in this area. Specifically, the aging populations, in developed countries around the world, have been charted to rise at an unprecedented rate (Kulik, Ryan, Harper, & George, 2014; Ortman, Velkoff, & Hogan, 2014). Furthermore, past and current surveys have revealed that chronic pain is consistently more prominent within elderly populations compares to younger populations(Andersson, Ejlertsson, Leden, & Rosenberg, 1993; Macfarlane, 2016; Tsang et al., 2008). To further underscore the social relevance of this research, chronic pain is costlier per year than cancer, diabetes and heart disease; accumulating an estimated total of $600 billion in The United States alone (Gaskin, & Richard, 2012). Therefore, understanding the patterns and specific mechanisms that contribute to chronic pain in the elderly would be highly beneficial to treating this costly ailment.

1.2 Chronic Pain

Chronic pain has been defined as pain that persists or reoccurs longer than 3 to 6 months (Treede et al., 2015). Chronic pain can also be classified as pain that persists past the healing stage of an injury. While acute pain is beneficial in alerting an organism to harm, chronic pain is detrimental and can result in several negative outcomes. For example, chronic pain can greatly decrease quality of life. Those who experience chronic pain often suffer from diminished

standards of physical, mental, and social wellbeing (Breivik, Collett, Ventafridda, Cohen, & Gallacher, 2006; Felce, & Perry, 1995; Jensen, Chodroff, & Dworkin, 2007; Zanocchi et al., 2008). It has been previously established that chronic pain is also comorbid with several other ailments such as depression and anxiety (McWilliams, Cox, & Enns, 2003; Tsang et al., 2008).

Investigation into the relationship between pain and age is clearly important due to the prevalence of chronic pain in elderly individuals and the ongoing rise of the elderly community. Examining this relationship is also extremely important due to pharmacological elements. Previous studies have revealed that some of the most common medications for pain relief are not as effective on elderly patients as they are on younger patients (Bernabei et al., 1998; Cleeland et al., 1994; Gagliese and Melzack, 1997). Moreover, the viability of traditional treatments for chronic pain overall, has been questioned due to issues of abuse and addiction (Ballantyne & Mao, 2003; Vest, Reynolds, & Tragesser, 2016). In sum, greater understanding of the relationship between pain and age is vital to improving quality of life and pain treatment for the vast majority of chronic pain sufferers.

1.3 Pain Affect

Pain has traditionally been evaluated using the sensory dimension; defined as the location and intensity of pain. However, pain is a multidimensional experience, incorporating affect and cognition, in addition to sensory components. Where, affect refers to the unpleasantness of pain or the emotional dimension of pain, and cognition refers to the mental processes used to evaluate pain (Melzack, 1999; Price, 2000). Pain affect can largely alter the experience and perceived intensity of pain; thus the current study was designed to evaluate this dimension in addition to sensory perceptions.

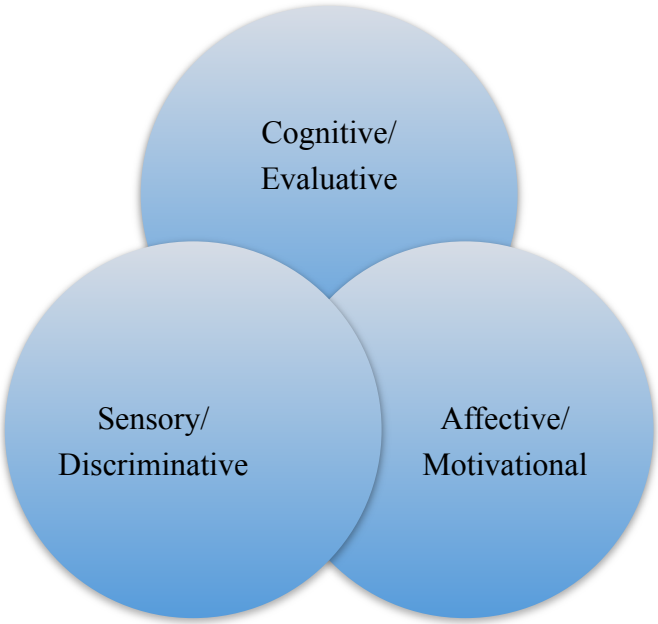


Figure 1.3.1 The multidimensional neuromatrix of pain (Melzack, 1999).

1.4 The Anterior Cingulate Cortex

The Anterior Cingulate Cortex (ACC) is a subcortical brain structure which has been implicated in both affective and cognitive processes (Devinsky, Morrell, & Vogt, 1995). In particular, it has been demonstrated to be a vital area for affective processing of pain (Fuchs, Peng, Boyette-Davis, & Uhelski, 2014). For example, anterior cingulotomy has been shown to significantly relieve pain and reduce pain affect without changing sensory ability to locate the area of pain (Pereira, Paranathala, Hyam, Green, & Aziz, 2014). In rodents, lesions on the rostral ACC reduce conditioned place avoidance but not acute pain behaviors, implicating the ACC in the affective component of nociception (Johansen, Fields, & Manning, 2001). As clearly evidenced, the ACC is an important structure associated in the experience of pain. Evaluating ACC activation across age groups could be crucial to determining differences in the affective dimension of the pain/ age dynamic. Thus, Immunohistochemistry will be performed on area

Cg1 and Cg2 of the anterior cingulate cortex. Previous research has suggested that Cg1 of the cingulate cortex (the dorsal ACC) is associated with evaluation of negative affect, and Cg2 of the cingulate cortex (the ventral ACC) is associated with regulation of limbic areas that respond to negative affect (Etkin, Egner, & Kalisch, 2011). The approximate coordinates of interest were AP+2.2mm from Bregma, AP+1.7mm from Bregma, and AP+1.6 from Bregma (see Figure 1.4.1). These coordinated were targeted due to findings from previous research, suggesting that this area of the ACC is associated with pain and avoidance (Gao, Ren, Zhang, & Zhao, 2004).

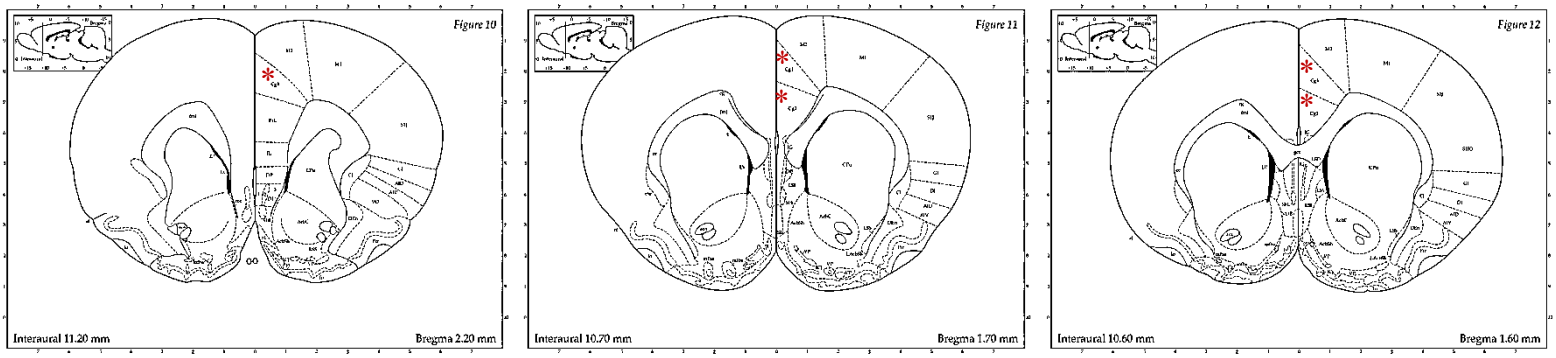


Figure 1.3.2. Images from the Rat Brain Atlas for Bregma coordinates AP+2.2mm, AP+1.7mm, and AP+1.6, respectively (Paxinos & Watson, 2006).

1.5 Clinical Research

Laboratory studies in clinical settings have revealed that pain behaviors and pain sensitivity are moderated by age (Lautenbacher, Peters, Heesen, Scheel, & Kunz, 2017). However, results have varied on the overall effect of age on pain. Some findings have indicated that pain sensitivity increases with age while others have indicated that pain sensitivity decreases with age (Bek, Uygur, Bayar, & Armutlu, 2002; Helme & Gibson, 2001). In addition, some studies have conflicting findings for mechanical or thermal thresholds; such that some studies have found differences across age and others have not (Heft, Cooper, O'Brien, Hemp, &

O'Brien, 1996; Heft & Robinson, 2010; Kenshalo Sr, 1986; Lautenbacher, Kunz, Strate, Nielsen, and Arendt-Nielsen, 2005; Marini et al., 2012; Pickering, Jourdan, Eschalier, and Dubray, 2002; Woodrow, Friedman, Siegelaub, & Collen, 1972). Despite slightly diverging threshold results, the overall trend within laboratory research indicates that pain sensitivity decreases with age (Lautenbacher, 2017). These laboratory studies indicate that elderly individuals perceive pain to a lesser degree than younger individuals. However, population data suggests a discrepancy with these findings; such that elderly individuals are seeking treatment for chronic pain at very high rates. Clearly pain, irrespective of age, can be a debilitating experience.

In addition to sensory results, laboratories have also investigated pain affect across age. Older adults in the lab have consistently shown less negative pain affect than younger individuals (Chao, Hsieh, Chiu, Tseng, & Chang, 2007; Gagliese & Melzack, 2003; Rustøen et al., 2005; Sherman & Robillard, 1960; Sherman & Robillard, 1964). These laboratory findings are again at odds with population data, which suggests that chronic pain sufferers are often diagnosed with comorbid affective ailments such as depression or anxiety, regardless of age (Herr, Mobily, & Smith, 1993; Ulbricht, Hunnicutt, & Lapane, 2016). One possible explanation for this discrepancy is laboratory studies often evaluate acute pain, while population statistics typically report information on chronic pain patients.

1.6 Preclinical Research

Relatively few studies have evaluated pain thresholds in old animals and even fewer have evaluated the affective dimension of pain. Of the acute pain evaluations, some have indicated greater sensitivity and lower thresholds in elderly animals while others have indicated the opposite trend (Jourdan et al., 2000; Jourdan et al., 2002; Ririe, Vernon, Tobin, & Eisenach, 2003; Zheng, Gibson, Khalil, Helme, & McMeeken, 2000). In comparison, neuropathic pain

studies have indicated older animals display less sensitivity than younger animals or do not develop allodynia/ hypersensitivity at all (Cruce, Lovell, Crisp, & Stuesse, 2001; Chung, Choi, Yoon, & Na, 1995; Tanck, Kroin, McCarthy, Penn, & Ivankovich, 1992). Unfortunately, due to the limited amount of preclinical assessments in elderly animals, overall patterns cannot be determined.

The ambiguity within the literature indicates that much is still unclear about the underlying mechanisms that are present in the age and pain dynamic. Greater comprehension of underlying mechanisms could lead to elucidation of conflicting results both within clinical and also within preclinical literature. To evaluate these biological processes, pain should be evaluated in the same way that it is experienced; through a multidimensional model. Thus, our understanding of pain processes in the elderly would be greatly enhanced by studies that evaluate pain across both affect dimensions of pain and sensory dimensions of pain. In addition, our understanding of the pain patterns would be greatly improved by evaluating both chronic and acute assessment of pain.

1.7 Purpose

The purpose of this experiment was to evaluate differences in the higher order and peripheral processes that occur during pain, across age. Previous literature has indicated that the aging process may alter the underlying mechanisms of pain. This experiment aimed to evaluate these differences using both behavioral testing and immunohistochemistry. In addition, the current study aimed to reveal whether nociceptive behaviors persist or change across acute pain to chronic pain.

CHAPTER 2

2.1 METHODS

To evaluate age differences in an arthritic pain model, several measures were assessed. The independent variables of the study were age (young, old), condition (acute pain, chronic pain), and treatment (arthritic model, sham). The dependent variables were sensory responses (mechanical thresholds, thermal thresholds) and affective responses (Place Escape-Avoidance Paradigm scores, c-fos expression in the ACC). All subjects underwent baseline testing prior to the experimental procedure. In addition, all animals were tested on exploratory behaviors in an Open-Field maze prior to, and after, experimental procedures.

2.2 Subjects

This study used eighty-four Sprague Dawley rats randomly selected from the University of Texas at Arlington vivarium. The experimental condition consisted of two groups; defined as young (3-6 months of age), or old (20 months of age or older). Within each age group animals were randomly assigned to a chronic pain condition, an acute pain condition, a chronic sham condition or an acute sham condition. See Figure 2.1 to view experimental design. Animals were initially double or triple housed, single housing occurred when cage-mates fulfilled the requirements of the study. All subjects were exposed to a 12:12 dark/light cycle, and food and water were be provided ad libitum. All procedures were conducted in accordance with the guidelines of the International Association for the Study of Pain and approved by the University of Texas at Arlington Institutional Animal Care and Use Committee.

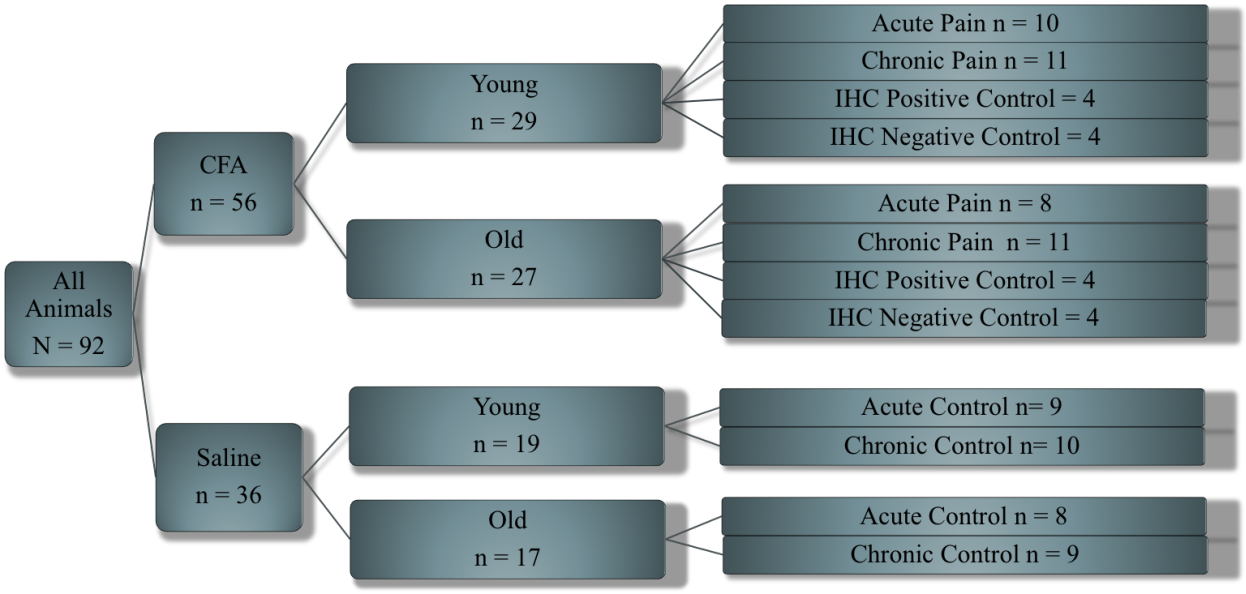


Figure 2.1. Experimental design for surgery and sham groups.

2.3 Procedure

Exploratory behaviors in the Open-Field Maze, baseline mechanical paw withdrawal thresholds (MPWTs), and baseline Hargreaves' Method thermal thresholds were measured prior to any experimental manipulations. A priori, it was determined that animals presenting tactile or thermal allodynia would be noted to avoid confounds in the experiment, no such animals were identified. All subjects then underwent induction of an arthritic model or a sham injection. The post-operative recovery period was two days in duration; long enough for the animals to recover. MPWTs and thermal thresholds were again measured, post injection, to evaluate hypersensitivity across age groups (evaluation occurred on the third day for the acute pain group and after a month for the chronic pain group). Place Escape-Avoidance Paradigm (PEAP) testing was also conducted to measure pain affect across ages. Lastly, immunohistochemistry was performed to evaluate c-fos levels in the Anterior Cingulate Cortex to evaluate protein activation across ages. All animals were weighed at the time of baseline testing and at the time of experimental testing.

2.4 Drugs

Chemicals used in this experiment included povidone-iodine, Complete Freund's Adjuvant (CFA), isoflurane, and pentobarbital. Povidone-iodine is an antiseptic that was applied liberally to the site of injection (before and after) to prevent infection. Animals were either injected with CFA or saline. CFA is an inactive from of *Mycobacterium Tuberculosis,* mixed with suspension antigens such as paraffin, which produces an elevated inflammatory reaction at the site of injection due to immunological responses (Fontes et al., 2017). The amount of CFA used for injection was .10mL for the experimental group. All animals were unconscious during injection due to Isoflurane inhalation. Isoflurane is a general anesthetic. The dosage of anesthesia was inhalation of 3% isoflurane in oxygen for induction and 2% isoflurane in oxygen for maintenance. After injection and behavioral testing, animals were anesthetized via pentobarbital injection for perfusion procedures. Pentobarbital is a barbiturate and depending on dosage can be used as a sedative or anesthetic. Anesthesia was induced by intraperitoneal injection of pentobarbital at 150mg/kg.

2.4 Open-Field Maze

Prior to and after injection, exploratory behaviors were measured using an Open-Field Maze, evaluating the mobility of each age group (see Figure 2.2). For this procedure, animals were placed in a circular chamber with a wooden base and aluminum metal walls (100cm diameter, 45cm height). The duration of the test was five minutes. Total distance traveled and mean velocity were quantified by Ethovision software. Rearing behavior was manually quantified at the time of the open field test.

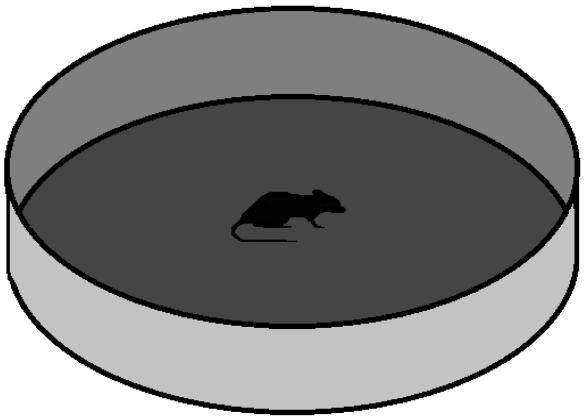


Figure 2.2. Open-Field chamber used to measure exploratory behaviors.

2.5 Mechanical Paw Withdrawal Thresholds (MPWT)

To evaluate mechanical thresholds, animals were placed upon a wire mesh platform within bottomless plexiglass chambers (see Figure 2.3). This apparatus allows for access to the base of each paw in order to stimulate and evaluate tactile sensation. Subjects were given ten minutes to habituate to the apparatus prior to stimulation. Mechanical thresholds were measured using von Frey monofilaments. The monofilaments are fibers of varying diameter and force that range from flexible to rigid (3.85mN, 5.68mN, 9.74mN, 18.39mN, 39.42mN, 77.30mN, 135.30mN, 251.34mN). Three separate trials were conducted for each hind paw, beginning with the 3.85mN von Frey filament. The von Frey filaments were applied to the left and right hind paw for approximately one second, respectively. If the animal did not respond by withdrawing or licking the stimulated paw then the next highest force filament was used. Conversely, if the animal did respond, then the next lowest force filament was used. Ambulation was considered an ambiguous response, in which case the stimulus was repeated. Stimulation of each hind paw

occurred until the highest force had been reached or until five total stimuli had been applied. This evaluation was conducted prior to and after injection.

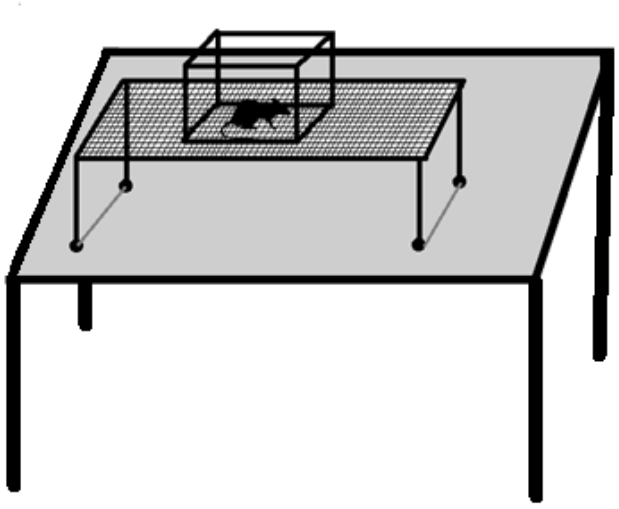


Figure 2.3. Mechanical Paw Withdrawal Threshold apparatus used to measure mechanical thresholds.

2.6 Hargreaves' Method Thermal Thresholds

To evaluate thermal thresholds, subjects were placed on a glass platform within a plexiglass chamber (see Figure 2.4). Beneath the glass platform there was a plantar test apparatus, which was moved freely for proper positioning. The plantar test apparatus was used to apply infrared heat to the left and right hind paw of each subject for three separate trials. The infrared heat gradually increased until the subject withdrew, at which point the apparatus automatically terminated the heat stimulation. Thresholds were measured in latency to withdraw (in seconds). If animals did not withdraw, the heat stimulation was terminated manually after a maximum of fifteen seconds, to avoid tissue damage. The average of each trial (three trials total)

was calculated for the left and right hind paw to calculate the threshold for each subject. This evaluation was conducted prior to and after injection.

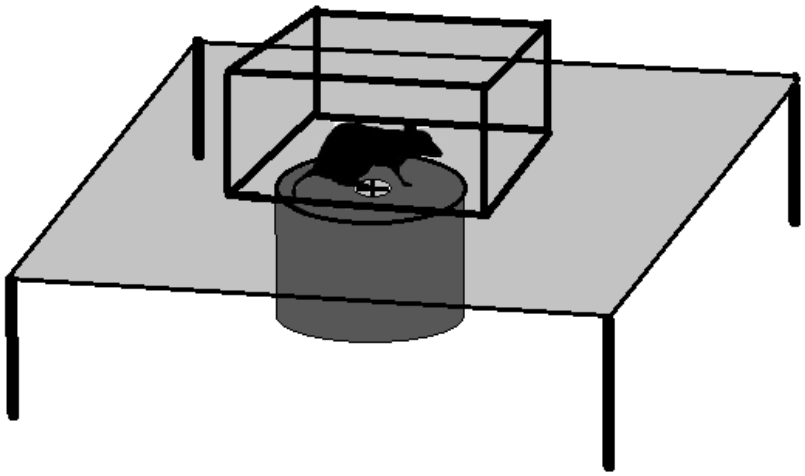


Figure 2.4. Plantar test apparatus used to evaluate thermal thresholds.

2.7 Limited Arthritic Model

The limited arthritic model proposed by Butler, Godefroy, Besson, and Weil-Fugazza, (1992) was adapted to induce inflammatory nociception (Wilson, Toepfer, Senapati, Wilson, & Fuchs, 2007). Animals were anesthetized with 3% isoflurane for induction and 2% for maintenance. Establishment of anesthesia was tested using a pinch stimulus applied to the hind paws and was confirmed through the absence of the withdrawal reflex. Breathing rate and reflexes were periodically assessed throughout the procedure to ensure proper depth of anesthesia. The injection site was cleaned, prior to injection, using povidone-iodine. Next, animals received an intra-articular (i.a.) injection in the left knee with a 28-guage needle. After injection, the site was cleaned, for a second time, using povidone-iodine. Experimental animals

received a .10ml injection of CFA and control animals received a .10ml injection of saline. Animals were then be placed under a heating lamp to regain consciousness from anesthesia.

2.8 Place Escape Avoidance Paradigm (PEAP testing)

The affective quality of pain was measured using the Place Escape Avoidance Paradigm (PEAP), developed by LaBuda and Fuchs (2000). Subjects were placed within a rectangular apparatus made of plexiglass with two chambers; one dark and one light (see Figure 2.5). The apparatus was positioned atop a wire mesh platform to allow access to the left and right hind paws of the subject. In this paradigm animals were able to move freely across the chambers. Previous research has shown that under normal circumstances rats prefer the dark side of the chamber. Affect is measured when animals shift their preference to the light chamber after experiencing pain stimuli in the dark chamber.

In the proposed study, animals were stimulated using a 476mN von Frey filament on the left hind paw (ipsilateral to injection) when they are in the dark chamber and on the right hind paw (contralateral to injection) in the light chamber. This stimulation was delivered for approximately one second, every 15 seconds. The total duration of the test was thirty minutes. Affect/avoidance was measured in the percentage of time that animals spent in the light chamber. Time spent in the light chamber was calculated for each five minute time interval (5 mins, 10 mins, 15 mins, 20 mins, 25 mins, 30 mins) and for the total duration of the test. A greater percentage of time spent in the light chamber revealed greater avoidance and indicated affect.

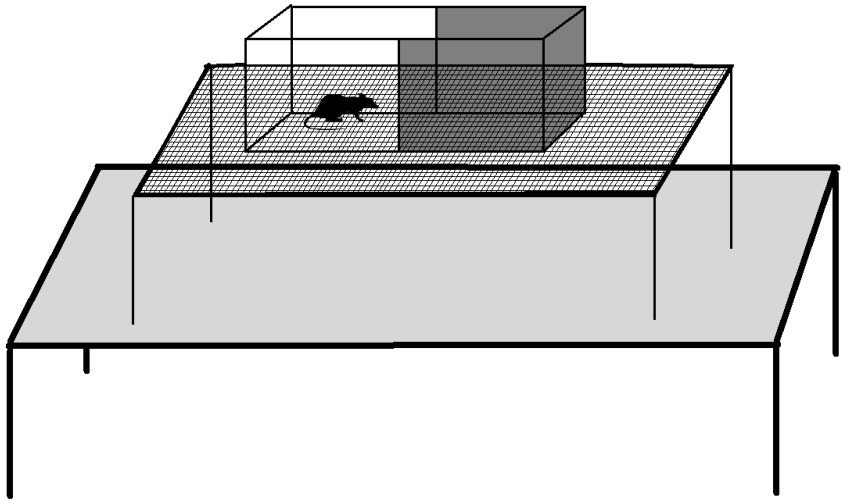


Figure 2.5. Place Escape-Avoidance Paradigm apparatus used to measure pain affect.

2.9 Immunohistochemistry

Following behavioral testing, animals were anesthetized and perfused intracardially as previously described by Perrotti et al., 2005. Animals were then be decapitated and the brain was extracted. Coronal slices were cut using a microtome and then stored in .1% sodium azide until the time of Immunohistochemistry. Coronal slices were collected for approximate Bregma coordinates for 2.5, 2.3, and 2.1. To begin staining, the tissue was first washed with 1X phosphate buffer saline (PBS) to remove excess components (three times). Endogenous peroxidase activity was inhibited with hydrogen peroxide and the tissue was, again, washed with 1 X PBS (three times). Blocking was then be performed for 45 minutes using .3% triton X, 3% normal goat serum (NGS), and 1X PBS. Next, the tissue was incubated in the primary antibody (c-fos) for 24 hours at room temperature or 48 hours at 4°C. Following incubation, the slices were washed three times with 1 X PBS. The tissue was then incubated in the secondary antibody for an hour and a half. Again, the tissue was washed three times with 1 X PBS. The Avidin-Biotin Complex (ABC) was then introduced for an hour and a half. Following ABC, the slices

were washed two times with 1 X PBS and then two times with Tris. Finally, DAB substrate was added to the sample in deionized water for approximately two to five minutes. Three final washes were performed in 1X PBS and then the slices were stored in 1X PBS until mounting. Cover slipping was performed after mounting and c-fos expression was quantified in the anterior cingulate cortex using Gen5 software.

CHAPTER 3

3.1 RESULTS

It was predicted that CFA injected subjects would have lower mechanical and thermal thresholds and less mobility (open field) than saline injected subjects. Additionally, it was predicted that there would be a significant interaction of treatment (CFA vs. saline) with age (old vs young) and time/condition (acute vs. chronic). For affective measures it was also predicted that there would be a significant interaction of treatment with age and condition.

3.4 Data Screening

Data were entered into IBM SPSS Statistics 23 and analyzed for violations of normality in continuous variables. Values for skew and kurtosis were within acceptable ranges for normality (-1 to 1 and -2 to 2, respectively). Graphical evaluation of histograms for each continuous variable confirmed acceptable levels of normality. Data collection for behavioral measures was complete and no missing variables were present. Some data were missing for immunohistochemistry (IHC) analysis due to tissue damage during extraction or failed staining. Where no data were available for IHC, the whole subject was removed from IHC analysis.

3.3 Open Field Testing

Evaluation of baseline differences via a one-way Analysis of Variance (ANOVA's) revealed significant differences between old and young animals for total distance traveled, velocity, and rearing. Specifically, younger animals indicated higher mobility and exploration than older animals in distance traveled, $F(1,74) = 54.439$, $p < .001$, in mean velocity, $F(1, 74) = 49.213$, $p < .001$, and in cumulative rearing, $F(1, 74) = 14.555$, $p < .001$. These results indicate, even prior to experimental manipulations, that the age groups differ in mobility and exploratory behaviors (see table 3.3.1).

Table 3.3.1

Estimated marginal means and standard errors by age in the open field maze.

	Old Subjects		Young Subjects	
Open Field Measures	*M*	*SE*	*M*	*SE*
Total Distance Traveled	2123.479	75.816	2907.311	74.169
Mean Velocity	7.141	.256	9.642	.248
Cumulative Rearing	17.361	1.517	24.600	1.173

Note. Total distance traveled measured in centimeters and velocity in centimeters per second.

A Multivariate Analysis of Variance (MANOVA) was conducted to evaluate differences in mobility and exploratory behaviors, following experimental procedures. A MANOVA test was conducted due to the theoretical similarity in the dependent variables and to reduce type one error. Evaluation of the dependent variables revealed that they were highly correlated, further supporting the use of a MANOVA (see table 3.3.2). Thus, the MANOVA evaluated mobility through the composite dependent variable (DV) scores for total distance traveled, mean velocity, and cumulative rearing. Independent measures for this test were age (young/old), treatment

(saline, CFA), and condition (acute/chronic). The assumption for equal error variance was not violated; however, the assumption for equal covariance was violated ($p < .001$). Therefore, the more conservative and robust values of Pillai's Trace (V) criterion are reported.

Table 3.3.2
Correlations between distance velocity and rearing in the Open Field Maze after injection.

		Total Distance	Mean Velocity	Cumulative Rearing
Total Distance	Pearson Correlation	1	.997**	.640**
	Sig. (2-tailed)		.000	.000
Mean Velocity	Pearson Correlation	.997**	1	.648**
	Sig. (2-tailed)	.000		.000
Cumulative rearing	Pearson Correlation	.640**	.648**	1
	Sig. (2-tailed)	.000	.000	

**. Correlation is significant at the 0.01 level (2-tailed).

The MANOVA revealed that there were significant main effects for age, $V = .323$, $F(3,66) = 10.480$, $p < .001$, and treatment $V = .290$, $F(3,66) = 8.980$, $p < .001$, on the composite DV for mobility. Where younger animals had higher mobility scores than older animals (see Table 3.3.3) and saline animals and higher mobility scores than CFA animals (see Table 3.3.4).

Table 3.3.3
Estimated marginal means and standard errors for mobility scores across age.

Dependent Variable	Age	*M*	*SE*
Total Distance	young	2471.964	106.888
	old	1623.882	113.340
Mean Velocity	young	8.289	.357
	old	5.427	.379
Cumulative Rearing	young	14.965	1.002
	old	8.897	1.062

Note. Total distance traveled measured in centimeters and velocity in centimeters per second.

Table 3.3.4

Estimated marginal means and standard errors for mobility scores across treatment.

Dependent Variable	Treatment	*M*	*SE*
Total Distance	saline	2107.912	112.737
	CFA	1987.935	107.524
Mean Velocity	saline	7.090	.377
	CFA	6.626	.359
Cumulative Rearing	saline	15.369	1.057
	CFA	8.493	1.008

Note. Total distance traveled measured in centimeters and velocity in centimeters per second.

It was also revealed that there was a significant interaction between age and condition on mobility assessments, $V = .200$, $F(3,66) = 5.489$, $p = .002$. Additionally, there was an interaction between age, treatment, and condition trending towards significance, $V = .097$, $F(3,66) = 2.361$, $p = .079$. Univariate statistics are shown in Table 3.3.5 and reveal the unique differences of age, condition, and treatment on total distance traveled, mean velocity, and cumulative rearing.

Table 3.3.5

Tests of Between-Subjects Effects

Main Effects and Interactions	Dependent Variables					
	Total Distance		Mean Velocity		Cumulative Rearing	
	F-statistic	*p*-value	*F*-statistic	*p*-value	*F*-statistic	*p*-value
Age	29.634	.000**	30.225	.000**	17.274	.000**
Treatment	0.593	0.444	0.794	0.376	22.175	.000**
Condition	4.312	0.042*	4.574	0.036*	4.678	0.034*
Age X Treatment	0.003	0.956	.000	0.991	0.185	0.669

Age X Condition	3.295	0.074	3.737	0.057	15.451	.000**
Treatment X Condition	0.444	0.508	0.356	0.553	2.125	0.149
Age X Treatment X Condition	4.301	0.042*	3.784	0.056	.000	0.999

**Significant at the .01 level

*Significant at the .05 level

3.4 Mechanical Paw Withdrawal Thresholds

Evaluation of baseline thresholds was conducted using a one-way ANOVA, revealing no significant differences in threshold levels between age groups for the right, $F(1,74) = 1.404$, $p = .240$, or left hind-paw, $F(1,74) = 1.418$, $p = .238$. These results indicate that, prior to experimental manipulation, the age groups had statistically similar mechanical threshold levels. To evaluate differences across age, treatment, and condition two Factorial ANOVA's were conducted. Factorial ANOVA's were used in place of a MANOVA due to the conceptual differences between the two DVs being measured (right hind-paw/ left hind-paw). Where the left hind-paw underwent manipulation and the right hind-paw did not.

Evaluation of mechanical thresholds for the left hind-paw was conducted using a 2X2X2 factorial ANOVA; where age (young/old), treatment (saline/CFA), and condition (acute/chronic) were the independent measures. The analysis revealed a significant main effect of treatment, $F(1,68) = 201.130$, $p < .001$, and a significant interaction between treatment and age, $F(1,68) = 14.763$, $p < .001$. The main effect of treatment revealed lower thresholds for CFA injected animals ($M = 8.493$, $SE = 1.008$) than saline injected animals ($M = 15.369$, $SE = 1.057$). Further investigation of the interaction, via estimated marginal means, suggests that thresholds were

lower for CFA injected animals, specifically for the young animals (see Figure 3.4.1). These results partially support the hypothesis, where treatment and age impacted threshold results. No effect of condition (acute pain/chronic pain) was present. However, condition was an important aspect of the experiment. Therefore, the impact of age and condition on CFA animals can be visualized in Figure 3.4.2 or in Table A1 in Appendix A.

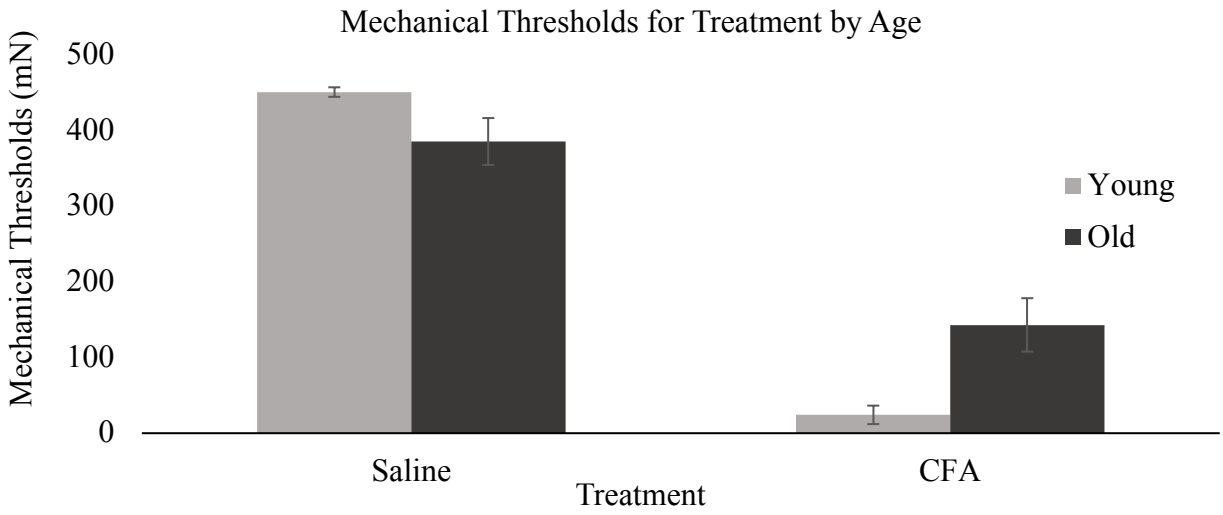


Figure 3.4.1. Shows the interaction between age and treatment on mechanical thresholds.

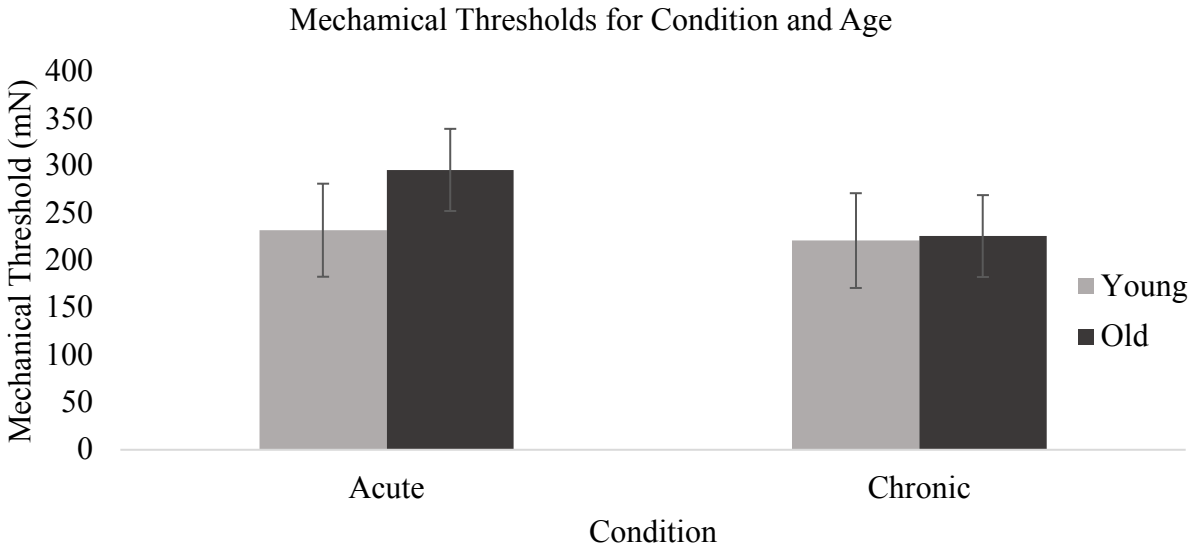


Figure 3.4.2. Shows no differences between age and condition on mechanical thresholds.

Evaluation of mechanical thresholds for the right hind-paw was also conducted using a 2X2X2 factorial ANOVA; where age (young/old), treatment (saline/CFA), and condition (acute/chronic) were, again, the independent measures. Contrary to prediction, there was a difference between groups, despite the lack of experiential manipulation on the right paw (no injection). The analysis revealed a main effect of age, $F(1,68) = 10.792$, $p = .002$, where older animals ($M = 382.729$, $SE = 16.366$) had significantly lower mechanical thresholds than younger animals ($M = 456.630$, $SE = 15.434$). This effect could possibly be explained by a confound of natural, endogenous pain due to old age.

3.5 Hargreaves' Method Thermal Thresholds

The evaluation of baseline thresholds was conducted using a one-way ANOVA, revealing no significant differences in threshold levels between age groups for the right, $F(1,74) = .201$, $p = .656$, or left hind-paw, $F(1,74) = .113$, $p = .737$. These results indicate that, prior to experimental manipulation, the age groups had statistically similar thermal threshold levels. Thermal thresholds were evaluated for both left and right hind-paw using separate 2X2X2 factorial ANOVA's. Where, in each analysis, age (young/old), treatment (saline/CFA), and condition (acute/chronic) were the independent measures.

The analysis of the left hind-paw revealed a main effect for treatment, $F(1,68) = 22.272$, $p < .001$ and age, $F(1,68) = 20.239$, $p < .001$. The main effect of treatment revealed, CFA injected animals ($M = 5.834$, $SE = .307$) had lower thresholds than saline injected animals ($M = 7.931$, $SE = .322$). The main effect of age revealed, young animals ($M = 5.883$, $SE = .305$) had lower thresholds than old animals ($M = 7.882$, $SE = .323$). There was also a significant interaction between treatment and age, $F(1,68) = 9.081$, $p = .004$, and an overall interaction between treatment, age, and condition, $F(1,68) = 5.534$, $p < .022$. The interaction reveals no

significant differences between saline treated animals. However, there are significant differences between each group for the CFA injected group (see Figure 3.5.1. for the total interaction and Figure 3.5.2 for the interaction in CFA animals only). Where younger animals had significantly lower thresholds for both acute and chronic conditions. Surprisingly, older animals in the chronic group had higher thresholds than older animals in the acute group.

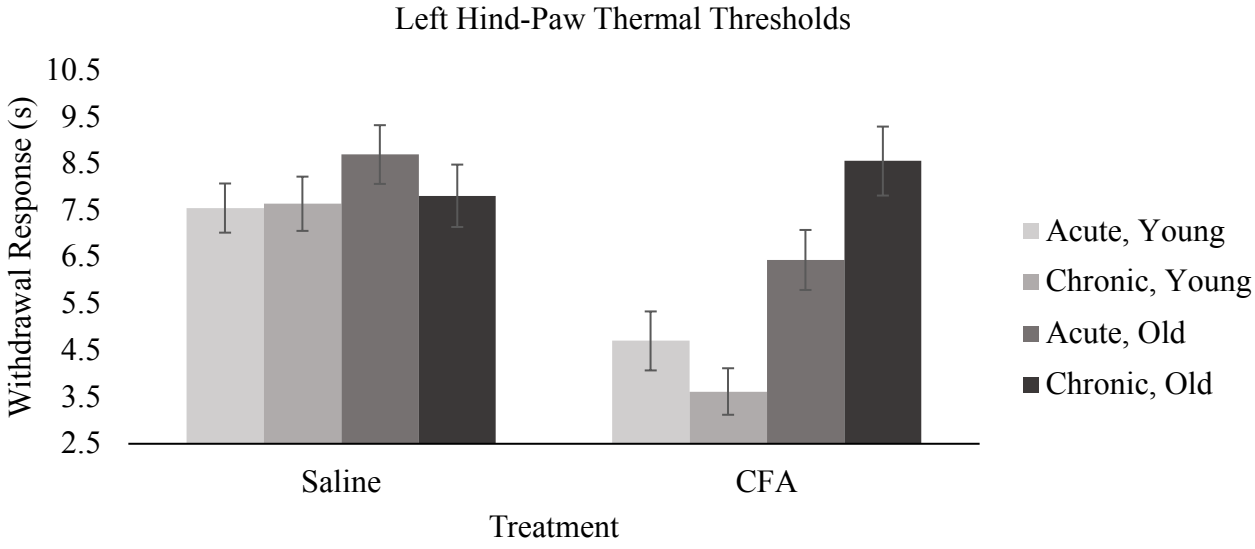


Figure 3.5.1. Showing the overall interaction between treatment, age, and condition.

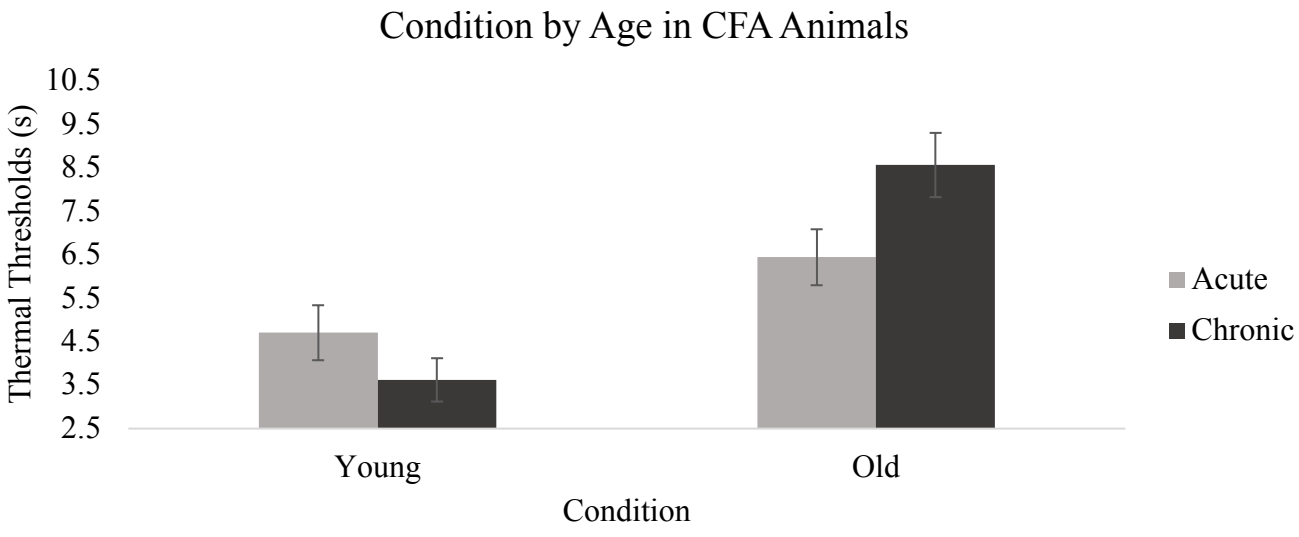


Figure 3.5.2. Showing the interaction between condition and age in CFA injected animals.

Evaluation of thermal thresholds on the right hind-paw revealed no significant interactions as expected. However, there was an unexpected significant main effect of condition, $F(1,68) = 5.103$, $p = .027$. Where, animals in the acute condition ($M = 7.740$, $SE = .341$) had lower thresholds than the animals in the chronic condition ($M = 8.788$, $SE = .315$). This effect of condition on the right hind-paw was unanticipated due to lack of experimental manipulation (no injection). These results may have been a consequence of practice effect. Such that, the acute group were initially exposed to the apparatus prior to injection and then exposed a second time after 48 hours. Whereas the chronic group were exposed to the apparatus prior to injection and then a second time after 30 days. Thus, it is possible that the acute group experienced practice effects from back-to-back exposure and the chronic group did not.

3.6 Place Escape/Avoidance Paradigm

The data from the PEAP tests were evaluated using a mixed model ANOVA; where the between subjects factors were age, treatment, and condition and the within subjects factor was intervals of time. The dependent measure was the percentage of time spent in the light chamber. The data did not violate the assumption of equal error variances but did violate the assumption of sphericity. Therefore, Greenhouse-Geisser corrected *F*-statistics were reported for a more conservative approach. There was no main effect of time for the within subjects effects. Interestingly, the overall interaction between time, treatment, age, and condition was approaching significance, $F(3.483, 236.845) = 2.479$, $p = .053$. See Figure 3.6.1 for the overall interaction and Figures 3.6.2 to visually compare only CFA injected animals. The results also revealed an interaction approaching significance between time, treatment, and age, $F(3.483, 236.845) = 2.495$, $p = .052$.

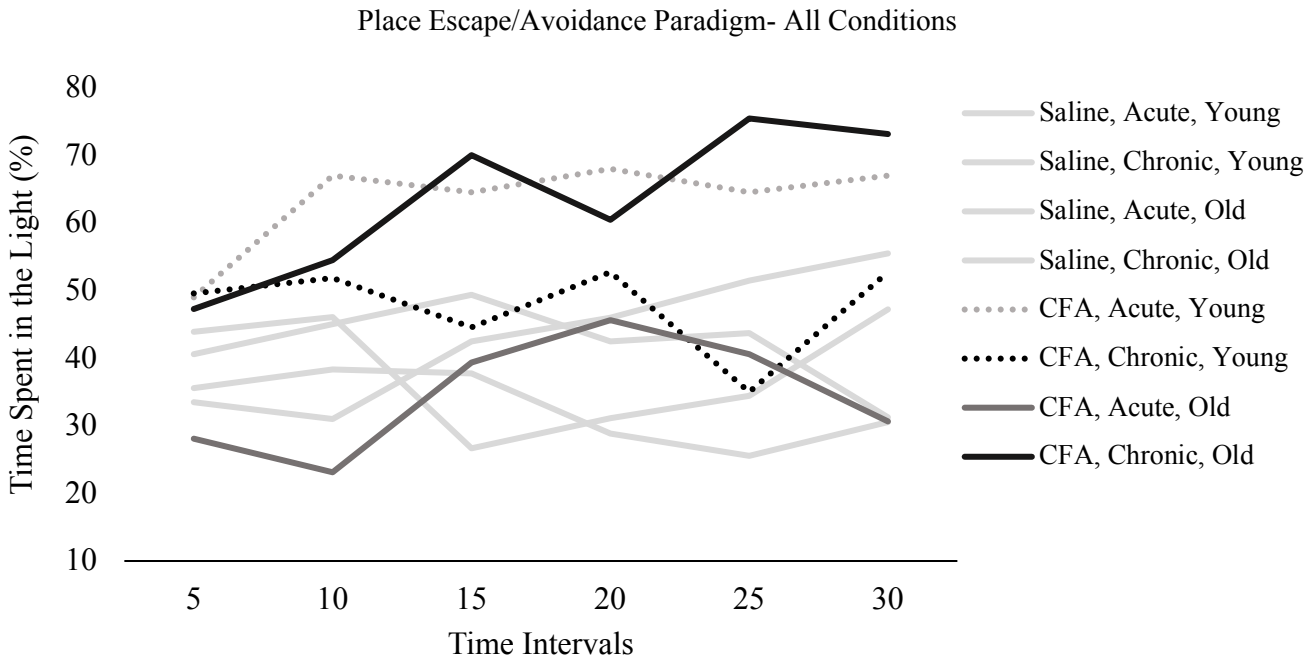


Figure 3.6.1. Shows the overall interaction of age, treatment, and condition across time.

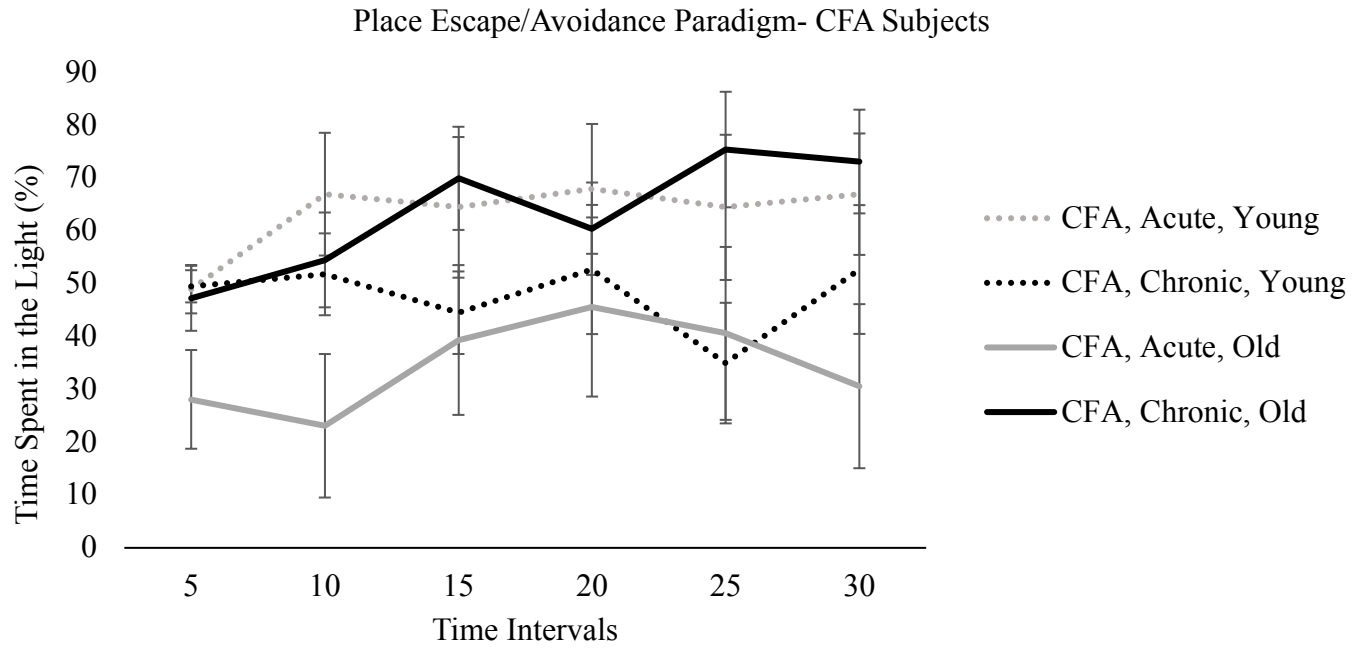


Figure 3.6.2. Shows the interaction between age, treatment, and time for CFA injected subjects.

Between subjects effects revealed a main effect of treatment on total time spent in the light chamber, $F(1, 68) = 3.938$, $p = .051$. Where CFA injected animals ($M = 52.282$, $SE = 4.582$) displayed more avoidance than saline injected animals ($M = 39.109$, $SE = 4.804$). Notably, there was an overall, significant interaction between age, treatment, and condition on total time spent in the light chamber, $F(1, 68) = 4.921$, $p = .030$ (see Figure3.6.3). The interaction reveals that the young animals in the acute pain group and the old animals in the chronic pain group avoided significantly more than animals injected with saline and old animals in the acute pain group.

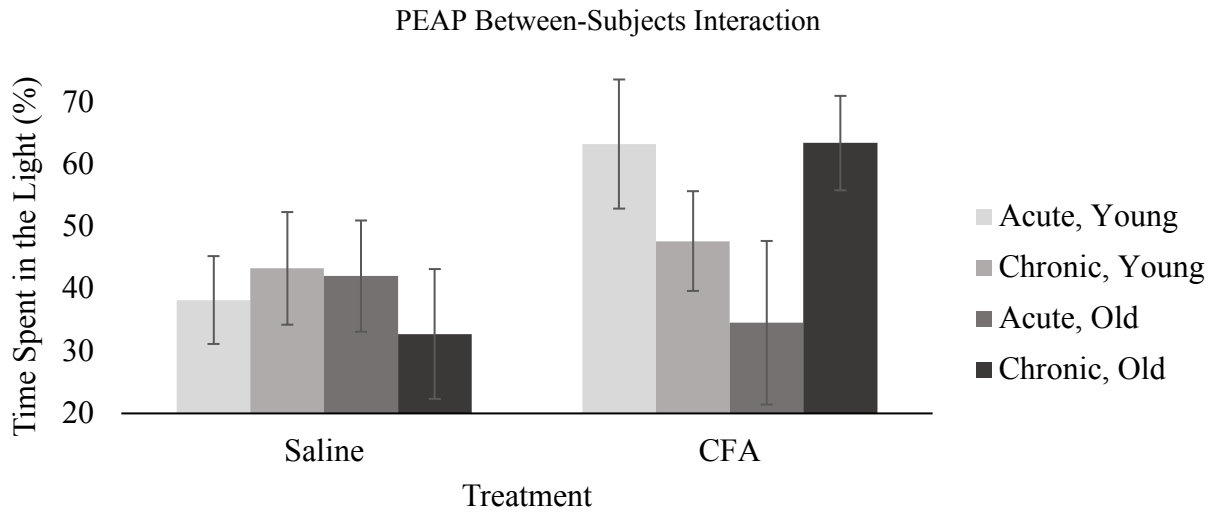
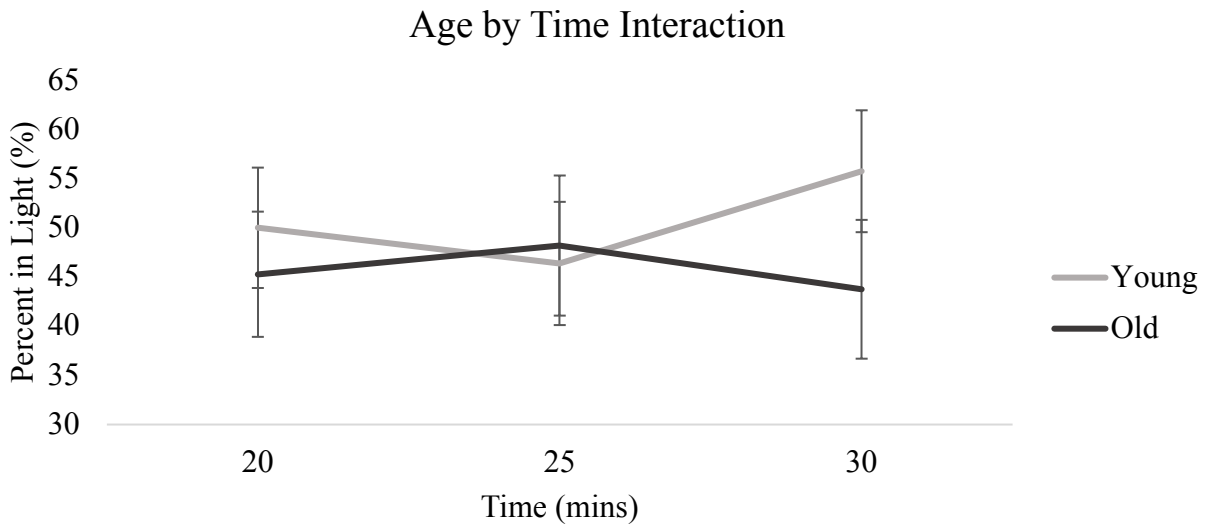


Figure 3.6.3. Illustrates the interaction between age, treatment, and condition on total percent of time spent in the light chamber.

The PEAP test involves the animals learning to escape/avoid, which typically stabilizes after the first fifteen minutes of the test. Therefore, in addition to the overall PEAP scores, analysis was conducted to evaluate just the last fifteen minutes of the paradigm. By analyzing the last fifteen minutes of the scores we can more accurately evaluate pure avoidance rather than evaluating the learning process in addition to avoidance. In this analysis a mixed model ANOVA

was still used; with the same between factors of age, treatment, and condition. The within factor was also the same, where subjects were evaluated at intervals of time. However, in this analysis we focus only on the last fifteen minutes. The analysis revealed a similar pattern of avoidance to the overall analysis. There was a marginally significant interaction between time and age, $F(1.674, 113.863) = 2.927$, $p = .067$, see Figure 3.6.4. Between subjects results revealed a significant main effect of treatment, $F(1, 68) = 3.912$, $p = .052$, where CFA animals ($M = 55.493$, $SE = 5.747$) avoided more than saline animals ($M = 39.023$, $SE = 6.026$). In addition, a significant interaction was seen between treatment, condition, and age, $F(1, 68) = 5.024$, $p =$



.028. See Figure 3.6.5 for interaction effects.

Figure 3.6.4. Illustrates the interaction between time and age during the last 15 minutes of the PEAP test.

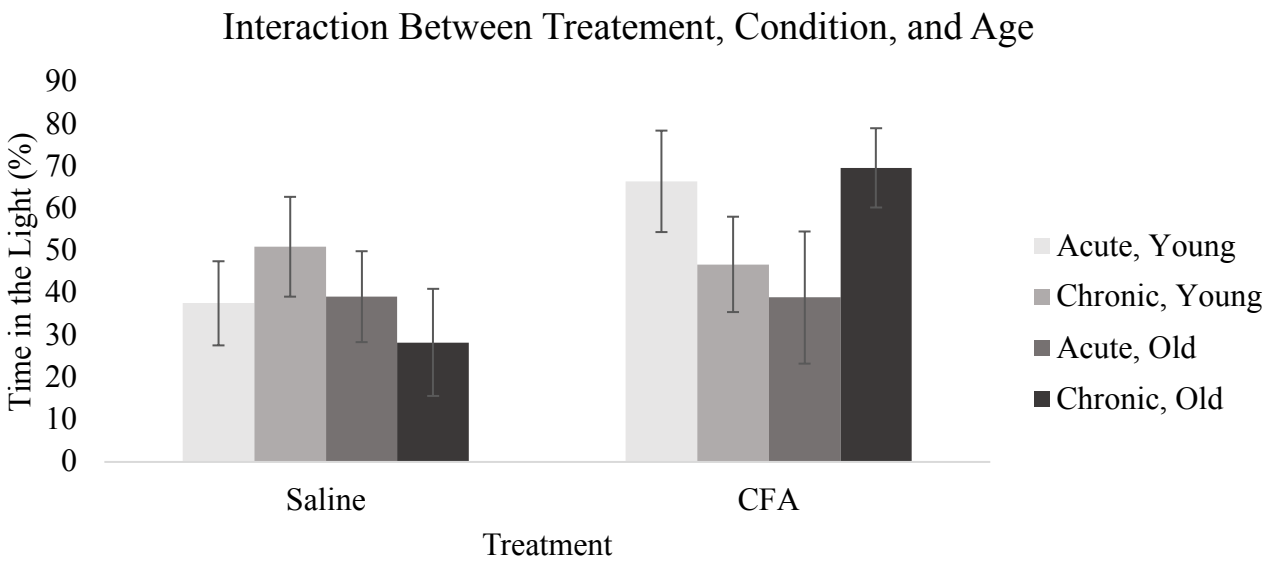


Figure 3.6.5. Illustrates the interaction between age, treatment, and condition on total percent of time spent in the light chamber during the last 15minutes of the PEAP test.

Due to differences in mobility (discussed in section 3.3), it is important to establish that differences in the PEAP tests were due to experimental methods and not a result of mobile ability. Therefore, number of crosses through the midline of the apparatus were assessed across age. A one way ANOVA, with age as the independent factor and crosses as the dependent measure, revealed no significant differences in crossing of the midline between age groups, $F(1, 74) = 2.360$, $p = .129$. These results suggest that mobility across age is unlikely to confound the results of the PEAP data.

3.7 Immunohistochemistry

The first analysis of variance conducted was a mixed model ANOVA to evaluate protein counts on the ACC area Cg1. Between-subjects factors were age, treatment, and condition, across the within-subjects factor of Bregma coordinates (+2.2mm, +1.7mm, +1.6mm). The assumption of equal covariance was not violated, however, the assumption of sphericity was. Therefore, more conservative *F*-statistics were reported after a Greenhouse-Geisser adjustment.

The analysis revealed a significant main effect of Bregma coordinate, $F(1.672, 102.012) = 17.702$, $p < .001$, where areas Bregma +1.7mm and Bregma +1.6mm had significantly higher protein expression ($p < .001$) than Bregma +2.2mm. See table 3.7.1 for means and standard errors. Positive and negative controls were also evaluated and revealed that staining was sufficiently due to primary antibody rather than non-specific binding.

Table 3.7.1
Means and standard error of protein expression in the Cg1 by Bregma coordinates.

Bregma	Mean	Std. Error
+2.2mm	5139.109	150.533
+1.7mm	5487.252	133.534
+1.6mm	5552.098	120.348

Note. Values reported are estimated marginal means and standard errors.

The mixed ANOVA also revealed a significant interaction between Bregma coordinate and treatment, $F(1.672, 102.012) = 4.321$, $p = .021$. Specifically, it was revealed that, in the most anterior portion of the Cg1 (+2.2mm), animals injected with CFA had significantly less c-fos expression (see Figure 3.7.1). See Table A2 in Appendix A for means and standard errors. Additionally, between-subjects results indicated a significant main effect of age, $F(1, 61) = 10.436$, $p = .002$. Where, older animals ($M = 4978.263$, $SE = 186.337$) had significantly fewer c-fos positive cells than younger animals ($M = 5807.377$, $SE = 176.488$).

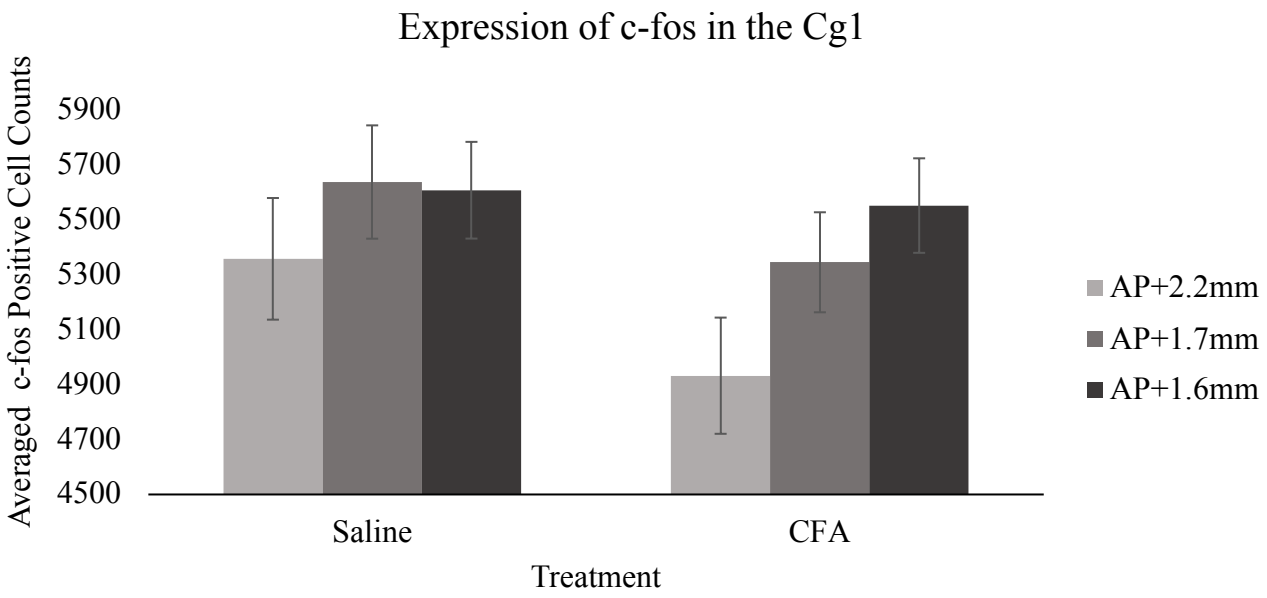


Figure 3.7.1. The number of cells positive for c-fos expression across Bregma coordinates and treatment.

Evaluation of the area Cg2 in the ACC was conducted through a mixed model ANOVA, where number of c-fos positive cells were compared across age, treatment, and condition. The within-subjects factor was Bregma coordinates (+1.7mm, +1.6mm). The analysis of Cg2 does not include the coordinate of +2.2mm, because at this point along the neural axis, the coordinate corresponds to the prelimbic area and not the ACC. In this analysis, the assumption of equal covariance was not violated but the assumption of sphericity was. Therefore, Greenhouse-Geisser corrected *F*-statistics were reported. There was an effect of Bregma coordinate trending towards significant, $F(1, 61) = 3.399$, $p = .070$. Where, Bregma +1.7mm had fewer c-fos positive cells ($p < .001$) than Bregma +1.6mm. See table 3.7.2 for means and standard errors.

Table 3.7.2
Means and standard error of protein expression in the Cg2 by Bregma coordinates.

Bregma	*M*	*SE*
+1.7mm	4402.297	129.974
+1.6mm	4516.119	112.435

Note. Values reported are estimated marginal means and standard errors.

Between-subjects results indicated a significant main effect of age, $F(1, 61) = 4.050$, $p = .049$. Where, older animals ($M = 4220.830$, $SE = 169.956$) had significantly fewer c-fos positive cells than younger animals ($M = 4697.586$, $SE = 160.973$). See Appendix B for analysis of Cg2 when coordinate 2.2mm is included.

Differences between coordinates in the Cg1 were not expected, therefore an exploratory analysis was conducted to evaluate differences between anterior and posterior areas of the ACC. To do this, areas +1.6 and 1.7 were collapsed together to quantify anterior counts. These counts were then compared to coordinate +2.2mm, which was defined as the anterior region. Justification for the collapse of coordinates +1.7mm and +1.6mm is twofold. Firstly, the ACC in these coordinates is similar based on both location and morphology. Secondly, no significant differences were found for c-fos positive cells across these coordinates. Henceforth, coordinate +2.2mm will be referred to as the anterior ACC and the average of coordinates +1.7mm and +1.6mm will be referred to as the posterior ACC. Area Cg2 of the ACC does not reach coordinate +2.2mm, therefore this analysis has been provided in Appendix C but has not been incorporated into the primary results.

A mixed model ANOVA was conducted to evaluate differences within subjects for anterior versus posterior c-fos positive cell averages across factors of age, treatment, and condition, within the Cg1. A significant difference was revealed between anterior and posterior regions of the Cg1, $F(1, 61) = 26.070$, $p < .001$, where the anterior region ($M = 5139.109$, $SE =$

150.533) had fewer c-fos positive cells than the posterior region (M = 5519.675, SE = 123.380). In addition, there was a significant interaction between treatment and area of the ACC (anterior/posterior), $F(1, 61) = 3.917$, $p = .052$, see Figure 3.7.2 and Table 3.7.3. Between subjects evaluations revealed a main effect of age, $F(1, 61) = 10.154$, $p = .002$. Where, average c-fos positive cells are fewer in the Cg1 for old animals (M = 4907.219, SE = 192.379) than for young animals (M = 5751.566, SE = 182.211).

Figure 3.7.2. Interaction between position within the Cg1 and treatment.

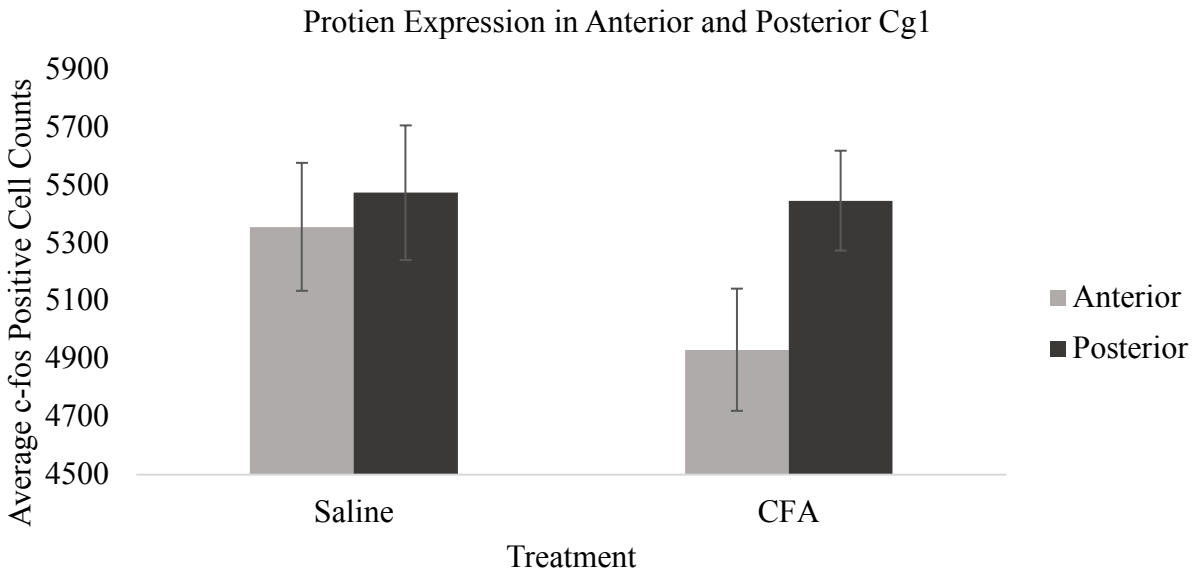


Table 3.7.3
Average c-fos positive cells by treatment and ACC coordinates.

	ACC Position	M	SE
Saline	Anterior	5371.962	222.093
	Posterior	5605.006	182.032
CFA	Anterior	4906.257	203.263
	Posterior	5434.345	166.599

Note. Values reported are estimated marginal means and standard errors.

3.8 Relationships

In order to evaluate the relationship between behavioral results and immunohistological results a series for regressions were performed. Firstly, mobility measures were regressed onto IHC results. Then mechanical and thermal thresholds were regressed onto IHC results. Finally, PEAP results were regressed onto IHC results. See Appendix D for the graphed relationships between behavioral measures and c-fos expression in the Cg1. See Appendix E for the graphed relationships between behavioral measures and c-fos expression in the Cg2.

Regression results for behavioral measures of mobility revealed that, distance traveled in the Open Field Maze was a marginally significant predictor for number of c-fos positive cells in the anterior Cg1, $\beta = .287$, $t(65) = 1.760$, $p = .083$. Similarly, mean velocity was also a marginally significant predictor for the number of c-fos positive cells in the anterior Cg1, $\beta = .001$, $t(65) = 1.742$, $p = .086$. Rearing was revealed to be the strongest mobility predictor for number of c-fos positive cells in the anterior Cg1, $\beta = .055$, $t(65) = 3.253$, $p = .022$. Mobility measures did not significantly predict c-fos positive cells for the posterior Cg1 or posterior Cg2. Evaluation of PEAP results and threshold data revealed no significant relationships or predictors.

CHAPTER 4

4.1 DISCUSSION

The purpose of this study was to evaluate the relationship between pain and age. In addition, there was interest in exploring the impact of chronicity on the factors of age and pain. This research has become increasingly necessary as the elderly population rises. Past clinical research has produced equivocal results on this relationship and past preclinical research is too limited and similarly convoluted to draw conclusions. Therefore, this study was designed to further evaluate the age/pain relationship by differentiating between sensory pain versus pain

affect and chronic pain versus acute pain. It was hypothesized that pain behaviors and affect would change across age and across the duration of pain (acute vs chronic). We predicted that acute pain would produce the most avoidance and the lowest thresholds in younger animals and the least avoidance and highest thresholds in older animals. We also predicted, that chronic pain would produce the opposite trend where older animals will avoid the most and have the lowest thresholds. In addition, we expected c-fos expression to follow similar trends; where c-fos positive cells would be most abundant in the young animals for acute pain, but most abundant in the old animals for chronic pain.

We expected these results because of several observations that have been made in the literature. Clinical laboratory findings have shown that the elderly have less negative affect and higher thresholds to acute pain, while population data has shown that the elderly who suffer from chronic pain have lower qualities of life and suffer more strongly from depression and anxiety (Turk, Okifuji, and Scharff, 1995). Suggesting, for clinical populations, that pain affect can change when evaluating acute pain versus chronic pain. In addition, elderly individuals have been shown to have decreased activity in areas of the brain that work to inhibit pain (Lariviere, Goffaux, Marchand, & Julien, 2007). It is important to note that these studies are clinical in nature and may not fully translate to preclinical models. However, due to the lack of preclinical research on pain and age, it was necessary to make predictions based on both clinical and preclinical findings.

Based on the literature available, we hypothesized that, for acute pain, older animals may have naturally degenerated peripheral pathways as a consequence of age, resulting in interruption of pain signals and reduced responsivity/affect. However, with chronic pain these pathways that have naturally degenerated in older animals could be strengthened and made more effective from

continual use. This, combined with a reduced ability to inhibit pain, could result in magnified responses from older animals at chronic levels.

In relation to what was predicted, the hypotheses were partially supported. Mechanical thresholds indicated that age did interact with pain. Where, younger animals had lower thresholds after CFA injection. This pattern has been previously seen in response to postoperative pain (Ririe et al., 2003). While, other studies of paw inflammation have seen no differences across age (Simón-Arceo et al., 2014) This suggests that age may have an effect on mechanical thresholds of certain models (postoperative and arthritic) but not others (plantar inflammation). Previous researchers have suggested that methodology may play a key role in the inconsistencies seen between studies evaluating the age and pain relationship (Gagliese, & Melzack, 2000). Interestingly, the older animals injected with saline had significantly lower thresholds than young animals. This could possibly be due to the older animals already experiencing age related pain issues. This same pattern was present in the right hind-paw evaluations. Where older animals had significantly lower thresholds despite the lack of experimental causes.

Thermal threshold results also served to partially support the hypotheses. The results indicated that there was an interaction between age, treatment, and condition. As predicted, in the young animals, chronic pain produced lower thresholds than acute pain. However, in older animals the opposite pattern was present. This indicates that time and chronicity may play a key role in the differences found between age groups and nociception. We also saw differences in patterns of pain processing between thermal and mechanical stimuli. This reiterates the importance of methodology when evaluating pain and age (Gagliese, & Melzack, 2000). Interestingly, clinical findings have also suggested and evaluated differences in mechanical and

thermal pain processing; further underscoring the importance of distinction between the two types of stimuli (Chakour, Gibson, Bradbeer, & Helme, 1996). Evaluation of the right hind-paw revealed a significant effect of condition, despite lake of experimental manipulation. As previously stated in the results, this could possibly be due to practice effects, where the acute group experienced practice effects from back-to-back exposure and the chronic group did not due to the length of time between testing.

The PEAP results were very similar to what was hypothesized. Results revealed that the older animals in the chronic pain group spent greater amounts of time avoiding than the older animals in the acute group. It was unexpected, though, that the younger animals in the acute group were more avoidant at times than the young animals in the chronic pain group. Acute differences between young and old animals replicated previous findings in our lab. Notably, threshold results revealed that older animals had less sensitivity to pain, while PEAP evaluations revealed that, in the chronic condition, older animals displayed greater avoidance. This supports clinical research, where older individuals tend to have higher thresholds, and also aligns with population data, where older individuals experience negative affect and comorbidities in conjunction with chronic pain.

The IHC data also provided mixed results. As expected, there was a significant interaction with treatment. However, this interaction revealed that c-fos expression was significantly lower in CFA animals for coordinates +2.2mm of the Cg1. The number of positive c-fos cells was highest in coordinates +1.7mm and +1.6mm of the Cg1. This interaction revealed no differences for the other coordinates across treatment, contrary to what was predicted. These results imply that there may be differential processing occurring across the ACC; in this case, greater activity was revealed in posterior coordinates of the ACC.

A main effect of age, for Cg1 and Cg2, revealed that older animals had lower levels of c-fos expression than younger animals, regardless of treatment or time. These results may suggest that the ACC of older animals is not as activated in response to inflammatory pain as younger animals. This reflects some of the unexpected results, where younger animals had lower thresholds than the older animals for chronic conditions. The results indicate that younger animals had, in general, lower thresholds and greater pain processing in the ACC. Suggesting, greater pain processing both at the peripheral and central level.

Evaluation of the relationships between behavioral measures and immunohistology revealed interesting results. The data indicated that mobility measures were more predictive of c-fos expression in ACC than threshold or PEAP results. Previous research has strongly linked the ACC with the processing of pain, particularly affective dimensions of pain (Donahue, LaGraize, & Fuchs, 2001). Indeed, past research has even revealed differences in c-fos expression in the ACC relating to pain condition (Uhelski, Morris-Bobzean, Dennis, Perrotti, & Fuchs, 2012).

There are three potential explanations for the unexpected results that were found. Firstly, prior exploration of the ACC has revealed that it is related to both processing of pain affect and motor initiation (Devinsky, Morrell, & Vogt, 1995). Specifically, context seems to have a large impact on ACC activation. It is possible that this relationship between pain and motor processing was detectible in the Open Field Maze; due to the greater range and potential for movement (whereas the same range of movement was not granted in other evaluations). The second explanation is, processing of pain affect may be more restricted to specific pathways of the ACC and was 'washed out' by evaluation of the entire coronal section of the ACC. Lastly, the unanticipated results may be due to the transient nature of c-fos protein. Between the time of pain processing and tissue collection the activity of the c-fos protein may have reduced;

preventing the detection of differences. Additionally, anesthesia used to euthanize the animals may have alleviated the pain stimulus, thus impacting c-fos expression.

One potential confound to the experiment could be the chronic aspect of the design. In humans, chronic pain has been defined as pain lasting or reoccurring for at least three to six months. For rats there is no such standardization. The proposed study defines chronic pain as persisting one month or longer. However, there is no way to confirm that this timeframe accurately represents a chronic state. Considering that for humans, chronic pain can occur at three months we believe it is reasonable to assume that a one-month timespan will be long enough to be considered chronic for animals. Particularly because rats have a substantially shorter life span than humans.

As previously discussed, endogenous chronic pain could also be a potential confound. Some of the older animals may have already been experiencing chronic pain from natural age-related issues. The onset of natural age related pain disorders is unknown in laboratory rodents. This could potentially skew the data being collected for the experimentally induced pain for both the treatment group and control group. Conversely, extinction of sensory nerves due to the injection of CFA could also be a potential confound. The CFA injection has been standardized to induce inflammation in the knee akin to what is seen in arthritic conditions. However, if any sensory nerves are negatively impacted this could result in loss of sensory input. This would present as the animal feeling diminished or no pain when in reality they have impaired sensory signaling.

The rise of the elderly population implies a rise in chronic pain sufferers. Greater understanding of pain patterns in the elderly could greatly benefit the establishment of treatment programs for this vulnerable group. Moreover, the elderly have been shown to react more slowly

to pain, which leads to greater probability of injury. Results from the current study suggest that chronicity in pain can be impactful for older animals relating to affect. Understanding of these issues could aid and educate programs for injury prevention and treatment. It would be specifically beneficial to know which types of pain are likely to lead to greater negative affect and comorbidities. For example, awareness of negative affect due to inflammatory pain versus neuropathic pain. Future research should aim to further analyze the underlying mechanisms of pain in the elderly. Focus on both peripheral and central processing, will be key to further understanding this complex relationship.

APPENDIX A

Table A1
Means and standard errors of age by condition.

Treatment	Young		Old	
	M	*SE*	*M*	*SE*
	232.388	49.248	296.20375	43.486
Chronic	221.397	50.156	226.1825	43.341

Table A2
Means and standard errors of the interaction between treatment and Bregma coordinate in Cg1.

Treatment	Bregma Coordinates	*M*	*SE*
Saline	+2.2mm	5371.962	222.093
	+1.7mm	5641.763	197.012
	+1.6mm	5568.248	177.558
CFA	+2.2mm	4906.257	203.263
	+1.7mm	5332.741	180.309
	+1.6mm	5535.949	162.504

APPENDIX B

Evaluation of the area Cg2 in the ACC was conducted through a mixed model ANOVA, where number of c-fos positive cells were compared across age, treatment, and condition. The within-subjects factor was Bregma coordinates (+2.2mm, +1.7mm, +1.6mm). Again, the assumption of equal covariance was not violated but the assumption of sphericity was. Therefore, Greenhouse-Geisser corrected *F*-statistics were reported. There was a significant effect of coordinate, $F(1.741, 106.195) = 22.009$, $p < .001$. Specifically, Bregma +1.7mm and Bregma +1.6mm had significantly more c-fos positive cells ($p < .001$) than Bregma +2.2mm. See Table B1 for means and standard errors.

Table B1
Means and standard error of protein expression in the Cg2 with the inclusion of coordinate +2.2mm .

Bregma	*M*	*SE*
+2.2mm	4042.656	136.444
+1.7mm	4402.297	129.974
+1.6mm	4516.119	112.435

The mixed ANOVA also revealed a significant interaction between Bregma coordinate and age, $F(1.672, 102.012) = 4.264$, $p = .021$. Specifically, it was revealed that, in the most anterior portion of the Cg2 (+2.2mm), older animals had significantly less c-fos expression (see Figure B1). Additionally, between-subjects results indicated a significant main effect of age, $F(1, 61) = 6.074$, $p = .017$. Where, older animals ($M = 4026.659$, $SE = 173.037$) had significantly fewer c-fos positive cells than younger animals ($M = 4614.055$, $SE = 163.891$).

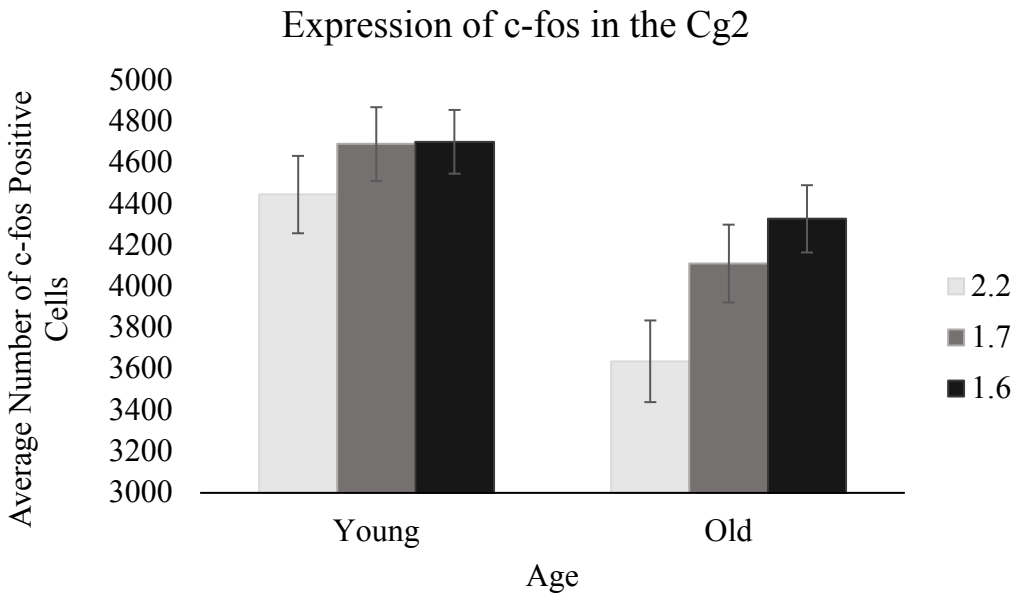


Figure B1. Showing the interaction between age and Bregma coordinates with the inclusion of coordinate +2.2mm.

APPENDIX C

A mixed model ANOVA was conducted to evaluate differences within subjects for anterior versus posterior c-fos positive cell averages; across factors of age, treatment, and condition, within the Cg2. A main effect of position was revealed, $F(1, 61) = 33.883$, $p < .001$, where the anterior region ($M = 4042.656$, $SE = 136.444$) had fewer c-fos positive cells than the posterior region ($M = 4459.208$, $SE = 117.044$). In addition, there was a significant interaction between age and area of the ACC (anterior/posterior), $F(1, 61) = 5.378$, $p = .024$, see Figure C1 and Table C1. Between subjects evaluations revealed a main effect of age, $F(1, 61) = 6.941$, $p = .011$. Where, average positive c-fos cells are fewer in the Cg1 for old animals ($M = 3929.574$, $SE = 177.116$) than for young animals ($M = 4572.290$, $SE = 167.754$).

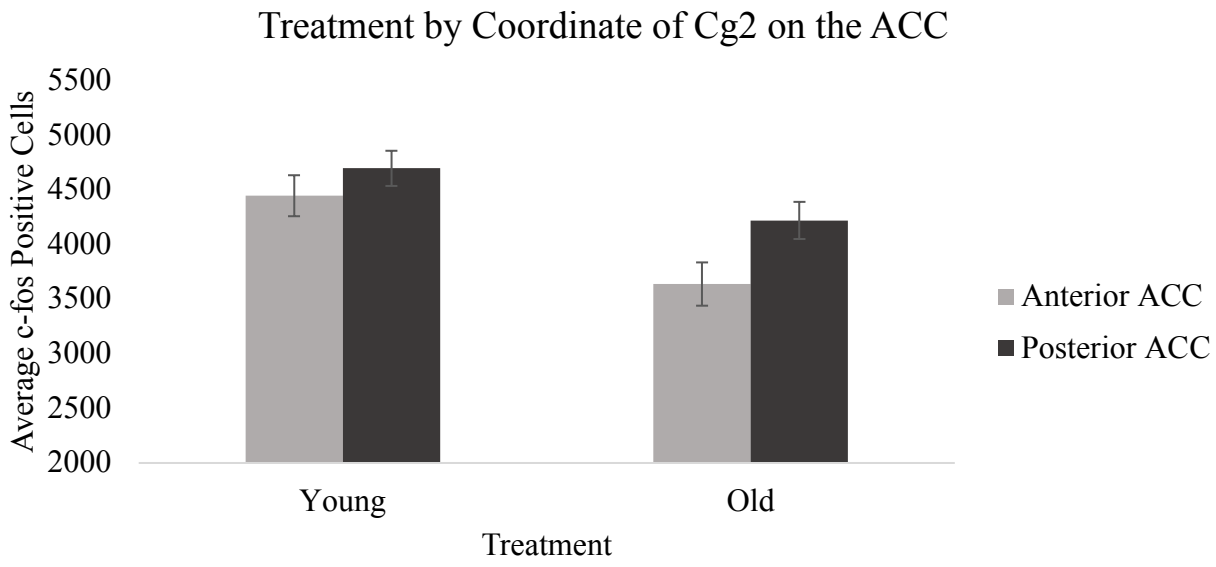


Figure C1. Showing the interaction between treatment and area of the ACC.

Table C1
Average c-fos positive cells by treatment and ACC coordinates in Cg2.

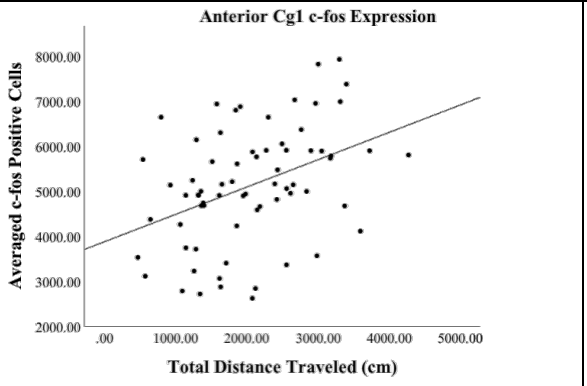
	ACC Position	M	SE
Young	Anterior	4446.994	187.654
	Posterior	4697.586	160.973
Old	Anterior	3638.317	198.126
	Posterior	4220.830	169.956

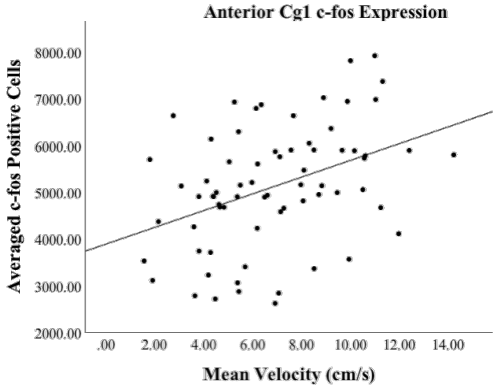
Note. Values reported are estimated marginal means and standard errors.

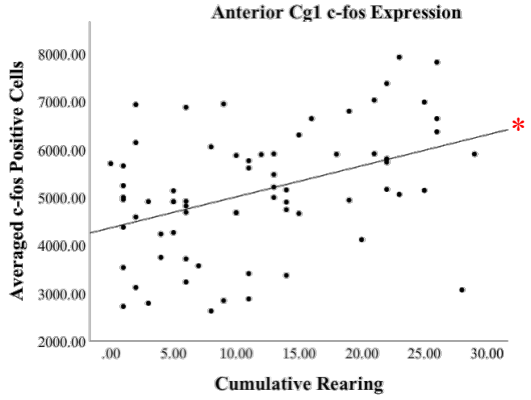
Appendix D

Regression results graphed for Cg1:

Mobility Measures Significantly Predict c-fos Expression

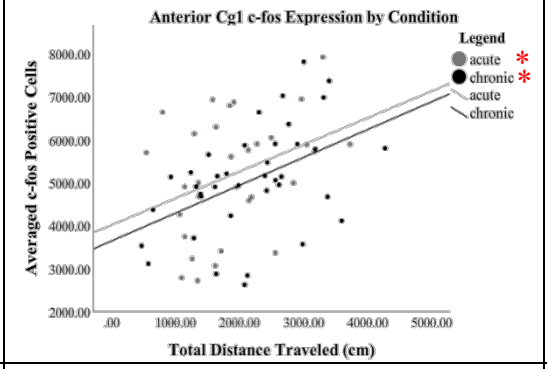


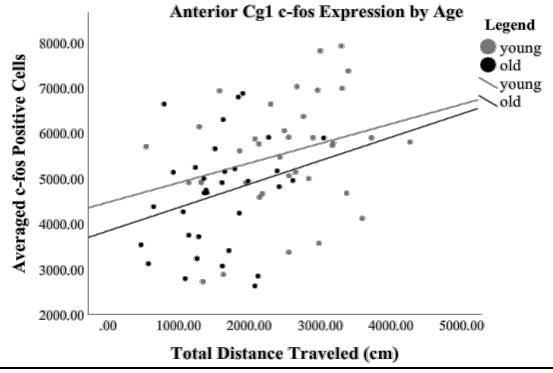




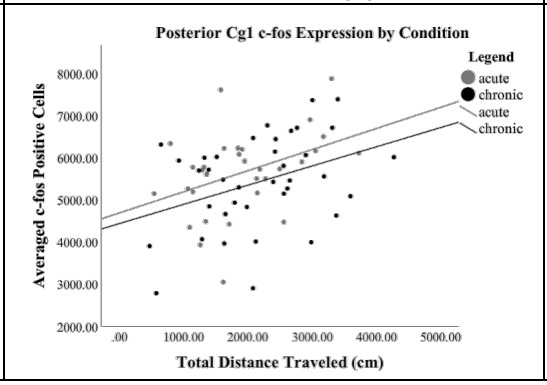
Total Distance Traveled and c-fos Expression in the Cg1

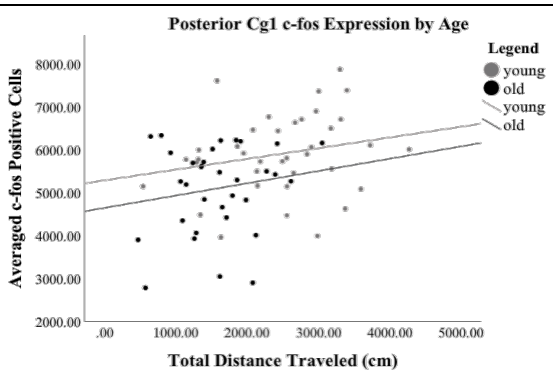
Anterior Cg1





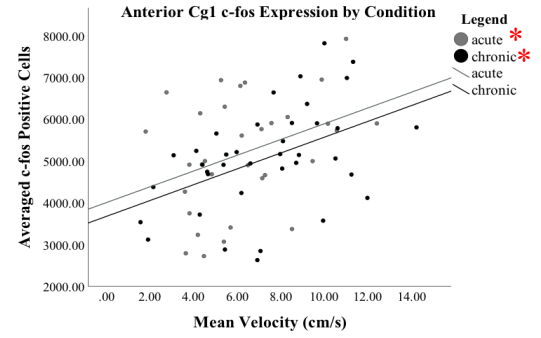
Posterior Cg1

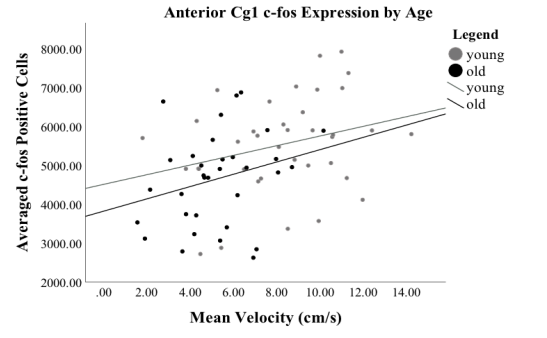




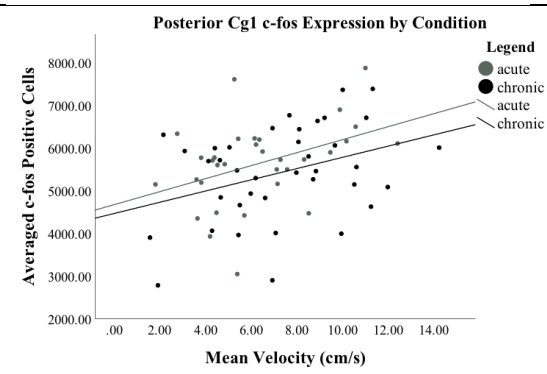
Mean Velocity and c-fos Expression in the Cg1

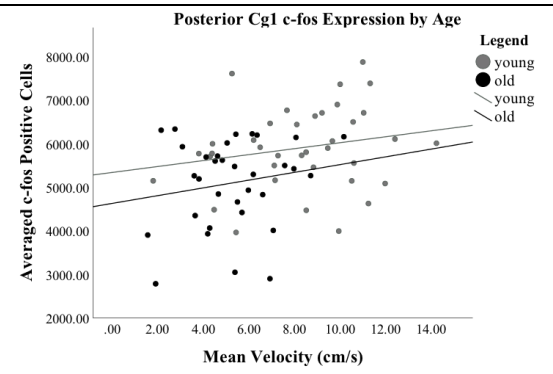
Anterior Cg1

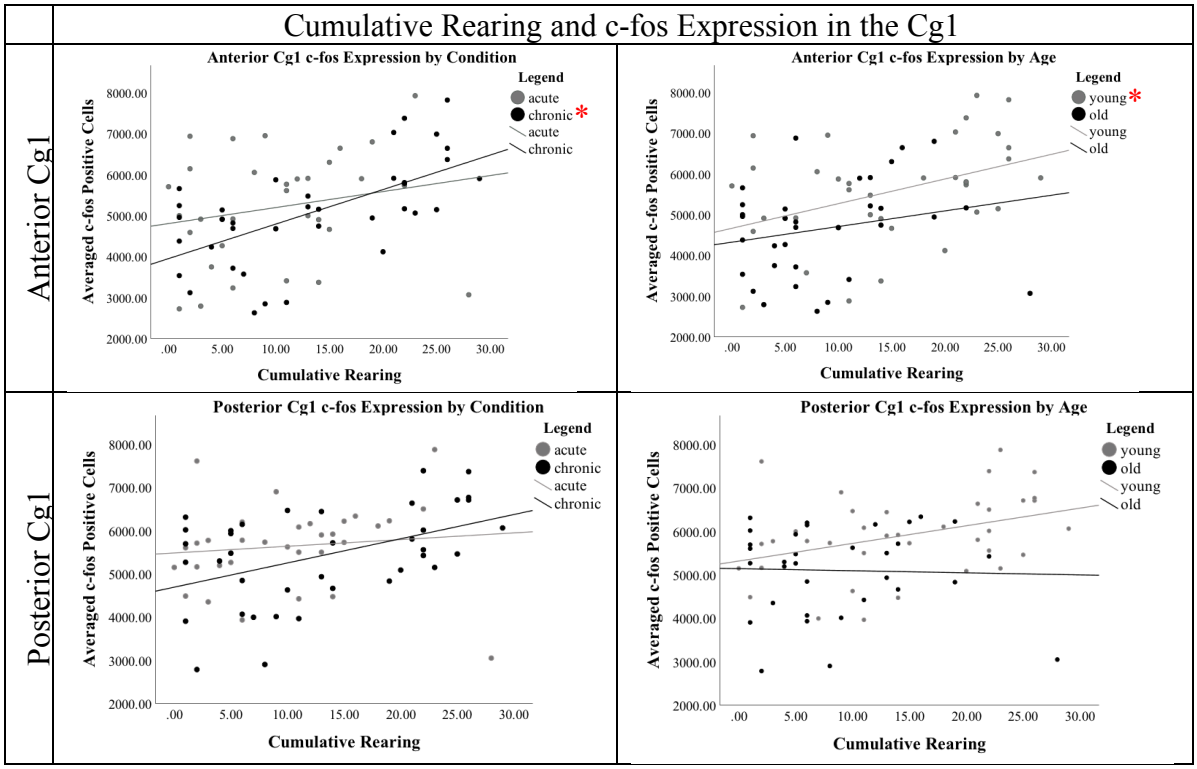


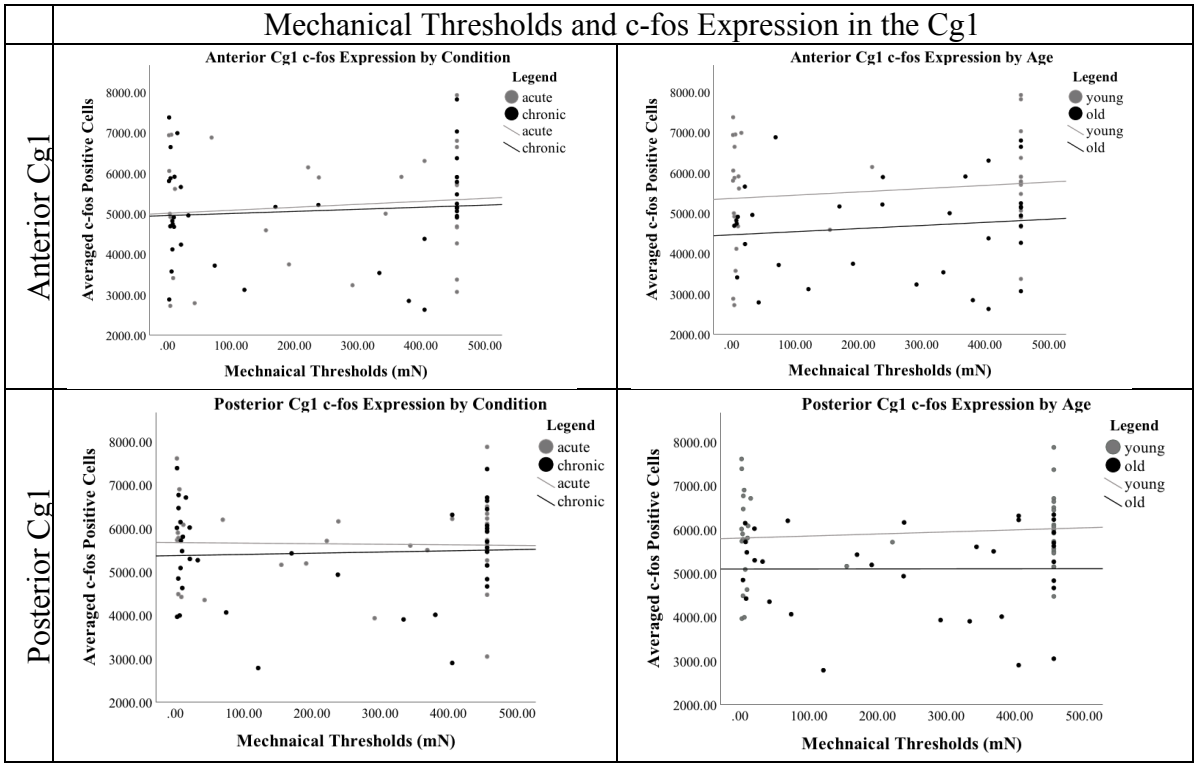


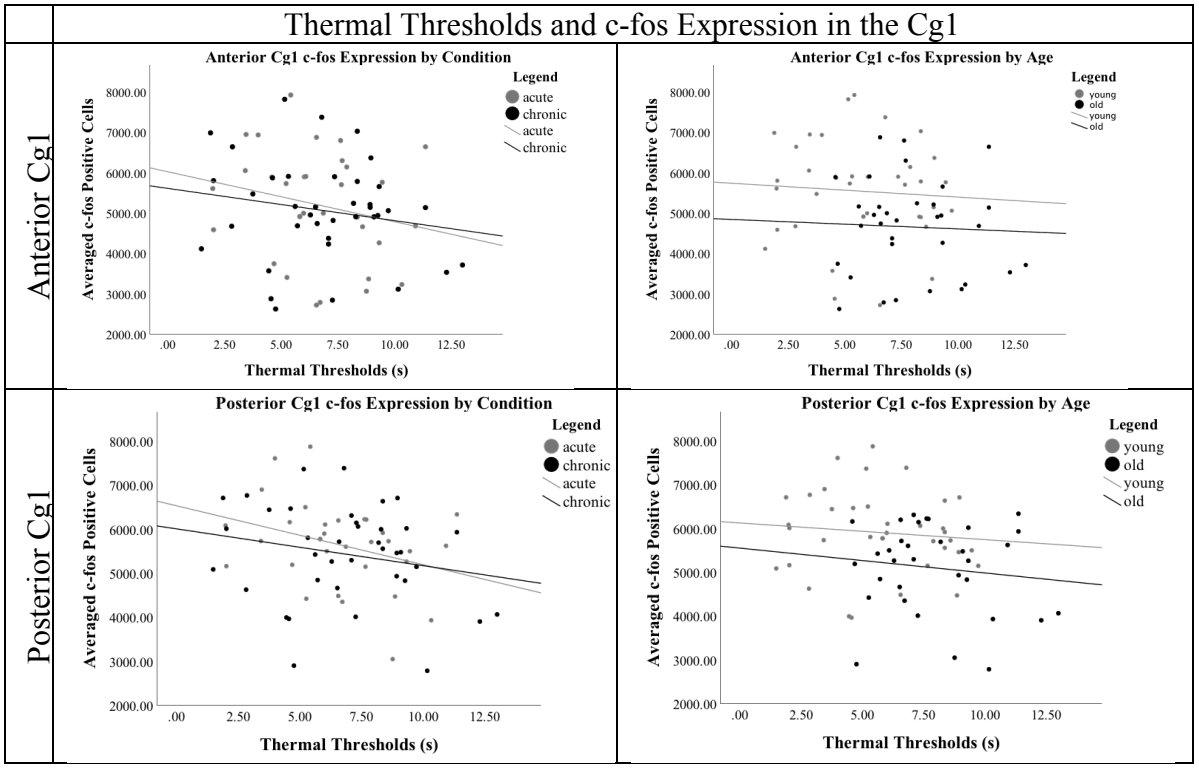
Posterior Cg1

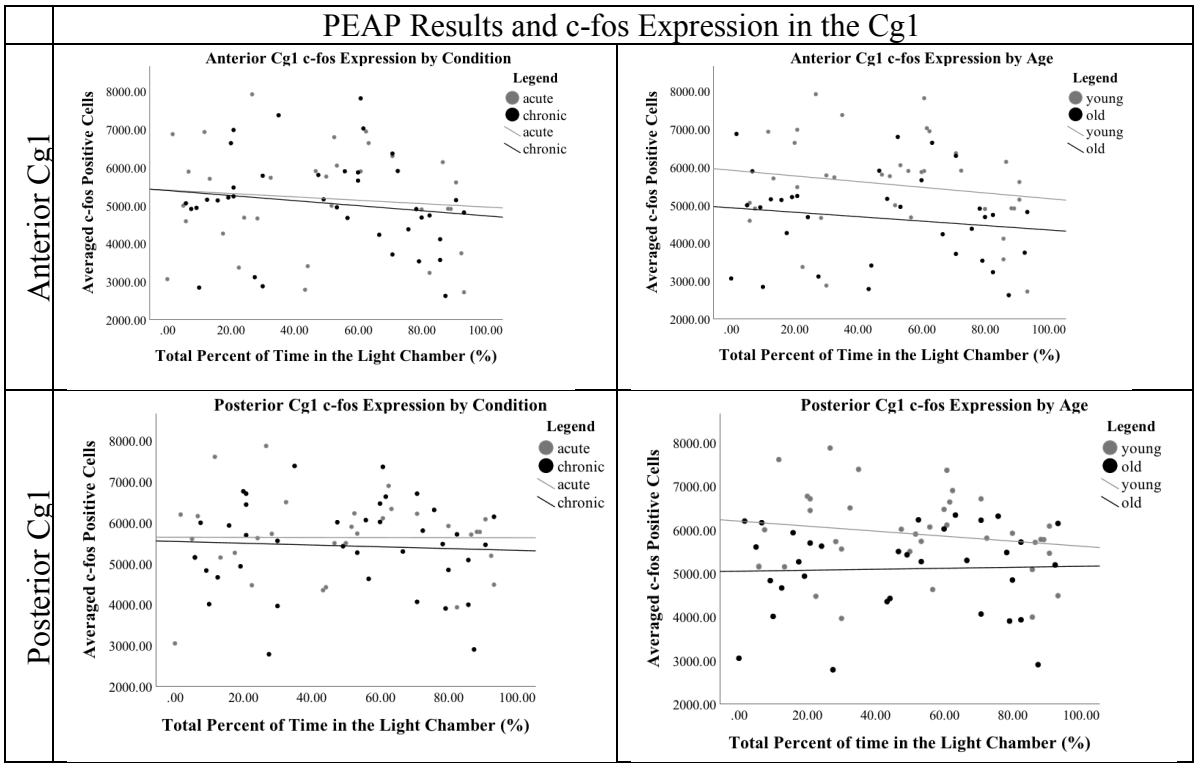





Cumulative Rearing and c-fos Expression in the Cg1
Anterior Cg1
Posterior Cg1
Anterior Cg1 c-fos Expression by Condition
Anterior Cg1 c-fos Expression by Age
Posterior Cg1 c-fos Expression by Condition
Posterior Cg1 c-fos Expression by Age
Averaged c-fos Positive Cells
Cumulative Rearing
Legend
acute
chronic
young
old
*

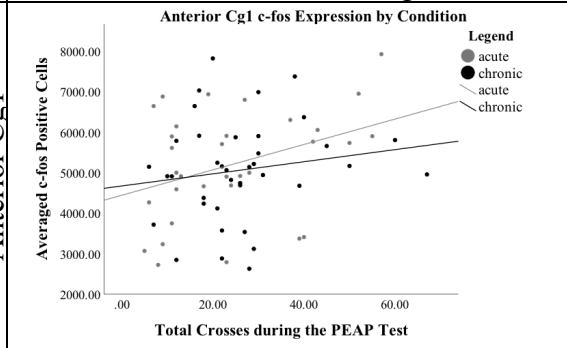

Mechanical Thresholds and c-fos Expression in the Cg1
Anterior Cg1
Posterior Cg1
Anterior Cg1 c-fos Expression by Condition
Anterior Cg1 c-fos Expression by Age
Posterior Cg1 c-fos Expression by Condition
Posterior Cg1 c-fos Expression by Age
Averaged c-fos Positive Cells
Mechnaical Thresholds (mN)
Legend
acute
chronic
young
old

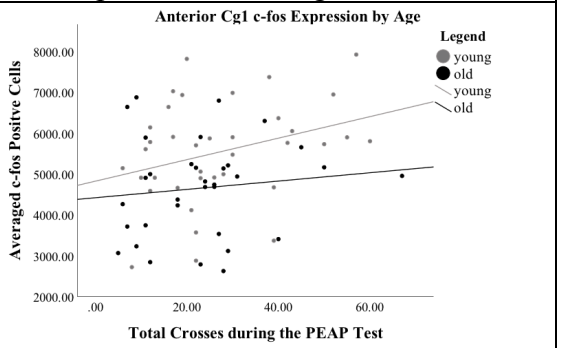

Thermal Thresholds and c-fos Expression in the Cg1
Anterior Cg1
Posterior Cg1
Anterior Cg1 c-fos Expression by Condition
Anterior Cg1 c-fos Expression by Age
Posterior Cg1 c-fos Expression by Condition
Posterior Cg1 c-fos Expression by Age
Averaged c-fos Positive Cells
Thermal Thresholds (s)
Legend
acute
chronic
young
old


PEAP Results and c-fos Expression in the Cg1
Anterior Cg1
Posterior Cg1
Anterior Cg1 c-fos Expression by Condition
Anterior Cg1 c-fos Expression by Age
Posterior Cg1 c-fos Expression by Condition
Posterior Cg1 c-fos Expression by Age
Averaged c-fos Positive Cells
Total Percent of Time in the Light Chamber (%)
Total Percent of time in the Light Chamber (%)
Legend
acute
chronic
young
old

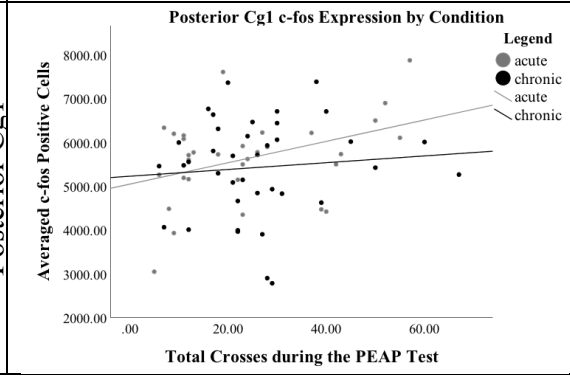
PEAP Crossing Results and c-fos Expression in the Cg1

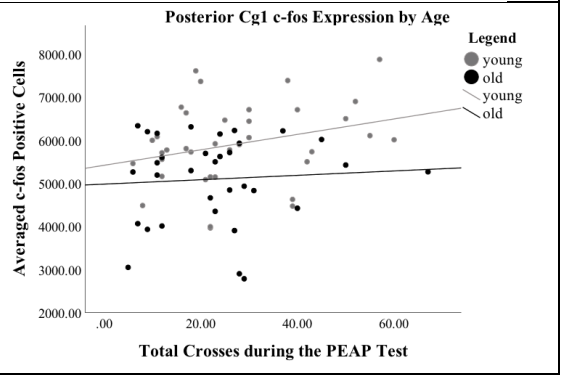
Anterior Cg1





Posterior Cg1

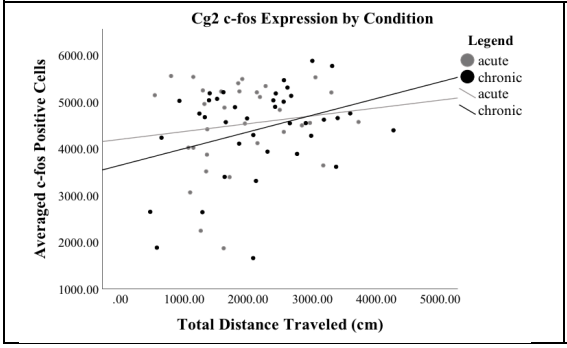


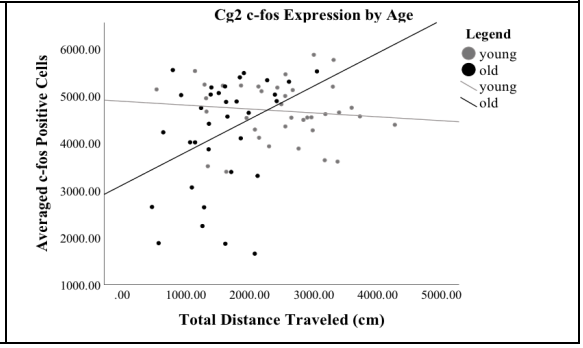


Appendix E

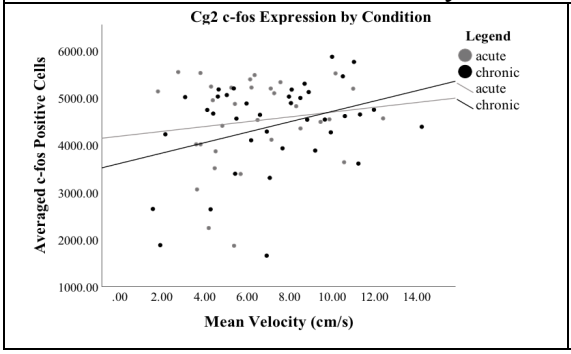
Regression results graphed for Cg2:

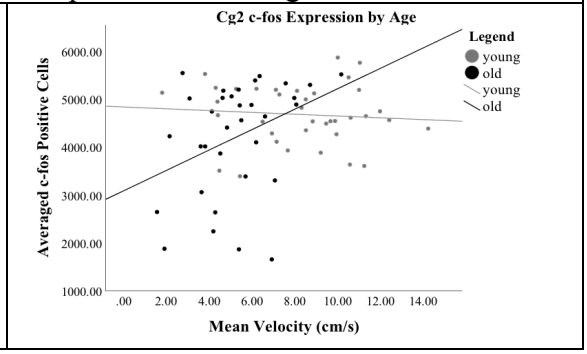
Total Distance Traveled and c-fos Expression in the Cg2



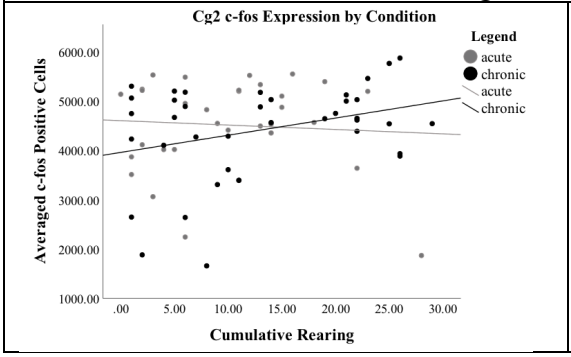


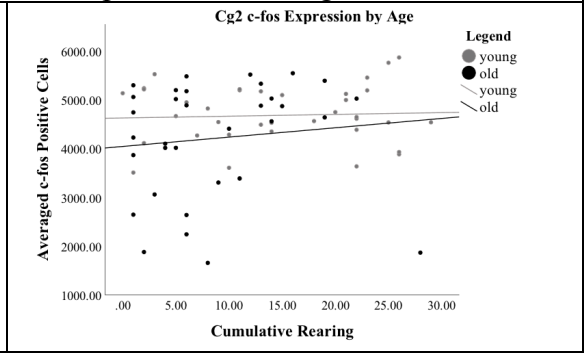
Mean Velocity and c-fos Expression in the Cg2



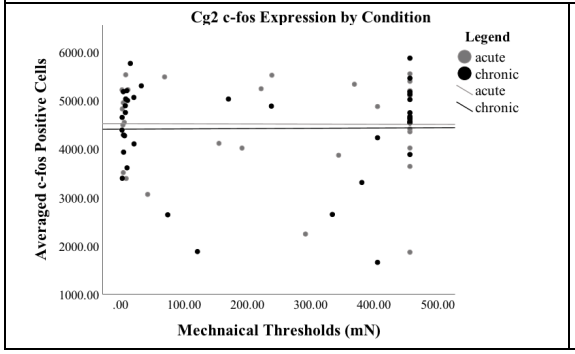


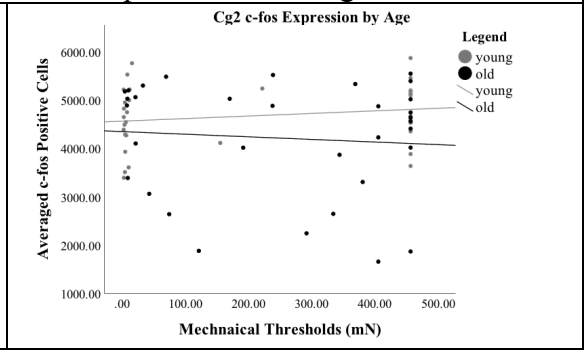
Cumulative Rearing and c-fos Expression in the Cg2



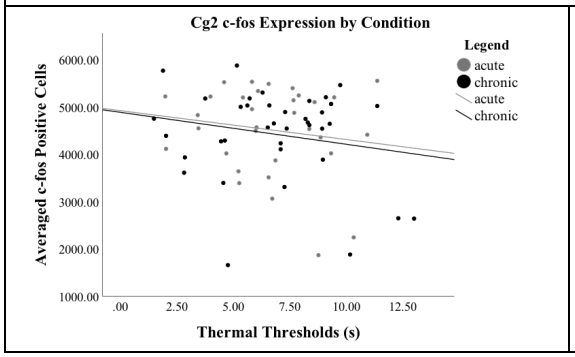


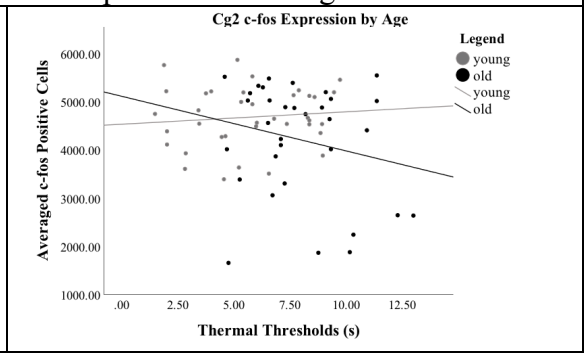
Mechanical Thresholds and c-fos Expression in the Cg2



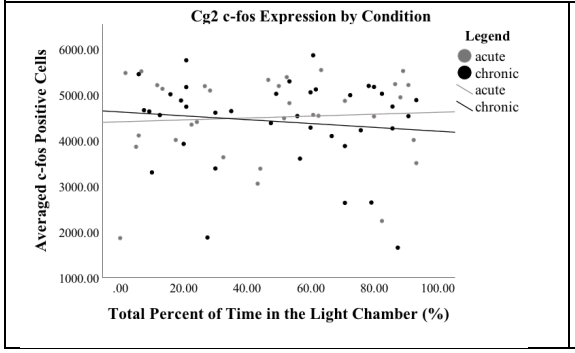


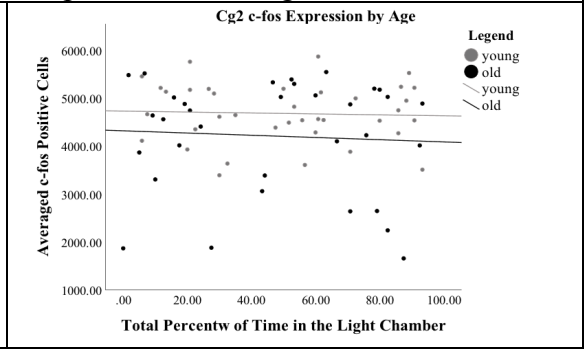
Thermal Thresholds and c-fos Expression in the Cg2



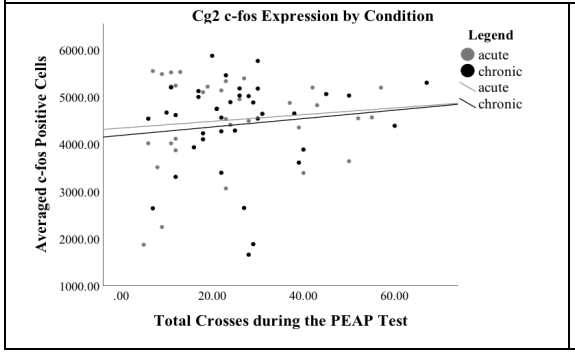


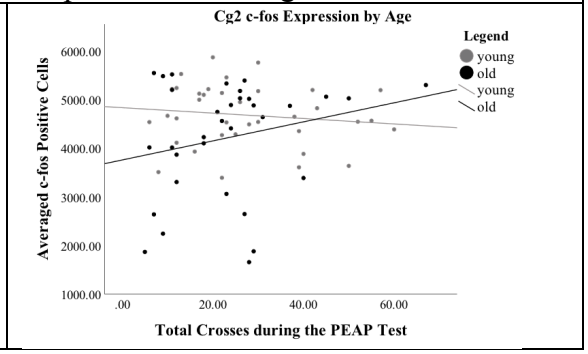
PEAP Results and c-fos Expression in the Cg2





PEAP Results and c-fos Expression in the Cg2





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