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A MULTIDIMENSIONAL PRECLINICAL INVESTIGATION OF FIBROMYALGIA MODELS: SUBCHRONIC SWIM AND BIOGENIC AMINE DEPLETION

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by

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DEDICATION

First and foremost, I give gratitude to the wonderful universe energy that allowed the following people into my life to make me a better person and scientist.

I dedicate this work to my father and brothers, my Sissy, and my grandmother, for their unconditional love, understanding, and always wanting to know the nuances of my work.

I dedicate this work to my friends, for always supporting and wishing nothing but my continued success.

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Abstract

A MULTIDIMENSIONAL PRECLINICAL INVESTIGATION OF FIBROMYALGIA MODELS: SUBCHRONIC SWIM AND BIOGENIC AMINE DEPLETION

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Pain is a multidimensional phenomenon, comprised of affective-motivational, cognitive-evaluative, and sensory-discriminative domains. In the presence of pain with no single determinable etiology, such as fibromyalgia, understanding the affective and cognitive dimensions of the disorder is crucial for adequate diagnosis and pain management. However, there is little empirical support for many of the primary animal models of fibromyalgia in replicating this disorder across all the dimensions of pain. Therefore, the current studies sought to evaluate two primary preclinical models of fibromyalgia – the reserpine and subchronic swim stress models – across all three pain dimensions and determine their predictive validity with an FDA-approved fibromyalgia pharmacologic, duloxetine (Cymbalta®). Further, these studies sought to combine these two preclinical models of fibromyalgia pain to determine if their compounded effect better replicates reported clinical manifestations and management profiles across the affective, cognitive, and sensory domains. Overall, the reserpine model was effective in producing mechanical hyperalgesia, and potentially time-dependent thermal hyperalgesia, but

ineffective in replicating anxiety- and depression-like behavior. The subchronic swim stress model was effective in producing mechanical hyperalgesia, and time-dependent thermal sensitivity, as well as trending effects of depression-like behavior, but no changes in anxiety-like behavior. The combination of these models produced mechanical sensitivity, and potentially time-dependent thermal sensitivity, alongside anxiety-like behaviors and trending depression-like behaviors. However, all models failed to produce any changes in cognitive function. The administration of duloxetine selectively alleviated effects within mechanical sensitivity and depression-like behaviors but may have offered adverse effects in measures of anxiety-like behavior and overall locomotion. Future research should aim to identify the contexts within which these individual models, and their combination, may best replicate the clinical multidimensionality of fibromyalgia.

Keywords: fibromyalgia pain, reserpine, subchronic swim, affect, cognition

1. Introduction

1.1 *Pain*

Pain is understood to be a multifaceted phenomenon, consisting of sensory-discriminative, affective-motivational, and cognitive-evaluative dimensions (Melzack & Casey, 1968). While each of these dimensions possesses the ability to be understood separately, pain operates across all of these facets on a neural level (Melzack, 1999). Research continuously supports the idea that each of these dimensions modulate and construct the pain experience as a whole, with each dimension exerting effects on the proceedings of the other. However, chronic pain in the absence of a clear pathophysiology lacks substantive attention and serves to benefit from evaluation of the pain state from a multidimensional perspective.

1.1.1 *Cognition and Pain*

Cognition is a complex phenomenon that is not easily defined. While most attempts to define cognition fail to incorporate all uses of the term, Byrne summarizes cognition as "all the activities and processes concerned with the acquisition, storage, retrieval and processing of information— regardless of whether these processes are explicit or conscious" (Bayne et al., 2019). Cognition is comprised of elements such as learning, executive function, perception, memory, decision-making, and attention— all of which are contributors to the subjective perception of pain (Khera & Rangasamy, 2021; Moriarty et al., 2011). Despite debate about the evolutionary pathway of "higher order" cognition, it is incontestable that human cognitive abilities are a foundational aspect of our adaptation and survival (Heyes, 2012). The relationship between pain and cognition is bidirectional, where cognition modulates the pain experience through factors such as appraisal, expectancy, and attention, while pain often results in deficits in

cognitive function (Bushnell et al., 2013; Kera & Rangasamy, 2021; Moriarty et al., 2011; Wiech, 2016).

Cognition and Pain in Humans

Cognitive attention tasks possess the ability to modulate the pain experience. Altering attention through cognitively demanding tasks or environmental distractors can reduce pain sensitivity (Khera & Rangasamy, 2021), whereas focusing on pain increases ratings of perceived pain intensity (Bushnell et al., 2013). It is postulated that neural networks comprising the attention matrix overlap with key pain processing pathways in humans, thus explaining the modulating effect observed during higher load or more engaging cognition (Wiech, 2016). However, there is also evidence for chronic pain states producing deficits in attentional switching and divided attention tasks (Khera & Rangasamy, 2021; Moriarty et al., 2011). These adverse cognitive effects span further into other domains of cognitive functioning, with chronic pain patients reporting issues with working memory, long-term memory, recall, and recognition (Moriarty et al., 2011). Decreases in volume of brain structures associated with higher order executive functioning, such as the dorsolateral prefrontal cortex (DLPFC), the insula, and the anterior cingulate cortex (ACC), are correlated with chronic pain states (Bushnell et al., 2013; Moriarty et al., 2011). These findings are substantiated by reported deficits in perceptual learning, reaction time, decision making, mental flexibility, concentration, and information processing speed (Moriarty et al., 2011). However, the observed effects on cognition are dependent upon the type of pain, its intensity and chronicity, task difficulty, age, and several additional factors (Moriarty et al., 2011; Phelps et al., 2021).

Cognition and Pain in Animals

The cognitive abilities of rats and mice have been investigated considerably more in recent years, with studies providing evidence for rodent episodic memory, prospective memory, social memories, cognitive bias, working memory, selective attention, metacognition, and extensive learning abilities (Mogil, 2019; Broschard et al., 2019; Poucet & Benhamou, 1997). Specifically, regarding rat cognition, there is continued evidence for complex spatial learning and memory systems within the hippocampal formation that serve to guide animal exploration and navigation (Geva-Sagiv et al., 2015; Poucet & Benhamou, 1997). These neural spatial representations are proposed to be created through the transformation of distal sensorial input, such as vision, and proximal sensorial input, such as somatosensation (Geva-Sagiv et al., 2015; Poucet & Benhamou, 1997). Within the context of high somatosensorial input, such as a pain state, cognitive deficits have been identified within animal research. Experimentally induced inflammatory and neuropathic pain has been shown to produce deficits in attention, decision-making, learning and memory, and cognitive flexibility (Hu et al., 2010; Boyette-Davis et al., 2008; Pais-Viera et al., 2009; Phelps et al., 2021; Moriarty et al., 2011; Low, 2013). Impairments in spatial learning and memory, often mediated by attention depending on the task employed, have been documented in preclinical neuropathic, inflammatory, and fibromyalgia-like pain (Moriarty et al., 2011; Kaur et al., 2019; Singh et al., 2020; Kaur et al., 2020; Singh et al., 2021; Murasawa et al., 2021, Argenbright et al., *submitted*).

1.1.2 *Affect and Pain*

The experience of emotion may be evoked by a physiological state, environmental stimuli, or cognitive processes, and is considered to be functionally adaptive (Gilam et al., 2020; Hanssen et al., 2017). Human survival is regarded as dependent on our ability to discriminate

between advantageous or dangerous stimuli and thus we depend heavily on motivational systems that distinguish between these factors. The approach/appetitive motivational system attends to stimuli that enhance survival, while the aversive/defensive system attends to that which is injurious (Rhudy, 2001). These affective-motivational systems are closely tied to emotion, such that positive valence emotions are attributed to the appetitive system while emotions of negative valence are attributed to the defensive system (Rhudy, 2001). Noxious stimuli, as a result, activate the defense system and result in motivational behaviors to avoid the experience, or synonymously, actuate the affective pain dimension (Rhudy, 2001; Salcido et al., 2018). When pain becomes chronic and creates a negative affective state beyond the realms of what is adaptational, it becomes a need, or a disruption in homeostasis, that must be attended to (Salcido et al., 2018; Hanssen et al., 2017).

Therefore, the experience of affect is closely dependent on cognitive processing. Neural substrates involved in the processing and production of emotions overlap with structures necessary for information processing and cognition (Forgas, 2008; Salcido et al., 2018). Psychological theories of emotion genesis have long been rooted in cognitive theories of appraisal, and psychologists have further expounded upon the role that affect has on memory acquisition and recall, priming, decision-making, attribution, and heuristics (Forgas, 2008). Similarly, to the relationship between cognition and pain, the outcomes between affect and pain are also bidirectional. Just as the experience of pain cannot be reduced to a noxious stimulus (Melzack & Casey, 1968; Gilam et al., 2020), emotion cannot conceptually be mitigated to a single emotional state (Gilam et al., 2020). The neural substrates responsible for processing the complex contributions to the experience of affect and pain sensation overlap, particularly in the anterior cingulate cortex (ACC) and insula (Gilam et al., 2020; Rainville, 2002). Further, this

activation of the ACC during pain may reflect regulation of behavioral and emotional responses to the pain, as well as cognitive strategies for pain coping (Rainville, 2002). This multidimensional overlap provides the framework for the emotional modulation of pain, or simultaneously for pain in producing deficits in affective states/processing.

Affect and Pain in Humans

In humans, there is evidence for quantitative pain assessment being predictive of negative affect (Georgopoulos et al., 2019). Negative emotional states increase the perceived unpleasantness of pain and alter ACC activation in the brain, without altering the intensity of the pain itself (Bushnell et al., 2013). These brain regions associated with pain processing are also activated in individuals observing another person experience pain, which can be altered by creating a positive or negative empathy state (Bushnell et al., 2013). Evidence has also been provided for negative affect reducing the experience of opioid analgesia, coupled with reductions in pain thresholds and endogenous pain inhibition (Edwards et al., 2016). Baseline scores of overall negative affect or pain-catastrophizing are predictive of temporal summation responses; higher scores of negative affect are associated with enhanced temporal summation and thus contribute to an enhanced pain state (Edwards et al., 2016). Generally, it is well documented that negative emotional mood states can instigate or intensify a more unpleasant pain experience (Hanssen et al., 2017).

Conversely, increased positive affect is associated with lower clinical pain reports across both acute and chronic manifestations, such that positive affect may even serve to buffer against fluctuations in day-to-day negative pain-related affect (Finan & Garland, 2015). Experimentally induced positive affect has also served as an efficacious intervention among various groups of persistent pain sufferers, with improvements being observed in measures of pain intensity, pain-

induced cognitive interference, depressive feelings, quality of life, and maladaptive coping strategies. These documented protective effects of positive affect offer insight into potential clinical benefits, as well as provide further evidence for the strong relationship between the affect, cognition, and sensation associated with a pain state (Hanssen et al., 2017; Finan & Garland, 2015).

Affect and Pain in Animals

Behavioral changes in animals are used to document the aversive nature of a pain state. Affective states in animals are elicited by rewards and punishers, or their predictors, where rewards create positive affect and punishers create negative affect (Mendl & Paul, 2020). Approach and avoidance behaviors driven by motivational systems, as described by Rhudy et al. (2001), are often employed to quantify these affective states. Formalin- or acetic acid-induced pain is associated with conditioned place-avoidance behaviors in rats, with the avoidance of the pain-associated environment observed for up to one month (Zhang et al., 2011). Neuropathic, inflammatory, and fibromyalgia-like pain is also associated with place/escape avoidance, wherein animals avoid a preferred location with perceived motivation to evade noxious stimulation (Zhang et al., 2011; LaBuda & Fuchs, 2000; Argenbright et al., 2023). Measures of anxiety and depression-like behaviors, through a multitude of paradigms, are often increased among animals experiencing fibromyalgia-, inflammatory-, and neuropathic-like pain, although these effects are dependent on the context of the experimental model and the timeline within affect is investigated (Kremer et al., 2020; Argenbright et al., *submitted*).

1.2 *Fibromyalgia*

Fibromyalgia (FM) is a female-prevalent chronic widespread pain disorder characterized by a spectrum of idiopathic pain symptoms including fatigue and disturbances in cognition, emotion,

and sleep (Henao-Perez et al., 2022). While fibromyalgia etiopathogenesis is unclear, the development of the disorder is primarily attributed to central sensitivity and abnormal processing of pain perception. Despite there being extensive work investigating the central and peripheral contributors to the development of FM, no single etiological determinate has been identified as the cause of this disorder (see Siracusa et al., 2021 for extensive review). Due to this lack of objective biomarkers, there has been a question about the "measurability" of FM (Girogi et al., 2022), despite the consensus regarding FM diagnostic criteria (Girogi et al., 2022; Wolfe et al., 2016). The American College of Rheumatology (ACR) criteria for the diagnosis of FM includes:

1) generalized pain in at least 4 of 5 regions;

2) symptom presence for at least 3-months;

3) a widespread pain index (WPI) score ≥ 7 and a symptom severity (SS) score ≥ 5, or a WPI score of 4 - 6 and a SS score ≥ 9;

4) diagnosis that does not exclude the presence of other clinical disorders (Wolfe et al., 2016).

Fibromyalgia Multidimensionality

The inclusion of the diagnostic criteria assessing WPI and SS score is different from previous iterations of FM diagnostic criteria (Wolfe et al., 1990), and underscores the increasingly recognized affective nature of the FM pain experience (Wolfe et al., 2010). Generally, anxiety, depression, sleep disturbances, and cognitive deficits are often characteristic of FM (Goldenberg, 2008; Ambrose, Gracely, & Glass, 2012; Aguglia et al., 2011; Henao-Perez et al., 2022; Berger et al., 2007; Walitt et al., 2015). Measures of SS score are effective at identifying the presence of these functional abnormalities and have been correlated with higher

anxiety and depression diagnoses, higher pain scores, and higher rates of sleep disturbances (Singh & Kaul, 2018; Bennett et al., 2007). The cognitive dysfunction associated with FM diagnosis, or "fibro fog", has also been well documented. Experimental assessments have confirmed patient reported deficits in memory, attention, and executive function (Kravitz & Katz, 2015; Sarzi-Puttini et al., 2020; Bennett et al., 2007; Mease et al., 2009). Despite the extensive work contributing to the characterization of the affective and cognitive dimensions of FM, little progress has been made in its overall management profile.

Treatment

Primary FM treatment methodologies employ a multidisciplinary approach (Giusti, Castelnuovo & Molinari, 2017; Sarzi-Puttini et al., 2020), and typically include a variety of specialists aimed at disorder management. The ACR (2021) strongly urges against the use of opioids, acetaminophen, and NSAIDs for FM, but encourages treatment to employ one of the three FDA-approved drugs for the treatment of FM – duloxetine (Cymbalta), milnacipran (Savella), and pregabalin (Lyrica) – in addition to physical exercise, cognitive-behavioral therapy, or other complementary alternative medicines such as acupuncture, chiropractic, and massage therapy (Forte et al., 2015; Chan, 2023). The European Alliance of Associations for Rheumatology (EULAR) offers similar recommendations for FM management, with the strongest suggestions geared towards aerobic and strength exercise regimens, cognitive-behavioral therapy (CBT), acupuncture, hydrotherapy, meditation and mindfulness, antidepressants, and anticonvulsants (MacFarlane et al., 2017). Other common FM treatment approaches involve muscle relaxants, antipsychotics, low-dose analgesics, hypnotics, and cannabis (Sarzi-Puttini et al., 2020). However, many of these treatment methodologies are dependent on the individual pain profile, selectively alleviate symptoms, and are associated with

many adverse effects (Sarzi-Puttini et al., 2020). For example, a meta-analysis revealed that duloxetine was most effective for improving pain and depression, while amitriptyline, a tricyclic "off-label" FM treatment, was most effective for improving sleep, fatigue, and patient quality of life (Farag et al., 2022). Dependent on the classification of the management approach, many FM treatments have yet to be investigated for their therapeutic benefits to pain and quality of life beyond that of 12 months (Mascarenhas et al., 2021). Further, many of these treatments are not financially and readily accessible to patients (Skaer, 2014). Review of the literature for treatments that are more at a patient's disposal, such as education and exercise therapies, has identified that these approaches only offer short term alleviation of pain, fatigue, depression, and anxiety (Hernando-Garijo et al., 2022; Mascarenhas et al., 2021). Therefore, there is a desperate need for investment towards a long-term, well-rounded FM treatment methodology.

1.2.1 *Preclinical Fibromyalgia*

It is postulated that the lack of robust treatment methodologies may be correlated with an inadequate understanding of the etiology of FM. Much of our understanding of the neurobiological processes underlying pain states, as well as their management, stems from preclinical evidence. Rodents particularly show a high degree of similarity to humans, and allow investigations of the genetic, molecular, cellular, sensory, and psychological complexities of pain (Soliman et al., 2022). Additionally, it is vital that animal models of pain states resemble the key facets of the human condition (Soliman et al., 2022). However, this has been a great challenge to FM researchers, given that there is no single etiological understanding of the disorder (Hung & Chen, 2015). A favorable preclinical model of FM possesses an ability to produce the commonly reported symptoms and comorbidities of FM through a prominent pathological mechanism, while simultaneously replicating outcomes of primary disorder management profiles (DeSantana

et al., 2013; Brum et al., 2022). While there are a handful of FM-like rodent models that selectively meet these criteria, none have yet to be investigated enough to provide strong empirical evidence for FM replication across the sensory, affective, and cognitive dimensions of pain — much less through a prominent pathological mechanism nor management profile.

Current Fibromyalgia Models

Some of the commonly employed preclinical models of FM-like pain include the reserpine model, the acidic saline model, the subchronic swim stress model, the cold stress model, the sound stress model, and the fatigue-enhanced muscle pain model (Hung & Chen, 2015; DeSantana et al., 2013; Argenbright et al., *submitted*). Recently, Argenbright et al. (2023, *submitted*) conducted a semi-systematic literature review to identify how well each of these models replicated the FM experience across the affective and cognitive dimensions of pain. While these models show evidence for producing behaviors that imply the sensory experience of pain, the literature failed to provide a clear consensus for how well these models replicate the affective and cognitive experiences (Argenbright et al., *submitted*). Review of the most common model, the reserpine model, yielded evidence for the ability of reserpine administration to produce reliable depression-like behaviors and cognitive deficits, but not anxiety-like behaviors. The acidic saline model was effective in producing negative affect, dependent on the paradigm employed, but results were mixed regarding cognitive deficits. Previous findings from our lab implied that the acidic saline model was effective for inducing place escape/avoidance behaviors, but not anxiety-like thigmotaxis (Argenbright et al., 2023). The subchronic swim stress model yielded mixed evidence for producing depression- and anxiety-like behaviors, with promise for producing cognitive deficits. The literature regarding the use of cold stress, sound stress, and

fatigue-enhanced muscle pain models were too sparse to draw clear conclusions about their ability to produce changes in affective and cognitive functioning.

Although results using many of these models are mixed, there is a high potential for usage of these models, or their variations, to produce the multidimensional phenotypic presentations associated with the clinical FM pain experience. For example, the reserpine model of FM-like pain induces chronic depletion of dopamine, norepinephrine, and serotonin, which coincides with biogenic amine-related disruptions often presented by FM patients (Nagakura et al., 2009). While assessments of the presence of depression-like behaviors are well reported within the use of this model, results are mixed regarding the ability of the model to produce anxiety-like behaviors among animals (Argenbright et al., *submitted*). Although there have been few studies done investigating reserpine model-associated cognitive deficits, significant deficits in fear conditioning (Souza et al., 2013) and spatial learning (Kaur et al., 2019; Singh et al., 2020; Kaur et al., 2020; Singh et al., 2021) have been reported. Within the subchronic swim stress model, decreases in central serotonergic efflux as a result of prolonged stress exposure have been reported (Quintero et al., 2000; Suarez-Roca et al., 2006a). However, previous studies have shown that combining this model with formalin insult results in higher levels of c-Fos-immunoreactive nuclei in the ipsilateral and contralateral lumbar dorsal horn (Quintero et al. 2003; Suarez-Roca et al., 2006b; Quintero et al., 2011), alongside decreased resting concentrations of GABA, increased resting concentrations of glutamate, and thus overactivation of NMDA receptors (Quintero et al., 2011) — mechanisms that are implicative of central sensitization. However, there are mixed reports on the ability of the subchronic swim stress model to replicate depression- and anxiety-like behaviors in animals. Cognitive deficits, as assessed in passive avoidance, have been reported in association with chronic swim stress

(Nazeri et al., 2014; Nazeri et al., 2016). While these two models have yet to be strongly empirically validated individually, a combination of the biogenic amine depletion and phenotypic presentations associated with the reserpine model may be further exacerbated by the reported mechanisms of central sensitization associated with the subchronic swim stress model. The proposed combination of these models is hypothesized to result in animals developing a more reliable multidimensional pain presentation through sensory, affective, and cognitive mechanisms.

Multidimensional Preclinical Investigations

Overall, the literature is severely lacking in rigorous investigations that assess the ability of current animal models to replicate the FM experience on a multidimensional level. Identifying a reliable animal model for FM-like pain reinstates the advantages held by preclinical research, wherein the opportunity is granted to assess the development and maintenance of pain, as well as systematically investigate causal relationships in a manner that is not feasible in humans (Soliman et al., 2022). The lack of a well-rounded understanding of FM animal models may contribute to the lack of development towards robust FM management, given that animal studies are a crucial contributor in identifying the risks and benefits of novel treatment methodologies. Therefore, it is vital that the evidence assessing the replicability and translatability of preclinical FM models be expanded.

1.3 *Rationale and Purpose*

The purpose of this study was to comparatively evaluate two established preclinical models of FM-like pain – the reserpine model and the subchronic swim stress model – in their ability to replicate the clinical manifestations reported by FM patients across the sensory, affective, and cognitive dimensions of pain. Based on results from previous studies, we sought to

determine if combining these models would compound the effects observed in each model independently to better replicate the multifaceted FM pain experience in animals. To determine this, animals were randomized to one of each of the following conditions: 1) reserpine or saline control injection and 2) subchronic swim or sham swim. To assess the predictive validity of the models, as well as their compounded outcome, animals were treated with duloxetine, an FDA-approved serotonin-noradrenaline reuptake inhibitor (SNRI), or a vehicle control. To determine the multidimensional characteristics of these models and their combination, animals were compared between groups within paradigms investigating the sensory, affective, and cognitive dimensions of pain. To assess evoked pain behaviors, animals underwent repeated tests of mechanical paw withdrawal thresholds (MPWTs) and thermal withdrawal latencies (TWLs; Hargreaves). To characterize potentially negative affect, the Elevated Plus Maze (EPM) was used to assess anxiety-like behaviors, while the splash test was used to assess depression-like behavior. To investigate cognitive deficits, animals underwent an olfactory discrimination test to assess learning and memory, as well as an object-location memory task to quantify spatial learning and memory, attention, locomotion, and thigmotaxic behavior.

1.3.1 *Specific Aims*

The specific aims of this study are as follows:

(1) To assess the multidimensional translatability of the reserpine model of FM-like pain.

Hypotheses: (1) Reserpine administration will produce mechanical and thermal sensitivity, (2) alongside depression-like behavior in the splash test and anxiety-like behaviors in the EPM. (3) Reserpine will produce cognitive deficits in the olfactory discrimination task and the object-learning memory task. (4) Reserpine

will increase thigmotaxic behavior but will not produce differences in overall locomotor activity.

(2) To assess the multidimensional translatability of the subchronic swim stress model of FM-like pain.

Hypotheses: (1) Subchronic swim will produce mechanical and thermal sensitivity. Subchronic swim animals will show increases in immobility time during hyperalgesia induction compared to sham swim animals. (2) Subchronic swim will produce depression-like behavior in the splash test and anxiety-like behaviors in the EPM. (3) Subchronic swim will produce cognitive deficits in the olfactory discrimination task and the object-learning memory task. (4) Subchronic swim will produce no differences in thigmotaxic behavior or overall locomotor activity.

(3) To evaluate the analgesic effect of duloxetine on affective, cognitive, and sensory dimensions of FM-like pain.

Hypotheses: (1) Duloxetine will attenuate mechanical and thermal sensitivity, depression- and anxiety-like behaviors, and cognitive deficits within both the reserpine and subchronic swims stress models.

(4) To combine the reserpine and subchronic swim stress models, characterize the multidimensional translatability, and assess predictive validity with duloxetine.

Hypotheses: (1) Reserpinized subchronic swim stress animals will produce increases in mechanical and thermal sensitivity, above that of reserpine or

subchronic swim alone. (2) Reserpinized subchronic swim will produce the greatest increases in depression-like behavior in the splash test, anxiety-like behavior in the EPM, and cognitive deficits in the olfactory discrimination and object-learning memory tasks. (3) Reserpinized subchronic swim will produce increases in thigmotaxic behavior but decreases in overall locomotor activity. (4) Duloxetine administration will attenuate all of these effects.

2. Methods

2.1 *Subjects*

For the scope of this study, 96 female Sprague Dawley rats were used. Animals were purchased from Charles River and ordered by weight (200-225g). Animals were singly housed and maintained on a 12-hour light/dark cycle, with access to food and water *ad libitum.* All procedures for this study were approved by the University of Texas at Arlington Institutional Animal Care and Use Committee (IACUC).

2.2 *Pain Condition*

Reserpine

Animals randomized to the reserpine pain condition (RES) were administered one injection of reserpine (1 mg/kg, s.c.) daily, from days 1-3, for a total of three consecutive injections (Nagakura et al., 2009). All animals not randomized to the reserpine condition (SALINE) received a subcutaneous saline control injection, adhering to the same procedure as reserpine administration.

Subchronic Swim

Animals randomized to the subchronic swim pain condition (SWIM) underwent three consecutive days of a forced swim test procedure (days 1-3). Animals were placed in a plastic cylinder (diameter 25.5 cm, height 50 cm), containing 25 cm of water. Animals were subjected to 10 minutes of swim stress on day 1, and 20 minutes of swim stress on days 2 and 3. Immobility time during the swim procedure was recorded across each of the swimming sessions, and quantified for time spent immobile, mobile, and highly mobile, using Ethovision tracking software. All animals not randomized to the swim stress condition (SHAM) underwent a daily sham swim procedure for 3 days. The sham swim procedure employed exposure time to the same apparatus as the swim stress procedure, but the cylinder contained no water.

Reserpinized Subchronic Swim

Animals randomized to the reserpine and subchronic swim stress pain condition (RES/SWIM) underwent both reserpine administration and the subchronic swim stress procedures as previously described. On days 1-3, animals were administered reserpine daily (1 mg/kg, s.c.) and allowed to rest for 10 minutes prior to undergoing the swimming procedure (10 minutes on day 1, 20 minutes on days 2-3).

2.3 *Drugs*

Reserpine (Fisher Science) was diluted to a final concentration of 1 mg/mL, and injected subcutaneously in a volume of .1 ml per 100g of animal weight. Reserpine administration occurred on days 1-3. Duloxetine hydrochloride (Millipore Sigma) was administered intraperitoneally (i.p.) at a dosage of 30 mg/kg on day 9.

2.4 *Procedure*

Experiment 1: A total of 59 animals were utilized within Experiment 1. Upon arrival, animals were allowed to habituate for at least 7 days. Beginning on day 1, animals underwent baseline MPWTs and Hargreaves' test of thermal withdrawal latency. Animals were randomized to a pain condition of reserpine administration (RES), subchronic swim (SWIM), a combination of both reserpine administration and subchronic swim (RES-SWIM), or their respective controls (Table 1). Induction into the respective pain model occurred on days 1-3. Following induction, animals were allowed to habituate for 3 days. On day 7, animals were habituated to the open field chamber for the object-location memory task for 10 minutes. During this time, thigmotaxic behavior and overall locomotion were recorded. On day 8, animals underwent pre-treatment MPWT and TWL tests. After pre-treatment thresholds are assessed, animals underwent an object-location memory task training session, wherein animals were exposed to the open field with four novel objects for 10 minutes. During this time, thigmotaxis and overall locomotion were recorded as well. On day 9 (test day), all behavioral tests were administered post-treatment. Within Experiment 1, all animals received a vehicle control treatment injection (Table 1, Row A) and were allowed to habituate for 60 minutes prior to behavioral testing. Post-treatment mechanical and thermal hyperalgesia were quantified with MPWT and TWL tests. Spatial learning and memory were quantified within the 10 minute object-location memory task, where locomotion and thigmotaxis were also recorded. Following the object-location memory task, animals underwent a 5-minute olfactory discrimination test, where time spent in the "familiar" compartment of the chamber was compared to time spent in the "unfamiliar" compartment of the chamber as an index of olfactory learning. Anxiety-like behaviors were assessed within the EPM, where greater time spent avoiding the open arms of the apparatus across the 10 minute

testing period were quantified as greater anxiety-like behavior. Depression-like behavior was evaluated using a 5-minute home cage splash test, where less time spent grooming after splash exposure to a sucrose solution was implicative of anhedonia-like behavior. Statistical analyses were conducted following Experiment 1 to evaluate differences in affect, cognition, and pain behaviors between animals across pain model conditions.

Experiment 2: A total of 43 animals were utilized within Experiment 2. The experimental timeline and procedures as described previously were replicated for Experiment 2. However, on day 9 (test day), prior to post-treatment behavioral assessments, all animals were administered duloxetine (30 mg/kg, i.p.) and habituated for 60 minutes prior to behavioral testing (Table 1, Row B). Statistical analyses were conducted following Experiment 2 to evaluate treatment efficacy for affect, cognition, and pain behaviors across experimental conditions.

Clarification of the experimental timeline for both Experiment 1 and Experiment 2 is presented in Figure 1.

	Pain Condition				
	N = 102	SAL/SHAM	RES/SHAM	SALINE/SWIM	RES/SWIM
Treatment Condition	**A:** DULOX. (30mg/kg)	*n* = 12	*n* = 12	*n* = 12	*n* = 7
	B: VEH. CONTROL	*n* = 14	*n* = 16	*n* = 12	*n* = 17

Table 1. Experimental conditions and sample size.

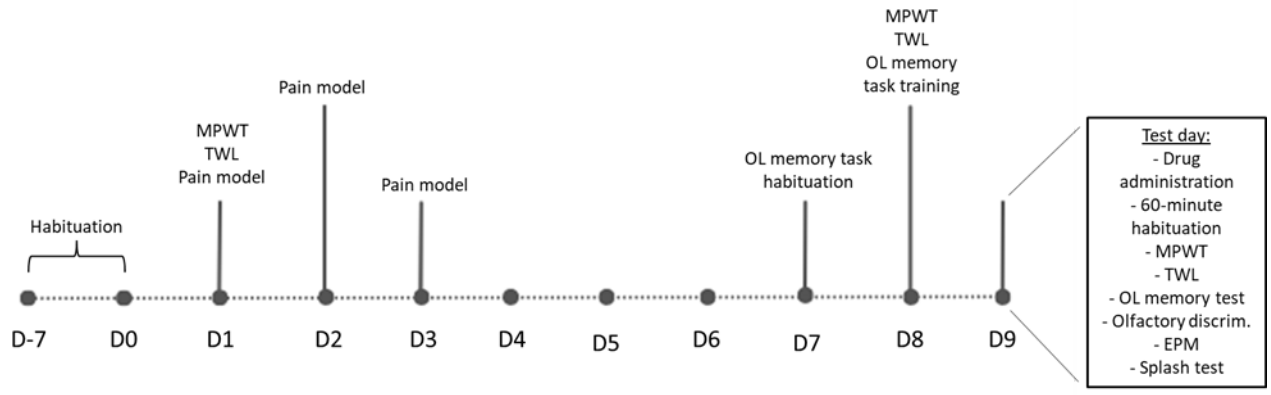


Figure 1. Experimental timeline.

2.5 *Sensory Dimension Tests*

Mechanical Paw Withdrawal Thresholds

To quantify mechanical hypersensitivity, animals were placed into a Plexiglass chamber with a wire mesh bottom that allows access to the plantar surface of the hind paw. Animals were allowed to habituate for 10 minutes. Mechanical sensitivity was assessed using von Frey monofilaments (3.85, 5.68, 9.74, 18.39, 39.42, 77.3, 135.3, and 251.34 mN) and quantified using the up-down method (Dixon, 1980). Each trial began with the 9.74 mN filament delivered to the left hind paw for approximately 1s, then to the right hind paw. If no withdrawal response was observed (i.e., licking or paw withdrawal), the next highest filament was used. If a withdrawal response was observed, the next lowest filament was used. This procedure was repeated until there was no response from the animal at the highest force (251.34) or until a total of 5 stimuli were administered. The 50% paw withdrawal threshold for each trial was calculated using the following formula: $[Xth]log = [vFr]log + ky$, where [vFr] is the force of the last von Frey used, $k = 0.2593$ is the average interval (in log units) between the von Frey monofilaments, and y is a value that depends upon the pattern of withdrawal responses. If an animal did not respond to the

highest von Frey monofilament (251.34 mN), then y = 1.00 and the 50% mechanical paw withdrawal response for that paw was calculated to be 456.63 mN. This test was conducted 3 times, with the score from each trial being averaged to determine the mean withdrawal threshold for the left and right hind paws of the animal. A combined mechanical threshold score was then averaged from the right and left paw values for each MPWT for statistical analysis. Measures of mechanical hypersensitivity were assessed on day 1 (baseline), day 8 (pre-treatment), and day 9 (post-treatment).

Thermal Withdrawal Latencies

To quantify thermal hypersensitivity, animals were placed into a Plexiglass chamber where they were allowed to habituate for 10 minutes. An infrared heat source was applied to the plantar surface of each hind paw, and the latency for the animal to withdraw from the stimulus was recorded. If the animal failed to withdraw from the stimulus after 20 seconds, the heat was removed from the hind paw to avoid damage, and the latency maximum was recorded as 20 seconds. Threshold testing was performed twice per paw, with at least 2 minutes separating each measurement. The average value of the 4 measures was calculated as the withdrawal latency for each animal. Measures of thermal hypersensitivity were assessed on day 1 (baseline), day 8 (pre-treatment), and day 9 (post-treatment).

2.6 *Affective Dimension Tests*

Depression-like Splash

To assess depression-like behaviors on test day (day 9), animals were placed in a dim-lit testing room and temporarily removed from their home cage and suspended for sucrose-splash administration. During suspension, animals were sprayed twice with a 10% sucrose solution on the dorsal coat before being quickly placed back in their home cage. After spraying the sucrose

solution, total grooming time, latency to the first grooming, and the number of grooms were recorded for a period of 5 minutes. Within this paradigm, depression-like behavior may be displayed as a decrease in total time spent grooming and number of grooms, or an increased latency to begin grooming.

Anxiety-like Elevated Plus Maze

To assess anxiety-like behaviors on test day (day 9), animals were placed in the center of a raised, plus-shaped, wooden platform, with two walled arms and two open arms. Exploratory behavior within the maze was recorded and quantified for 5 minutes. Measures included total time spent in the open-arms and the closed-arms of the maze, in addition to total number of entries into each the open- and closed-arms. In this paradigm, anxiety-like behavior is assumed when an animal avoids the open-arms of the maze significantly more than the closed-arms.

2.7 *Cognitive Dimension*

Olfactory Discrimination

To quantify potential differences in olfactory discrimination on test day (day 9), animals were placed in a plexiglass chamber with two identical compartments, separated by a divided opening. One half of the chamber contained “familiar” bedding, or bedding from the same animal’s home-cage that has been occupied for at least 48-hours prior, while the other half of the chamber contained “unfamiliar” bedding, or fresh bedding which no animal has previously occupied. The time spent in each compartment (familiar vs. unfamiliar) over a 5-minute period was recorded as an index of olfactory discrimination. In this paradigm, accurate olfactory discrimination is displayed by the animal spending more time in the familiar compartment of the chamber.

Object-Location Memory Task

To assess spatial learning and memory, and locomotion and thigmotaxic behavior, animals underwent an object-location memory task in an open field. On day 7, animals were placed in the center of a circular open field chamber with a wooden base and aluminum sheet metal walls to habituate for 10 minutes. A video tracking system (Ethovision) was used to record and quantify the distance traveled within the center and perimeter of the apparatus to evaluate locomotion and thigmotaxic behavior during initial habituation exposure to the chamber. On day 8 (pre-treatment), animals were exposed to a 10-minute training session where they were placed into the same open field chamber with either four or two similar, yet novel, objects in the center of the chamber that were roughly equidistant from each other. Animals were recorded for quantification of locomotion and thigmotaxis during this training phase. On test day (day 9; post-treatment), animals were placed back into the same open field chamber for 10 minutes, where their locomotion and thigmotaxic behavior were recorded. During this exposure, two of the equidistant objects in the center of the apparatus were switched in location, while the other two objects maintained their location from the training phase. The time spent exploring (i.e., sniffing, running around, touching, climbing) the two objects placed in novel locations compared to the objects in the same spatial locations was recorded as the index of object-location discrimination. The object-location discrimination index was calculated using the following formula: [(time spent exploring the objects moved to a novel location / the total time spent exploring all objects) x 100]. Additional analyses were conducted to determine any potential main effects of object-location condition (two vs. four objects) on subsequent behavior tests, as well as determine if there were any differences in object preference.

2.8 *Statistical Analysis*

To analyze pre-treatment bodyweight, MPWTs, TWLs, and locomotive behaviors, a mixed repeated measures analysis of variance (ANOVA) was used with time as the within-subjects variable and experimental condition (pain condition) as the between-subjects variable. To analyze post-treatment behavior among MPWTs, TWLs, EPM, Splash Test, Olfactory Discrimination, and Object-Location Memory task, separate ANOVAs were used to determine potential differences between experimental conditions (pain and treatment condition). All *post hoc* analyses were conducted using Fisher's LSD and group values are presented as mean (*M*) and standard error (*SE*).

3. Results

3.1 Bodyweight

Bodyweight fluctuation values, an indication of overall body condition of animals, were calculated by determining the difference in the daily body weight (g) of each animal when compared to baseline weight values on Day 1. Values calculated to be positive indicated a significant increase in body weight when compared to Day 1 values, while negative values indicated a significant loss in body weight when compared to bodyweight on Day 1.

3.1.1 *Pre-treatment (Days 1-9)*

To evaluate for any potential differences in bodyweight fluctuation between groups as a result of pain condition (Figure 2), a mixed model repeated measures ANOVA was used with pain condition was the between-subjects variable and time prior to treatment (days 1-8) was used as the within subjects variable. A significant main effect of time was identified, $F(8, 776) = 21.757$, $p < .001$, $\eta p2 = .183$ alongside a significant main effect of pain condition, $F(3, 97) =$

3.567, $p = .017$, $\eta p2 = .099$. A significant time by pain condition interaction was also identified, $F(24, 776) = 1.704$, $p = .019$, $\eta p2 = .050$.

When probing for specific differences over time in bodyweight fluctuation, Fisher's LSD post-hoc analyses revealed that when collapsed across pain conditions and compared to baseline bodyweight on Day 1, animals had a significantly higher bodyweight gain across Day 4 ($M = 1.855$, $SE = .506$) through Day 9 ($M = 5.787$, $SE = .656$). Specifically, there were no differences in bodyweight fluctuation from Days 1-3. However, animals had a significant increase in weight across Day 4 and Day 5 ($M = 2.492$, $SE = .565$). There was another significant increase in weight on Days 6 ($M = 5.355$, $SE = 1.164$) and Days 7 ($M = 4.791$, $SE = .594$). However, there was a significant decrease in the amount of weight fluctuation compared to Day 1 from Day 7, to Day 8 ($M = 3.401$, $SE = .641$). Weight gain fluctuation significantly increased again from Day 8 to Day 9 ($M = 5.787$, $SE = .656$).

When probing for specific differences in bodyweight fluctuation across pain conditions, post-hoc analyses revealed that SAL/SHAM animals ($M = .899$, $SE = .888$) had significantly smaller gains in bodyweight compared to RES/SHAM ($M = 4.57$, $SE = .872$) animals. However, there was a significant difference in bodyweight fluctuation between RES/SHAM and SAL/SWIM ($M = 1.43$, $SE = .925$) animals. Additionally, SAL/SWIM animals showed no significant differences in bodyweight gain compared to SAL/SHAM animals, while differences when compared to RES/SWIM ($M = 3.149$, $SE = .925$) animals were trending.

When probing for specific differences between groups over time within the significant interaction effect, no significant differences were identified prior to Day 4. Specifically on Day 4, SAL/SHAM ($M = 0.750$, $SE = .996$) animals began to show a significant difference in bodyweight gain when compared to RES/SHAM ($M = 4.20$, $SE = .978$) animals. Additionally,

RES/SHAM animals showed a significant weight gain from Day 1 when compared to SAL/SWIM (*M* = 0.836, *SE* = 1.037) animals. On Day 5, continued effects were observed, with SAL/SHAM animals (*M* = 0.895, *SE* = 1.113) showing a significant weight gain difference from Day 1 compared to RES/SHAM (*M* = 5.226, *SE* = 1.092) animals, while RES/SHAM animals showed an additional significant weight gain from Day 1 when compared to SAL/SWIM animals (*M* = 1.454, *SE* = 1.158). On Day 6, the only significant difference between groups identified was between SAL/SHAM (*M* = 1.762, *SE* = 2.292) and RES/SWIM (*M* = 6.467, *SE* = 2.249) animals, where RES/SWIM animals showed a significantly higher weight gain fluctuation. On Day 7, Day 8, and Day 9, patterns in weight fluctuation reverted to that which was observed for Day 4 and Day 5, with SAL/SHAM animals (Day 7: *M* = 3.438, *SE* = 1.17; Day 8: *M* = 1.685, *SE* = 1.262; Day 9: *M* = 3.43, *SE* = 1.292) showing a significantly lower weight fluctuation from RES/SHAM (Day 7: *M* = 7.863, *SE* = 1.148; Day 8: *M* = 6.459, *SE* = 1.238; Day 9: *M* = 9.63, *SE* = 1.268) animals, where RES/SHAM animals showed an additional significant weight gain difference from SAL/SWIM animals (Day 7: *M* = 3.017, *SE* = 1.217; Day 8: *M* = 1.238, *SE* = 1.313; Day 9 = 3.588, *SE* = 1.345).

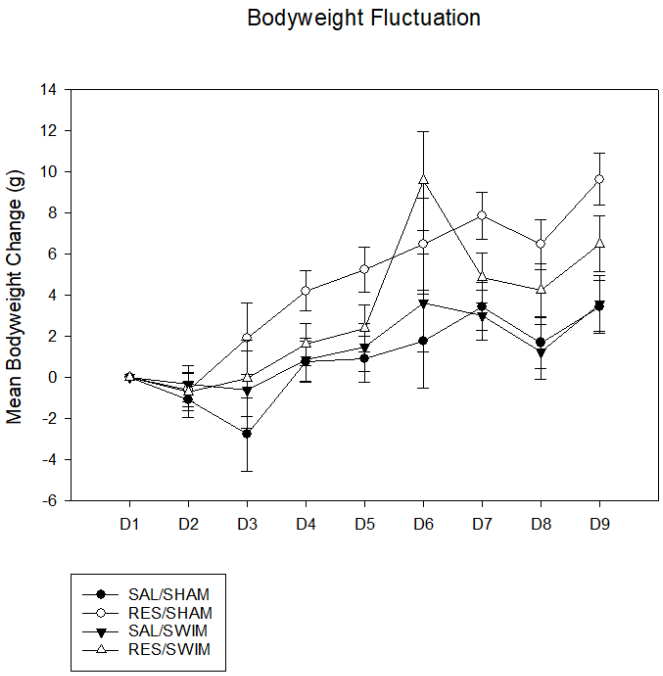
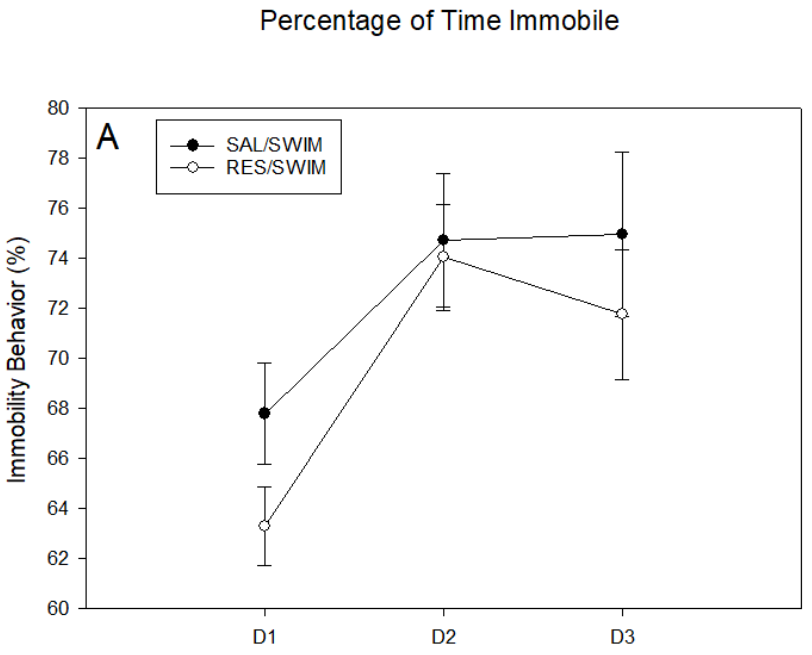


Figure 2. Bodyweight fluctuation from Day 1 between groups over time.

3.2 Immobility

To determine if there were any differences in percentage of time spent immobile over time during repeated exposure to the forced swim paradigm (Figure 3A), a repeated measures mixed model ANOVA was used with time as the within subjects variable and swim condition (RES/SWIM or SAL/SWIM) as the between subjects variable. There were no significant main effects of swim condition, $F(2, 45) = 1.246$, $p = .27$. Additionally, there were no significant interaction effects between time and swim condition, $F(2, 90) = .448$, $p = .64$. However, there was a significant main effect of time identified, $F(2, 90) = 11.077$, $p < .001$, $\eta p2 = .198$. Post-hoc analyses revealed that animals showed significantly more immobility behavior from Day 1 ($M = 65.537$, $SE = 1.28$) to Day 2 ($M = 74.377$, $SE = 1.705$) and Day 3 ($M = 73.350$, $SE =$

2.087), regardless of reserpine administration. However, there were no differences in immobility behaviors between Day 2 and Day 3 (Figure 3B).



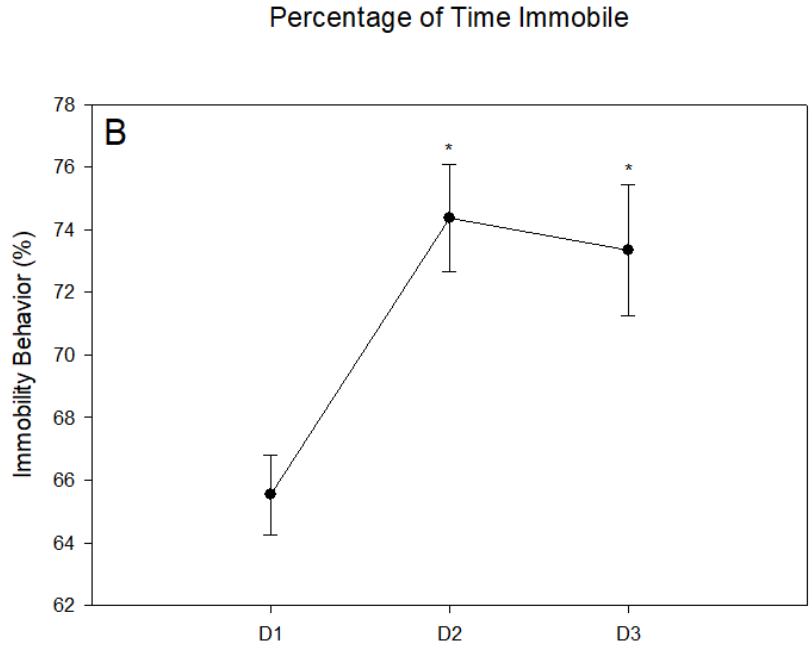


Figure 3. Percentage of time spent immobile during subchronic swim stress induction. (A) Percentage immobility behavior over time across swim conditions. (B) Percentage of time spent immobile across all animals exposed to swim stress. * Compared to D1 at $p < .001$

3.2 Mechanical Paw Withdrawal Thresholds

3.2.1 *Baseline to Pre-treatment*

To evaluate the efficacy of each experimental condition in producing mechanical hypersensitivity from baseline to pre-treatment across all animals within Experiment 1 and Experiment 2, a repeated measures mixed-model analysis of variance (ANOVA) was used with time as the within-subjects variable, and pain condition as the between-subjects variable. A significant main effect of both time, $F(1, 97) = 208.375$, $p < .001$, $\eta p2 = .682$, and pain condition, $F(3, 97) = 9.267$, $p < .001$, $\eta p2 = .223$, were identified. Additionally, a significant time by pain condition interaction was identified, $F(3,97) = 21.405$, $p < .001$, $\eta p2 = .398$.

To probe for specific differences within main effects, all post-hoc analyses were conducted using Fisher's LSD. Within the main effect of time, mean mechanical thresholds were significantly higher at baseline ($M = 427.301$, $SE = 4.70$) than at pre-treatment ($M = 308.106$, $SE = 8.239$). Within the significant main effect for pain condition, animals randomized to the SAL/SHAM condition had significantly higher mean thresholds than all other groups ($M = 414.254$, $SE = 10.404$). However, there were no significant differences identified between mean thresholds for animals randomized to the RES/SHAM ($M = 361.419$, $SE = 10.209$), SAL/SWIM ($M = 353.01$, $SE = 10.828$), or RES/SWIM ($M = 342.124$, $SE = 10.828$) conditions.

To evaluate significant between group differences in mean mechanical hypersensitivity over time, post-hoc analyses were conducted using Fisher's LSD. At baseline measures, there were no significant differences identified between groups prior to stress and injection exposure.

However, at pre-treatment measures (Figure 4), SAL/SHAM control animals maintained significantly higher mean thresholds than all other groups (M = 406.448, SE = 16.216), as expected. At pre-treatment, both SAL/SWIM (M = 293.069, SE = 16.879) and RES/SHAM (M = 289.276, SE = 15.913) animals maintained reduced mechanical thresholds that were not significantly different from each other. However, animals randomized to the RES/SWIM condition displayed the most reduced mechanical thresholds (M = 243.629, SE = 16.879), with statistical significance being identified when compared to SAL/SWIM animals, and trending significance when compared to RES/SHAM animals (p = .052).

3.2.2 *Post-treatment*

To determine the potential efficacy of duloxetine administration in alleviating reduced mechanical thresholds across groups, a one-way ANOVA was used with pain and treatment condition (SAL/SHAM, RES/SHAM, SAL/SWIM, RES/SWIM, each with SAL or DULOX treatment) as the independent variable. A significant main effect of pain and treatment condition was identified, $F(7, 93) = 10.149$, $p < .001$, $\eta p2 = .433$.

3.2.2.1 Saline Treatment

To identify specific differences in mean thresholds between groups following treatment (Figure 5), post-hoc analyses were conducted using Fisher's LSD. Specifically, within animals administered a saline control treatment, SAL/SHAM/SAL (M = 395.629, SE = 22.759) animals exhibited the highest thresholds between groups. However, animals in the both the SAL/SWIM/SAL (M = 273.591, SE = 24.583) and RES/SHAM/SAL (M = 317.279, SE = 21.289) groups displayed significantly reduced thresholds when compared to SAL/SHAM/SAL controls, although mean thresholds between these groups were not different from each other. As expected, animals randomized to the RES/SWIM/SAL group (M = 219.771, SE = 20.654)

generally displayed the greatest reduction in mechanical thresholds. Mean thresholds for RES/SWIM/SAL animals were reduced when compared to SAL/SHAM/SAL and RES/SHAM/SAL animals. However, mean mechanical thresholds among this group were not significantly reduced when compared to SAL/SWIM/SAL animals ($p = .092$).

3.2.2.2 Duloxetine Treatment

When evaluating differences among animals administered duloxetine (Figure 5), as expected, animals within the SAL/SHAM/DULOX ($M = 419.605$, $SE = 24.583$) group. However, there were also no significant differences identified between SAL/SHAM/SAL and SAL/SHAM/DULOX animals when compared to both RES/SHAM/DULOX ($M = 415.916$, $SE = 25.676$) and RES/SWIM/DULOX ($M = 399.799$, $SE = 32.186$) animals.

Saline vs. Duloxetine

When evaluating differences among animals administered duloxetine compared to those administered a saline control treatment (Figure 5), as expected, no differences in thresholds were identified between SAL/SHAM/SAL and SAL/SHAM/DULOX ($M = 419.605$, $SE = 24.583$) animals. As further expected, there were no significant differences identified between SAL/SHAM/SAL, SAL/SHAM/DULOX, RES/SHAM/DULOX ($M = 415.916$, $SE = 25.676$), and RES/SWIM/DULOX ($M = 399.799$, $SE = 32.186$) animals. Contrary to our hypotheses, the only group that displayed significantly reduced mean mechanical thresholds when compared to controls after duloxetine treatment, were animals randomized to the SAL/SWIM/DULOX group ($M = 327.870$, $SE = 24.583$). Specifically, SAL/SWIM animals that were treated with duloxetine showed no difference in mechanical thresholds compared to SAL/SWIM animals treated with a saline control.

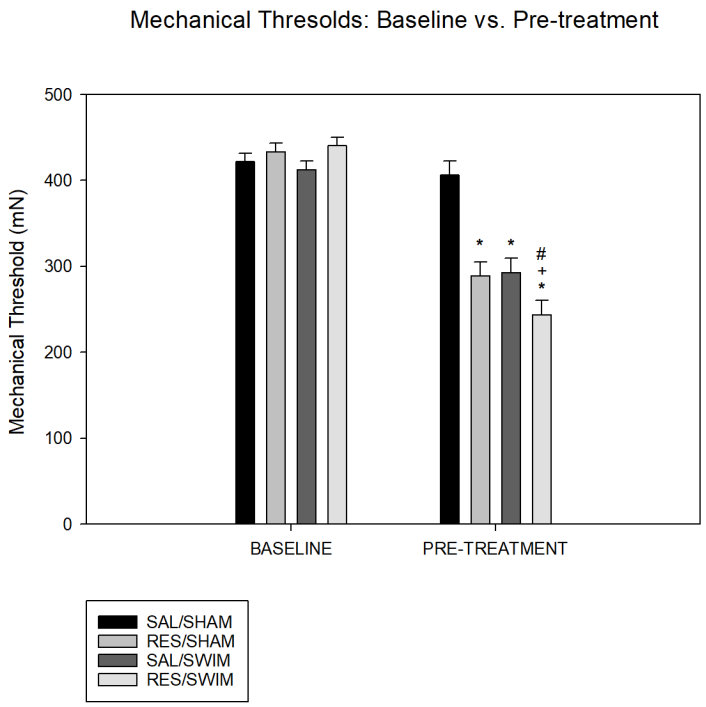


Figure 4. Mechanical paw withdrawal thresholds at baseline and pre-treatment. * Compared to SAL/SHAM at $p < .001$; + compared to RES/SHAM at $p = .052$; # compared to SAL/SWIM at $p = .041$

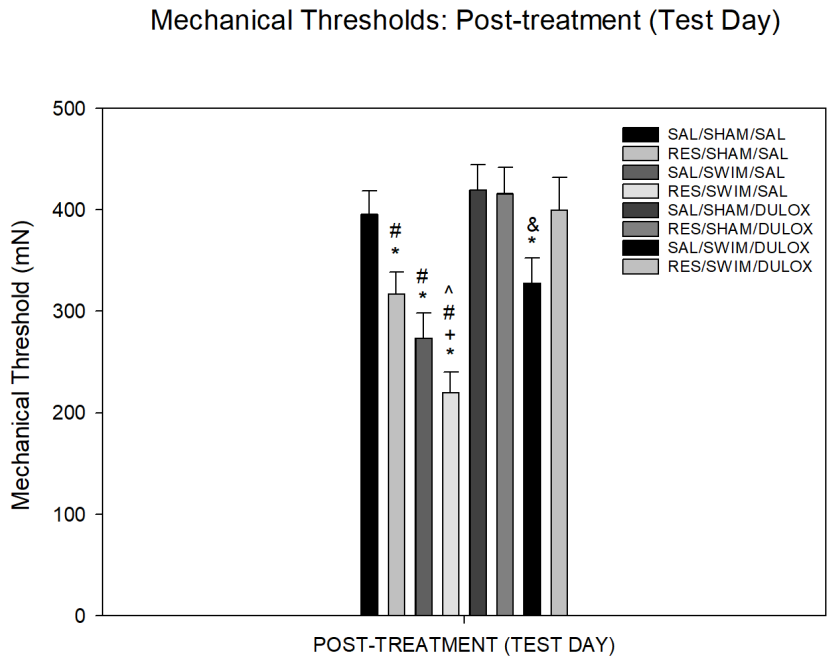


Figure 5. Mechanical paw withdrawal thresholds at post-treatment. * Compared to SAL/SHAM at $p < .05$; + compared to RES/SHAM at $p < .001$; # compared to SAL/SHAM/DULOX, RES/SHAM/DULOX, RES/SWIM/DULOX at $p < .05$; & compared to SAL/SHAM/DULOX and RES/SHAM/DULOX at $p < .05$; ^ compared to SAL/SWIM/DULOX at $p = .001$

3.3 Thermal Withdrawal Latencies

3.3.1 *Baseline vs. Pre-treatment*

To determine potential statistical differences in TWLs between groups at baseline and pre-treatment (Figure 6), a repeated measures mixed-model ANOVA was used with time (baseline vs. pre-treatment) as the within subjects variable and pain condition as the between-subjects variable (SAL/SHAM, RES/SHAM, SAL/SWIM, RES/SWIM). A significant main effect of time was identified, $F(1, 96) = 16.207$, $p < .001$, $\eta p2 = .144$, while a significant main

effect of condition was not found, $F(3, 96) = 1.524$, $p = .213$. However, a significant time by pain condition interaction was identified, $F(3,96) = 3.204$, $p = .027$, $\eta p2 = .091$.

When collapsed across groups to evaluate differences in mean latencies over time, TWLs were found to be significantly higher at baseline ($M = 12.611$, $SE = 0.259$) than pre-treatment ($M = 11.503$, $SE = 0.231$). However, when probing for specific differences in latencies between groups over time, no differences were found between groups at baseline latency measures. Animals randomized to the RES/SHAM (BL: $M = 12.668$, $SE = 0.507$; PT: $M = 11.492$, $SE = 0.453$) and SAL/SWIM (BL: $M = 12.925$, $SE = 0.528$; PT: $M = 10.515$, $SE = 0.471$) groups showed significant reductions in thermal latencies from baseline to pre-treatment. No differences were observed over time among SAL/SHAM or RES/SWIM animals. However, at pre-treatment, SAL/SHAM ($M = 12.736$, $SE = 0.453$) animals exhibited significantly higher latencies than animals in the SAL/SWIM ($M = 10.515$, $SE = 0.472$) and RES/SWIM ($M = 11.27$, $SE = 0.472$) groups. No other significant differences were identified at pre-treatment.

3.3.2 *Post-treatment*

To determine the potential efficacy of duloxetine administration on TWLs across groups (Figure 7), a one-way ANOVA was used with pain and treatment condition (SAL/SHAM, RES/SHAM, SAL/SWIM, RES/SWIM, each with SAL or DULOX treatment) as the independent variable. However, no significant main effect of pain and treatment condition was found, $F(7,92) = 1.028$, $p = .417$. In general, no significant differences were identified between animals administered duloxetine or saline. However, probing of between-group relationships to identify potentially meaningful relationships between groups, albeit non-significant, identified a trending relationship among SAL/SWIM/DULOX ($M = 11.36$, $SE = .745$) animals. Specifically, these animals displayed post-treatment withdrawal latencies that were reduced (trending

significance) compared to SAL/SHAM/SAL (M = 11.237, SE = 0.689; p = .077), SAL/SHAM/DULOX (M = 11.36, SE = 0.745; p = .069), and RES/SHAM/DULOX (M = 11.32, SE = 0.778; p = .081) animals.

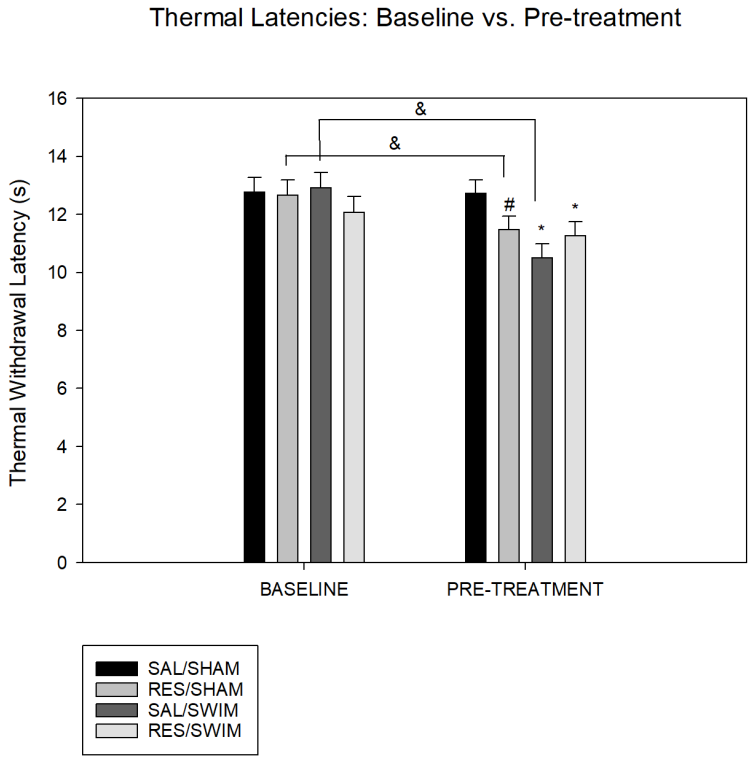


Figure 6. Thermal withdrawal latencies at baseline and pretreatment. * Compared to SAL/SHAM at $p < .05$; # compared to SAL/SHAM at $p = .055$; & difference over time at $p < .05$

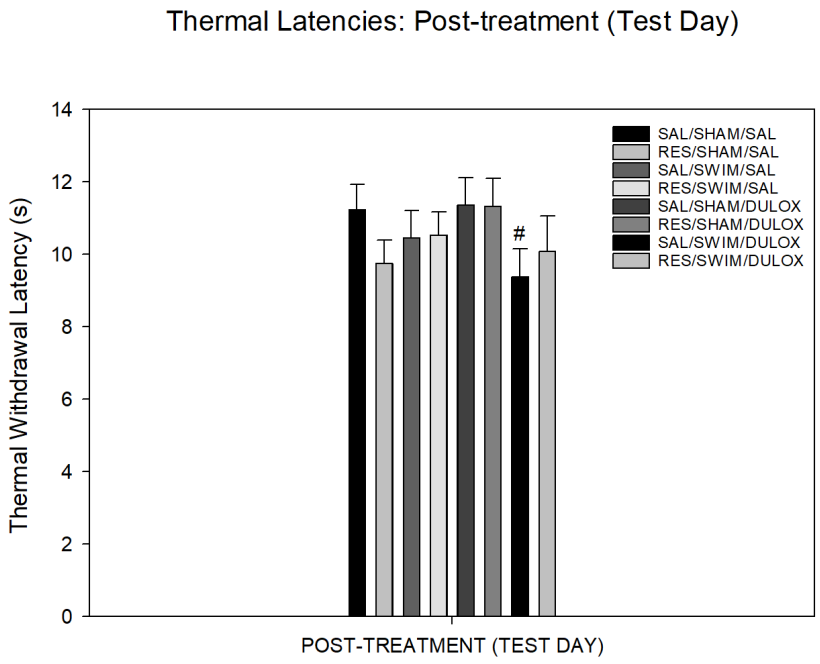


Figure 7. Thermal withdrawal latencies at post-treatment. # trending significance compared to SAL/SHAM/SAL, SAL/SHAM/DULOX, RES/SHAM/DULOX

3.4 Thigmotaxis

3.4.1 *Habituation vs. Pre-treatment/Training*

To determine potential statistical differences in distance traveled in the center of an open field between groups at habituation and pre-treatment/training (Figure 8), a repeated measures mixed-model ANOVA was used with time (habituation vs. training) as the within subjects variable and pain condition as the between-subjects variable (SAL/SHAM, RES/SHAM, SAL/SWIM, RES/SWIM). An expected significant main effect of time was identified, $F(1, 97) = 66.960$, $p < .001$, $\eta p2 = .408$. However, there was no main effect of pain condition identified, $F(3, 97) = 0.538$, $p = .657$, nor a significant time by pain condition interaction, $F(3, 97) = 0.080$, $p = .971$.

Within the significant main effect of time, there was an expected significant decrease in the distance traveled in the center of the arena from habituation (M = 1383.443, SE = 54.732) to pre-treatment (M = 933.908, SE = 44.269). However, no other meaningful thigmotaxis behaviors were identified.

3.4.2 *Post-treatment/Test*

To determine if duloxetine exerted an effect on locomotion in the center of the open field between groups (Figure 9), a one-way ANOVA was used with pain and treatment condition (SAL/SHAM, RES/SHAM, SAL/SWIM, RES/SWIM, each with SAL or DULOX treatment) as the independent variable. However, contrary to our hypotheses, no significant main effect of pain and treatment conditions were found, $F(7, 93) = 1.181$, $p = .321$.

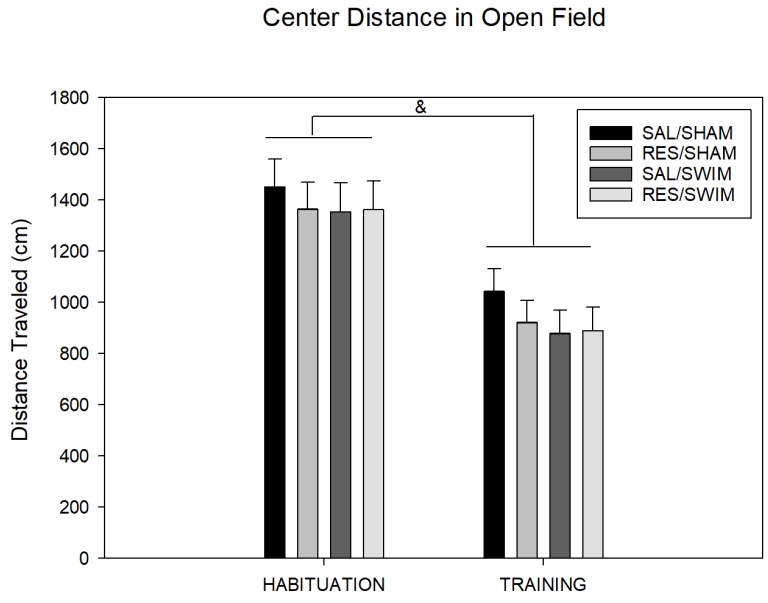


Figure 8. Distance traveled in the center of the open field apparatus during habituation and training phases of the object-location memory task. & within-subjects effect at $p < .001$

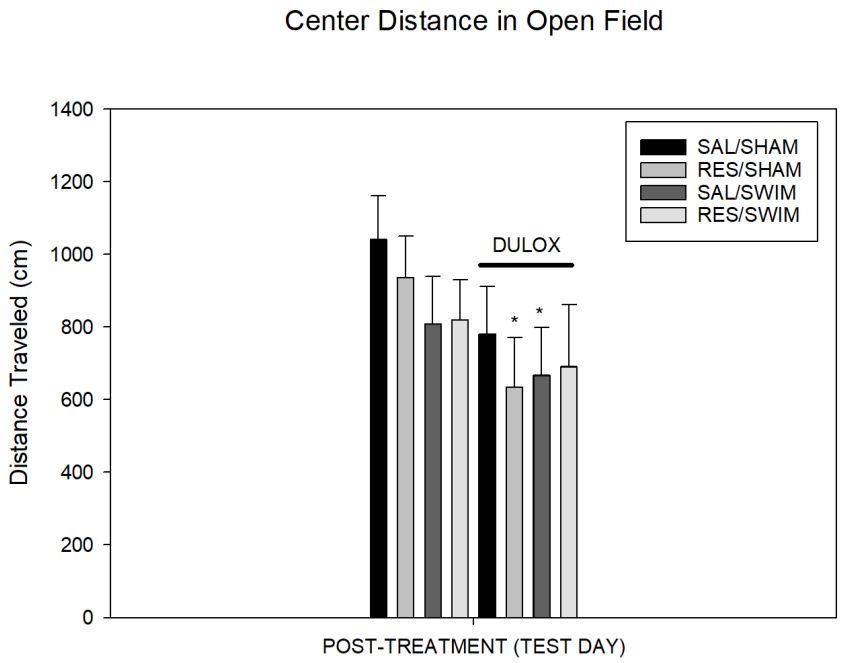


Figure 9. Distance traveled in the center of the open field apparatus during test phase of the object-location memory task. * Compared to SAL/SHAM at $p < .05$

3.5 Distance Traveled in an Open Field

3.5.1 *Habituation vs. Pre-treatment/Training*

To determine potential statistical differences in total distance travelled in an open field between groups at habituation and pre-treatment (Figure 10), a repeated measures mixed-model ANOVA was used with time (habituation vs. training) as the within subjects variable and pain condition as the between-subjects variable (SAL/SHAM, RES/SHAM, SAL/SWIM, RES/SWIM). A significant main effect of time was found, $F(1,97) = 42.584$, $p < .001$, $\eta p2 = .305$. However, there were no significant main effects found for pain condition, $F(3,97) = 0.724$, $p = .54$, nor was a significant interaction effect found, $F(3, 97) = 0.238$, $p = .87$. Specifically, when collapsed across groups, animals travelled significantly more during the habituation phase

(*M* = 6404.398, *SE* = 101.695) than the training phase (*M* = 5543.201, *SE* = 154.784). When evaluating interaction post-hoc analyses for potential meaningful relationships between groups, albeit nonsignificant, the only relationship identified was between RES/SWIM and RES/SHAM animals, where during habituation, RES/SWIM (*M* = 6680.234, *SE* = 208.345) animals travelled significantly more than RES/SHAM (*M* = 6102.164, *SE* = 196.43) animals. However, neither of these groups travelled significantly more or less than controls.

3.5.2 *Post-treatment/Test*

To determine if duloxetine exerted an effect on overall locomotion between groups (Figure 11), a one-way ANOVA was used with pain and treatment condition (SAL/SHAM, RES/SHAM, SAL/SWIM, RES/SWIM, each with SAL or DULOX treatment) as the independent variable. A significant main effect of pain and treatment condition was found, $F(7, 93) = 7.28$, $p < .001$, $\eta p2 = .354$.

Specifically, all animals administered duloxetine (SAL/SHAM: *M* = 3792.817, *SE* = 409.554; RES/SHAM: *M* = 3013.50, *SE* = 427.765; SAL/SWIM: *M* = 3101.372, *SE* = 409.554; RES/SWIM: *M* = 3535.831, *SE* = 536.232) travelled significantly less during the test phase than animals administered a saline control treatment (SAL/SHAM: *M* = 5520.133, *SE* = 379.173; RES/SHAM: *M* = 5308.341, *SE* = 354.684; SAL/SWIM: *M* = 5352.703, *SE* = 409.554; RES/SWIM: *M* = 5209.036, *SE* = 344.094). However, among animals who were administered duloxetine, and among animals who were administered saline, there were no significant differences in overall locomotion between groups as a function of pain condition.

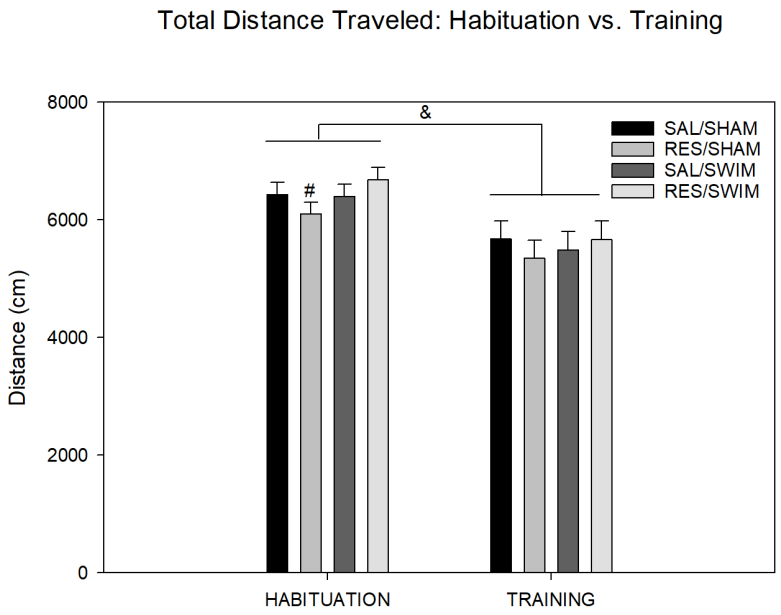


Figure 10. Total distance traveled in the open field apparatus during habituation and training phases of the object-location memory task. [#] compared to RES/SWIM at $p < .05$; [&] within-subjects difference over time at $p < .05$

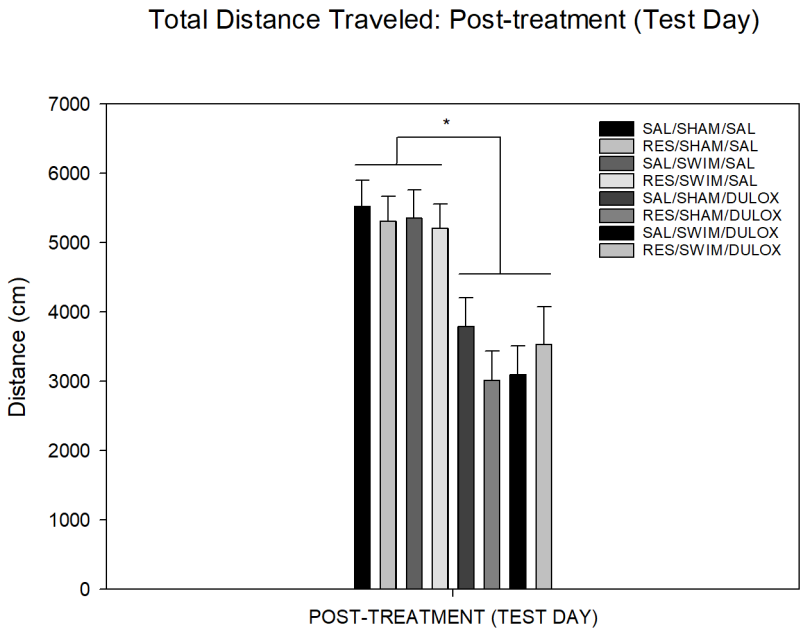


Figure 11. Total distance traveled in the open field apparatus during habituation and training phases of the object-location memory task. * Between-subjects difference between groups at $p < .05$

3.6 Velocity in an Open Field

3.6.1 *Total Average Velocity: Habituation vs. Pre-treatment/Training*

To determine if there were differences between groups in average total velocity in the open field chamber across time, a repeated measures mixed-model ANOVA was used with time (habituation vs. training) as the within subjects variable and pain condition as the between-subjects variable (SAL/SHAM, RES/SHAM, SAL/SWIM, RES/SWIM). A significant main effect of time was identified, $F(1, 97) = 52.797$, $p < .001$, $\eta p2 = .352$. However, there was not a significant main effect of pain condition identified, $F(3, 97) = 0.848$, $p = .471$, nor a time by pain condition interaction, $F(3, 97) = 0.441$, $p = .724$. Within post hoc analyses of the identified main

effect of time, animals travelled significantly faster at first exposure to the chamber (habituation) (M = 10.867, SE = 0.18) compared to the training phase (M = 9.255, SE = 0.259).

3.7.2 *Total Average Velocity: Post-treatment/Test*

To determine if duloxetine exerted an effect on overall velocity in an open field between groups, a one-way ANOVA was used with pain and treatment condition (SAL/SHAM, RES/SHAM, SAL/SWIM, RES/SWIM, each with SAL or DULOX treatment) as the independent variable. However, there was no significant between-subject effect of pain and treatment condition identified, $F(7, 93) = 0.695$, $p = .676$.

3.6.3 *Average Center Velocity: Habituation vs. Pre-treatment/Training*

To determine if there were differences between groups in average total velocity in the center of the open field chamber across time, a repeated measures mixed-model ANOVA was used with time (habituation vs. training) as the within subjects variable and pain condition as the between-subjects variable (SAL/SHAM, RES/SHAM, SAL/SWIM, RES/SWIM). As was observed in measures of total velocity, a significant main effect of time was found, $F(1, 97) = 50.758$, $p < .001$, $\eta p2 = .344$. However, there was not a significant between-subjects effect of pain condition, $F(3, 97) = 1.255$, $p = .294$, nor a time by pain condition interaction, $F(3, 97) = 1.157$, $p = .330$. Similarly as to was observed among patterns of total average velocity, animals traveled significantly faster in the center of the open field at initial habituation exposure (M = 18.499, SE = .694) than at pre-treatment (M = 13.065, SE = .419).

3.6.4 *Average Center Velocity: Post-treatment/Test*

To determine if duloxetine exerted an effect on overall velocity in an open field between groups, a one-way ANOVA was used with pain and treatment condition (SAL/SHAM, RES/SHAM, SAL/SWIM, RES/SWIM, each with SAL or DULOX treatment) as the independent variable.

Similarly to patterns observed in analyses of total average velocity, there was no significant main effect of pain and treatment condition on average velocity in the center of the open field, $F(7, 93) = 0.798$, $p = .591$.

3.7 Olfactory Discrimination

To determine potential differences in percentage of time spent in the familiar and unfamiliar compartments of the olfactory discrimination chamber (Figure 12), a MANOVA was used with pain and treatment condition as the independent variable and percentage of time spent in each the familiar and unfamiliar compartments as the dependent variables. No significant main effect of pain and treatment condition was found of percentage of time spent in either the unfamiliar, $F(7, 92) = 0.566$, $p = .781$, or the familiar, $F(7, 92) = 0.613$, $p = .744$, compartments of the chamber.

To evaluate the impact of pain and treatment conditions on the total number of crosses between compartments within the olfactory discrimination task (Figure 13), a one-way ANOVA was used with pain and treatment condition as the independent variable and total number of crosses in the chamber as the dependent variable. There were no significant effects found as a result of pain and treatment condition, $F(7,92) = 1.837$, $p = .091$. However, animals in the RES/SHAM/SAL ($M = 12.231$, $SE = .874$) produced a trending effect for higher number of crosses compared to SAL/SWIM/DULOX ($M = 9.7$, $SE = .996$), RES/SWIM/SAL ($M = 9.529$, $SE = .764$), RES/SWIM/DULOX (8.286, $SE = 1.19$), and SAL/SHAM/DULOX ($M = 8.417$, $SE = .909$).

Based on the graphical presentations of total number of crosses and previous data implying a decrease in locomotive activity associated with duloxetine, and exploratory one-way ANOVA was used to evaluate the impact of treatment on total number of crosses in the chamber.

There was a main effect of treatment condition found when collapsed across pain model conditions, $F(1,92) = 5.475$, $p = .021$, $\eta p2 = .057$. Specifically, animals treated with duloxetine ($M = 8.854$, $SE = 0.495$) crossed significantly less across the compartments of the olfactory discrimination chamber compared to animals treated with a saline control ($M = 10.404$, $SE = 0.44$), implicative of significant changes in exploratory behavior and locomotion as a result of duloxetine administration (Figure 14).

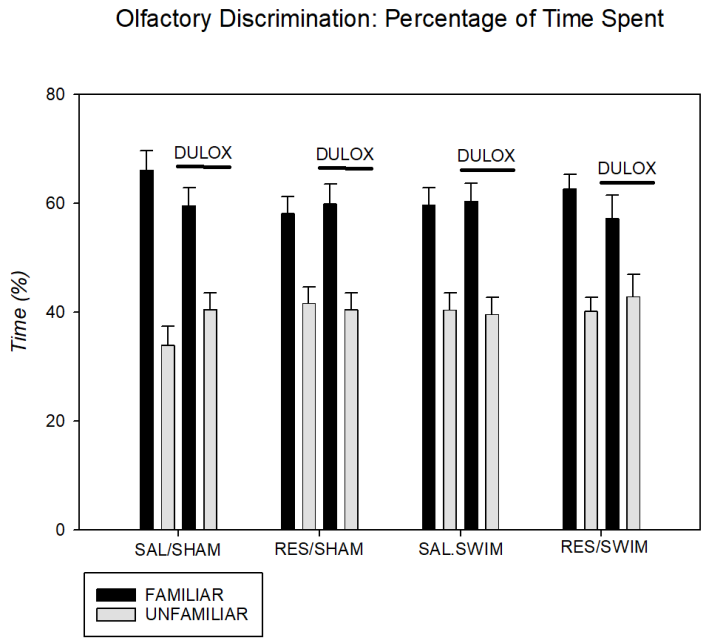


Figure 12. Percentage of time spent in the familiar and non-familiar compartments of the chamber during the olfactory discrimination task. No significant differences were identified.

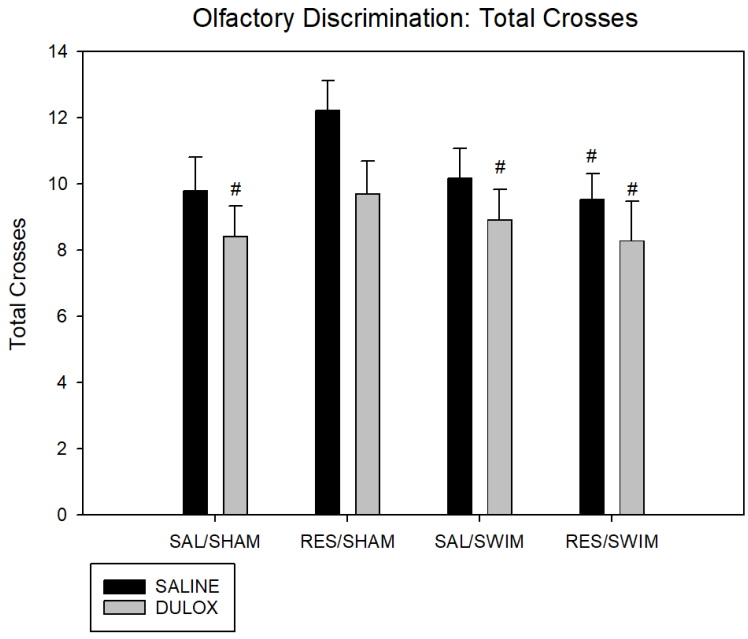


Figure 13. Total number of crosses during the olfactory discrimination task. No significant main effects were identified. # compared to RES/SHAM/SAL at $p < .05$

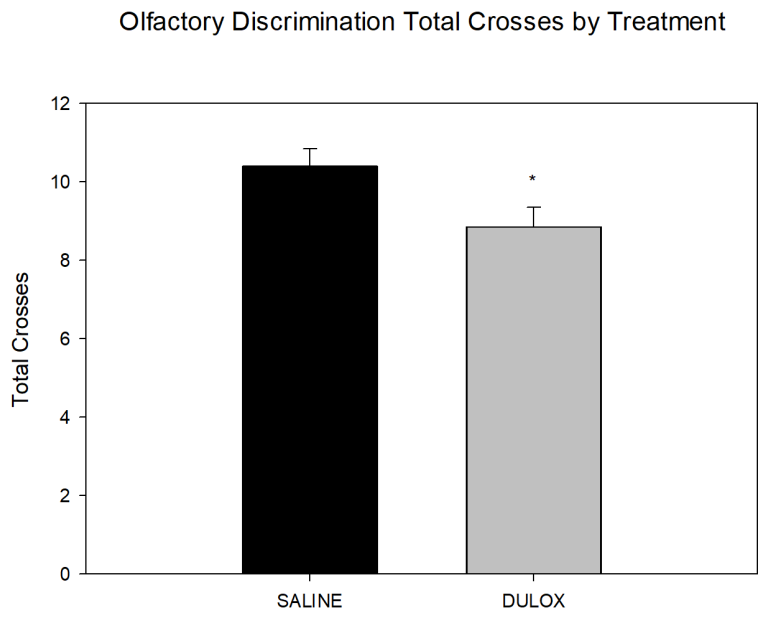


Figure 14. Total number of crosses during the olfactory discrimination task within treatment conditions. * Compared to SALINE at $p = .02$

3.8 Elevated Plus Maze

To evaluate the impact of pain and treatment condition on behaviors in the EPM, a MANOVA was used with pain and treatment condition as the independent variable, and number of entries into the open arms, number of entries into the closed arms, time spent in the open arms, and time spent in the closed arms as dependent variables. A significant between-subjects main effect was found for number of entries into the open arms, $F(7, 93) = 2.741$, $p = .012$, $\eta p2 = .171$, time spent in the closed arms of the chambers, $F(7,93) = 3.849$, $p = .001$, $\eta p2 = .225$, and time spent in the open arms of the chambers, $F(7, 93) = 2.303$, $p = .033$, $\eta p2 = .148$. There was no significant main effect found for number of entries into the closed arms of the chambers, $F(7, 93) = 1.187$, $p = .318$.

Post hoc analyses for entries into the open arms (Figure 15A) revealed that SAL/SHAM/SAL animals (*M* = 7.143, *SE* = .714) entered the open arms of the chamber significantly more than RES/SWIM/SAL (*M* = 4.647, *SE* = .648), SAL/SHAM/DULOX (*M* = 3.83, *SE* = .771), RES/SHAM/DULOX (*M* = 4.182, *SE* = .805), AND SAL/SWIM/DULOX (*M* = 4.833, *SE* = .771) animals. Additionally, RES/SHAM/SAL animals (*M* = 6.812, *SE* = .668) entered the open arms of the chamber significantly more than RES/SWIM/SAL, SAL/SHAM/DULOX, RES/SHAM/DULOX animals.

Post hoc analyses for time spent in the closed arms of the chamber (Figure 16B) revealed that SAL/SHAM/SAL (*M* = 171.429, *SE* = 10.338), RES/SHAM/SAL (*M* = 164.625, *SE* = 9.67), and SAL/SWIM/SAL (*M* = 171.00, *SE* = 11.166) animals spent significantly less time in the closed arms than RES/SWIM/SAL 212.529, *SE* = 9.382), SAL/SHAM/DULOX (*M* = 209.333, *SE* = 11.166), RES/SHAM/DULOX (207.455, *SE* = 11.663), AND RES/SWIM/DULOX (*M* = 215.286, *SE* = 14.62) animals.

Post hoc analyses of time spent in the open arms of the chamber (Figure 16A) revealed that animals in the SAL/SHAM/SAL condition (*M* = 77.429, *SE* = 9.772) spent significantly more time in the open arms of the chamber than animals in the RES/SWIM/SAL (*M* = 37.059, *SE* = 8.868), SAL/SHAM/DULOX (40.50, *SE* =10.55), RES/SHAM/DULOX (*M* = 46.818, *SE*= 11.024), and RES/SWIM/DULOX (*M* = 41.857, *SE* = 13.819) animals. Animals in the RES/SHAM/SAL condition spent significantly more time in the open arms compared to RES/SWIM/SAL and SAL/SHAM/DULOX animals.

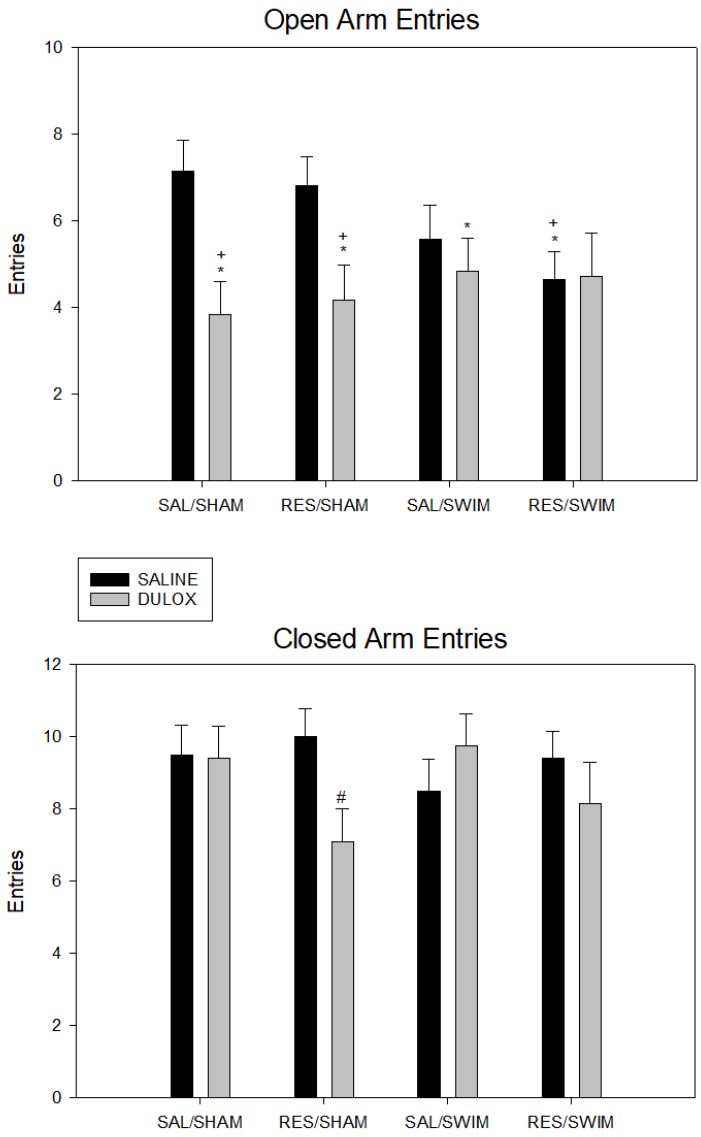


Figure 15. Entries into the open (A) and closed (B) arms of the EPM. (A): * compared to SAL/SHAM/SAL at $p < .05$; + compared to RES/SHAM/SAL at $p < .05$. (B): No significant main effects found. # compared to RES/SHAM/SAL and SAL/SWIM/DULOX at $p < .05$

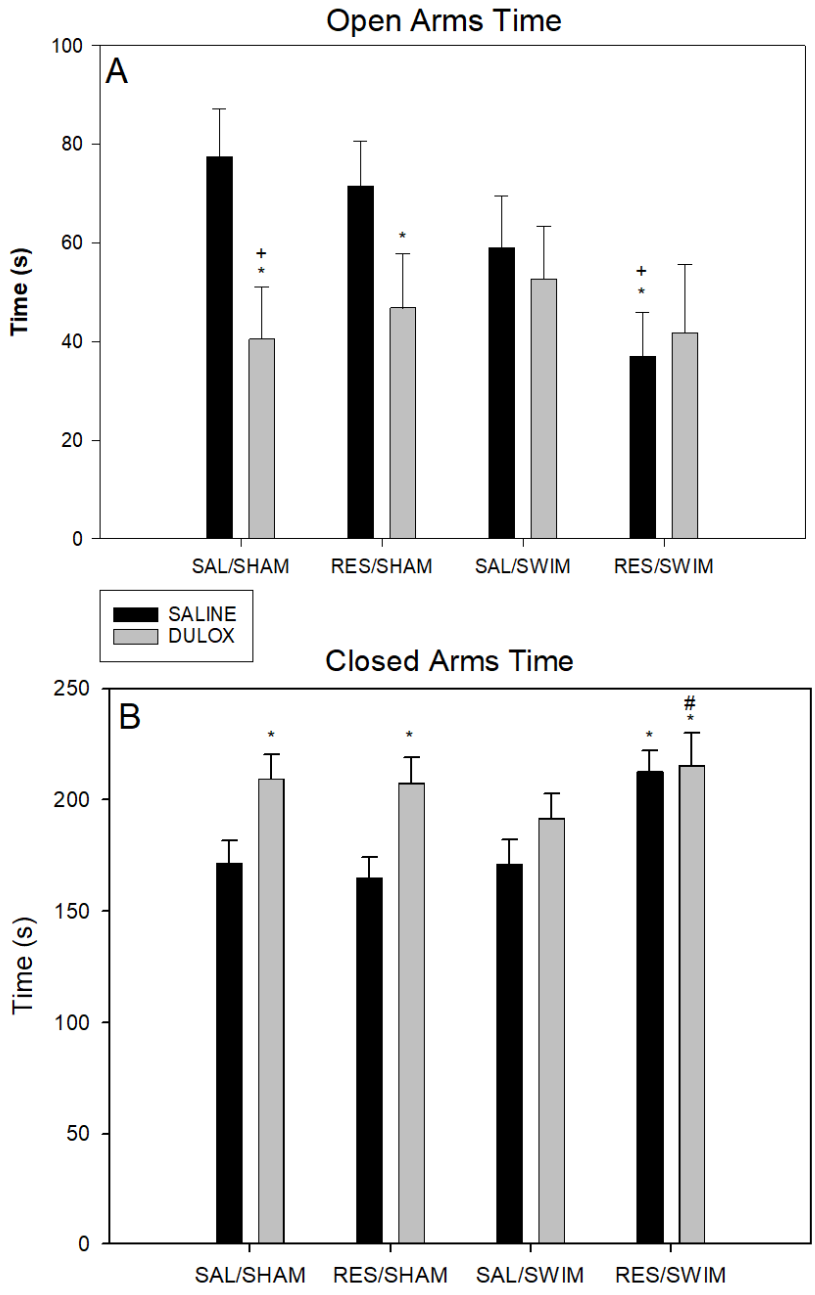


Figure 16. Time spent in the open (A) and closed (B) arms of the EPM. (A): * compared to SAL/SHAM/SAL at $p < .05$; + compared to RES/SHAM/SAL at $p < .05$. (B): * compared to SAL/SHAM/SAL, RES/SHAM/SAL, and SAL/SWIM/SAL at $p < .05$; # compared to RES/SHAM/SAL at $p < .05$

3.9 Splash Test

To evaluate potential differences in depression-like behavior among animals following treatment, a MANOVA was used to determine the effects of pain and treatment condition on total time spent grooming, number of grooming sessions, and latency to begin grooming. There were no significant main effects of pain and treatment condition on total grooming time, $F(7, 83) = 0.662$, $p = .704$ (Figure 17A), or number of grooming sessions, $F(7, 83) = 1.395$, $p = .218$ (Figure 17C). However, there was a trending main effect of pain and treatment condition on latency to begin grooming, $F(7, 83) = 1.910$, $p = .078$, $\eta p2 = .139$.

Specifically, within latency to begin grooming (Figure 17B), animals in the RES/SHAM/SAL condition ($M = 84.913$, $SE = 22.582$) began grooming significantly sooner than animals in the SAL/SWIM/SAL ($M = 177.818$, $SE = 24.55$), RES/SWIM/SAL ($M = 149.412$, $SE = 19.748$), and RES/SWIM/DULOX ($M = 164.143$, $SE = 30.775$) groups. Additionally, animals in the SAL/SWIM/SAL group took significantly longer to begin grooming than animals in the RES/SHAM/DULOX ($M = 103.00$, $SE = 27.141$) condition. While no differences were observed between SAL/SHAM/SAL animals ($M = 112.09$, $SE = 24.55$) or any other groups, SAL/SWIM/SAL animals displayed a trending increase in latency to begin grooming when compared to control and SAL/SHAM/DULOX ($M = 97.50$, $SE = 23.505$) animals, implicative of an increase in depression-like behavior that was not attenuated by duloxetine ($M = 115.636$, $SE = 24.55$).

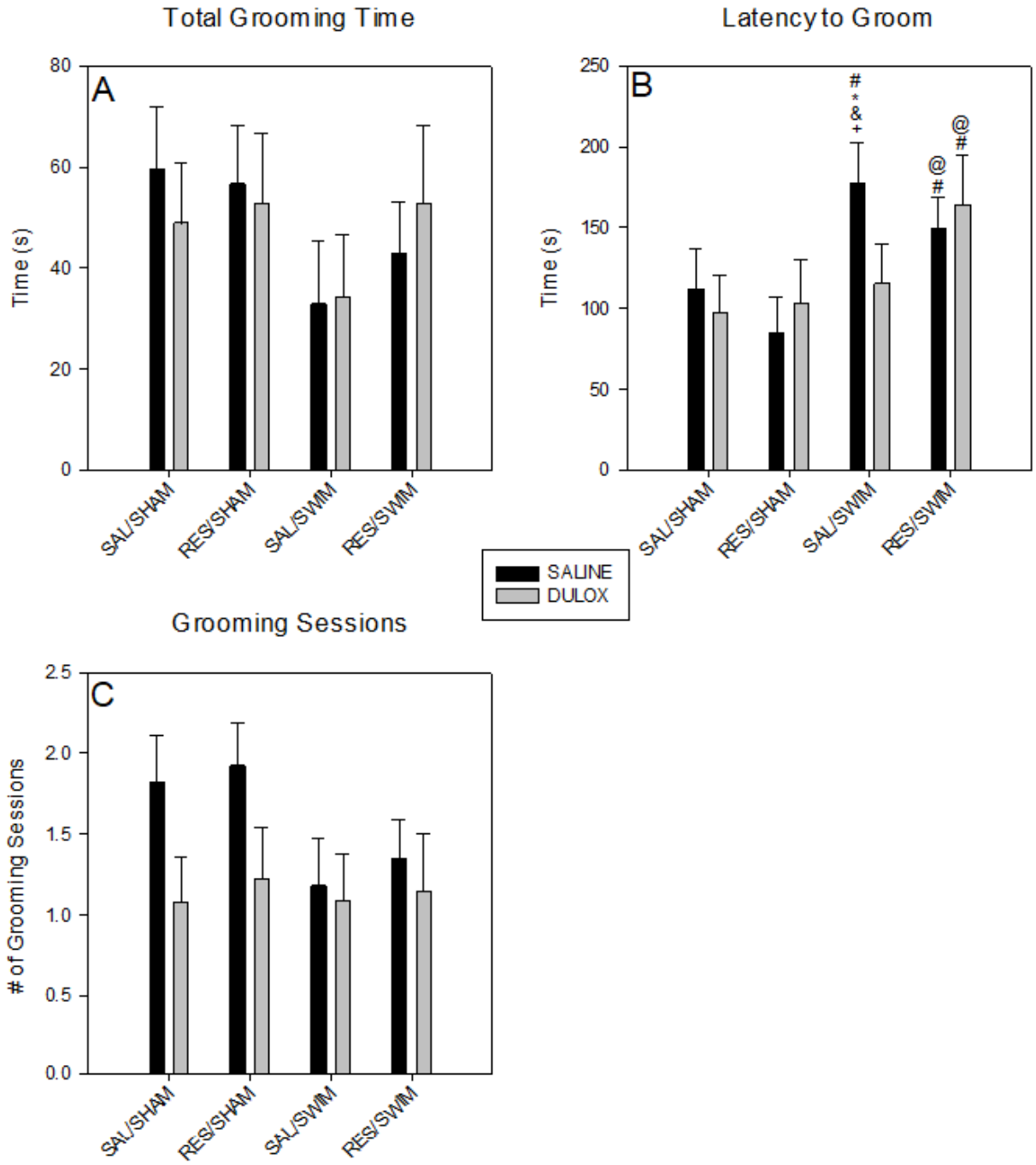


Figure 17. Grooming behavior during the splash test. (A) Total grooming time as a function of pain and treatment condition. No significant main effects found. (B) Latency to begin grooming as a function of pain and treatment condition. (C) Total number of grooming sessions as a

function of pain and treatment condition. # compared to RES/SHAMM/SAL at $p < .05$; + compared to RES/SHAM/DULOX at $p < .05$, & compared to RES/SHAM/SAL and SAL/SHAM/DULOX at $p < .05$, * trending effect compared to SAL/SHAM/SAL and SAL/SWIM/DULOX, @ trending effect compared to SAL/SHAM/DULOX

3.10 Object-Location Memory Task

3.10.1 *Training Phase*

To determine if there were any statistical differences between the amount of time animals explored objects during the training phase of the Object-Location Memory task, an ANOVA was used with both pain condition and object-location condition as the independent variables (Figure 18). There were no significant differences in exploration or potential acquisition behavior as a result of pain condition, $F(3, 93) = .678$, $p = .568$. However, there was a significant main effect of object-location condition (two vs. four objects) on the amount of time spent exploring objects during the training phase, $F(1, 93) = 15.169$, $p < .001$, $\eta p2 = .14$. As would be expected, animals in the two-object condition spent significantly less time exploring objects upon introduction ($M = 41.686$, $SE = 9.609$) compared to those exposed to four objects ($M = 86.038$, $SE = 6.112$). However, this is likely due to the fact that the animals in the two-object condition had fewer objects to explore than those in the four-object condition, and a repeated measures approach would be likely to nullify this significant difference.

3.10.2 *Test Phase*

To determine if there were any statistical differences between the amount of time animals spent exploring objects during the test phase of the Object-Location Memory task, an ANOVA

was used with both pain/treatment condition and object-location condition (two vs. four objects) as independent variables (Figure 19). There were no main effects of pain and treatment condition, $F(7, 88) = 1{,}208$, $p = .307$, or object-location condition, $F(1, 88) = .073$, $p = .787$, on the total time spent exploring objects during the test phase. There was also no significant interaction effect identified, $F(4, 88) = .431$, $p = .786$. The lack of main effect of object-location condition on total time spent exploring the objects during the test phase reinforces the previous assessment that the increased exploration of animals exposed to the 4-object paradigm in the training phase was likely a result of a mere increase in number of objects available to explore.

To determine if there were any significant differences in object-location discrimination index on test day between pain conditions exposed to both the two and four object conditions (Figure 20), an ANOVA was used. There were no significant main effects found for pain and treatment condition, $F(7, 86) = 0.773$, $p = .611$, or object-location condition, $F(1, 86) = 2.641$, $p = .108$. There were also no significant interactions identified, $F(4, 86) = .963$, $p = .432$. Overall, there were no differences found in discrimination index as a result of pain and treatment condition, or number of objects animals were exposed to.

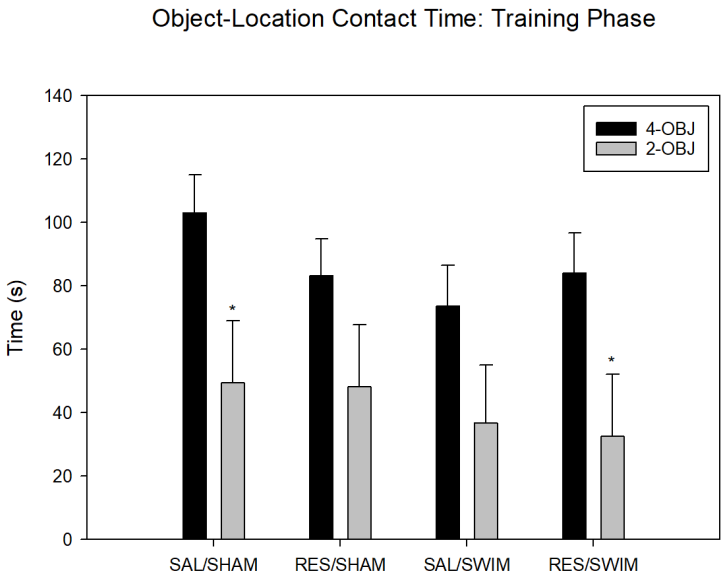


Figure 18. Mean object contact time during the training phase of the object-location memory task as a function of object exposure condition. * Compared to 4-OBJ counterpart within the same pain condition at $p < .05$

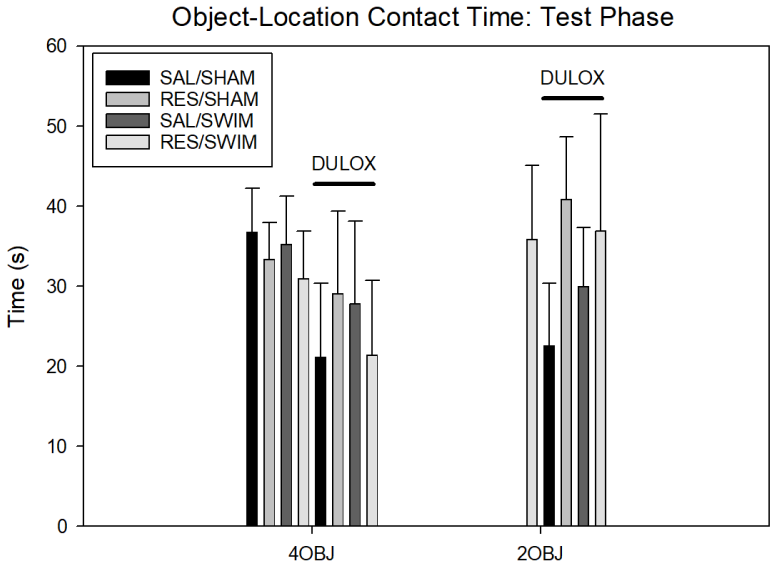


Figure 19. Mean object contact time during the test phase of the object-location memory task as a function of object exposure condition. No significant effects identified.

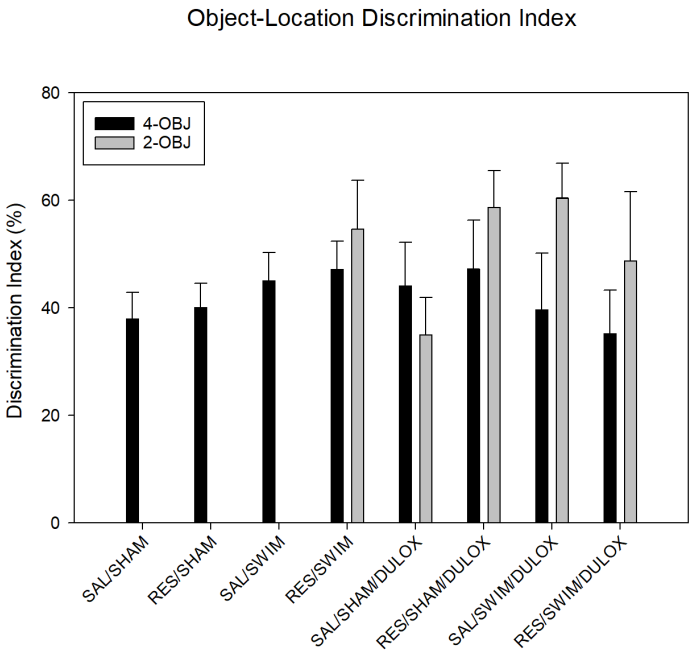


Figure 20. Discrimination index during the test phase of the object-location memory task as a function of object exposure condition. No significant effects identified.

3.10.3 *Secondary Analysis for Object-Location Condition on Subsequent Behavioral Tests*

In order to determine if there were any effects of the different conditions in the object-location task (two vs. four objects) on subsequent behavioral paradigms on Test Day, secondary analyses were conducted with object-location condition as an additional independent variable in each of the previous primary analyses for EPM, Olfactory Discrimination, and Splash Test.

3.10.3.1 Object-Location and Elevated Plus Maze

There were no main effects of object-location condition (two vs. four objects) on number of entries into the open arms, $F(1, 88) = .530$, $p = .469$, or the closed arms, $F(1, 88) = .229$, $p = .633$, of the EPM. There were also no main effects found on time spent in the open, $F(1, 88) = .380$, $p = .539$, or the closed arms of the chamber, $F(1, 88) = .566$, $p = .454$.

Further, there were no significant interaction effects between pain/treatment condition and object-location condition found for entries into the open arms, $F(4, 88) = 1.289$, $p = .281$, or the closed arms, $F(4, 88) = .774$, $p = .545$, nor time spent in the open arms, $F(4, 88) = .657$, $p = .623$, or closed arms of the chamber, $F(4, 88) = 1.388$, $p = .245$.

3.10.3.2 Object-Location and Olfactory Discrimination

There were no main effects of object-location condition (two vs. four objects) on the percentage of time animals spent in the familiar, $F(1, 80) = .031$, $p = .860$, or the unfamiliar, $F(1, 80) = .185$, $p = .668$, compartments of the chamber. There was also no interaction effect of pain/treatment condition and object-location condition on percentage of time spent in the familiar, $F(4, 80) = 1.061$, $p = .381$, or the unfamiliar, $F(4, 80) = .986$, $p = .420$, compartments of the chamber.

There were no main effects of object-location condition on total number of crosses in the Olfactory Discrimination chamber, $F(1, 80) = 2.86$, $p = .095$. Additionally, there was no interaction effect between pain/treatment condition and object-location condition on total number of crosses in the chamber, $F(4, 80) = 1.54$, $p = .198$.

3.10.3.3 Object-Location and Splash Test

There was no main effect of object-location condition (two vs. four objects) on the amount of time animals spent grooming, $F(1, 78) = .075$, $p = .785$, number of grooming sessions, $F(1, 78) = .209$, $p = .649$, or latency to begin grooming, $F(1, 78) = 3.429$, $p = .068$, $\eta p2 = .042$. It is likely that the trending effect observed in latency to begin grooming was a result of differences in sample sizes, as animals in the 2-object condition ($M = 104.327$, $SE = 17.588$) trended towards

a shorter latency to begin grooming compared to 4-object condition animals (M = 140.214, SE = 11.71), with N = 27 for the 2-object condition and N = 64 for the 4-object condition.

Additionally, no interaction effects between pain/treatment condition and object-location condition were identified for grooming time, $F(4, 78) = .620$, $p = .649$, number of grooming sessions, $F(4, 78) = .435$, $p = .783$, or latency to begin grooming, $F(4, 78) = .494$, $p = .740$.

4. Discussion

The fibromyalgia pain experience is understood to be a multidimensional pain presentation, with representations characterizing this disorder spanning across the affective, cognitive, and sensory dimensions of pain (Goldenberg, 2008; Ambrose, Gracely, & Glass, 2012; Aguglia et al., 2011; Henao-Perez et al., 2022; Berger et al., 2007; Walitt et al., 2015). The complexity of this type of pain presentation has contributed to challenges in etiological understanding, diagnosis and management, both on a clinical and preclinical level. Generally, preclinical pain research offers significant advantages for physiological and behavioral advancements and management of many disorders. However, with the complex multifaceted presentation of fibromyalgia, it has been challenging to determine the reliability of the existing preclinical models of fibromyalgia to fully replicate this clinical experience (Argenbright et al., *submitted*; Brum et al., 2022). With these challenges, there were three primary goals of the current study: (1) to elucidate the multidimensional strengths and weaknesses of two animal models of fibromyalgia pain — the reserpine model and the subchronic swim stress model, (2) to investigate the development of a new approach for modeling FM-like pain preclinically by combining these two pre-existing models of FM-like pain in animals, and (3) to predictively validate an FDA-approved drug for the treatment of fibromyalgia, duloxetine, within each of these models individually and in their combination.

One of the previously cited weaknesses associated with the use of the reserpine model of fibromyalgia includes discrepant symptomatology in regard to body weight alterations among animals (Brum et al., 2022). The current study showed that while there were some between group and time-related interactions highlighted for fluctuations in bodyweight, animals induced into all pain models maintained a healthy body condition and did not display any downward trends in weight that might imply an incorrect reserpine dosage or adverse events (Brum et al., 2022). Additionally, there were no meaningful differences implicative of adverse body conditions associated with the stress-based subchronic swim model, or the combination of this model with reserpine injection. In analyses of immobility behavior during the induction of the subchronic swim condition, the addition of a reserpine injection did not exacerbate helplessness behaviors. However, all animals subjected to forced swim test procedures showed an increase in helplessness behaviors, implicative that the swim exposure was effective in inducing stress-related effects.

Exploration within the current study into the mechanical and thermal presentations of both the subchronic swim stress and reserpine models yielded mixed results. As was hypothesized, animals randomized to the reserpine condition and the forced swim condition exhibited decreased MPWTs following induction into the respective pain model (Day 8). Additionally, reserpine animals and subchronic swim animals subsequently treated with a vehicle control treatment maintained their reduced mechanical thresholds compared to control animals (Day 9). These findings of decreased mechanical thresholds are consistent with previous studies investigating both the reserpine (Arora et al., 2011; Hubner de Souza et al., 2014; Tamano et al., 2016; Siemian et al., 2019; Fusco et al., 2019; Dagnino et al., 2020; Xu et al., 2013; Klein et al., 2014; Blasco-Serra et al., 2015; Klein et al., 2016; Oliveira et al., 2016; Nagakura et al., 2018;

Dagnino et al., 2019; Brusco et al., 2019; Nagakura et al., 2019; Yao et al., 2020; Fischer et al., 2020; Brum et al., 2020; Kang et al., 2020; Salat & Furgala-Wojas, 2021; Mendes et al., 2021; Ferrarini et al., 2021; Martins et al., 2022; Álvarez-Pérez et al., 2022; Zhao et al., 2022; Kaur et al., 2019; Singh et al., 2020; Kaur et al., 2020; Singh et al., 2021; Hernandez-Leon et al., 2019) and the subchronic swim stress models (Li et al., 2017; Zhang et al., 2020; Xue et al., 2020). When evaluating for differences in TWLs, animals randomized to the reserpine condition and the subchronic swim condition exhibited an expected reduction in thresholds compared to controls following induction into the respective pain model (Day 8), consistent with previous findings (Hubner de Souza et al., 2014; Fusco et al., 2019; Miyahara et al. 2021; Klein et al., 2014; Klein et al., 2016; Oliveira et al., 2016; Sousa et al., 2018; Dagnino et al., 2019; Yao et al., 2020; Fischer et al., 2020; Mendes et al., 2021; Elkholy et al., 2021; Álvarez-Pérez et al., 2022; Zhao et al., 2022; Zhang et al., 2020; Xue et al., 2020; Nazeri et al., 2014; Nazeri et al., 2016). However, contrary to our hypotheses, these reductions in thermal latencies were not maintained among reserpine animals or subchronic swim stress animals treated with a saline control treatment (Day 9). While these results were not expected, this may be a result of a limited time course of the model, which is supported by the majority of studies identifying significant reductions having had evaluated thermal thresholds 24 hours to 3 days after induction (Hubner de Souza et al., 2014; Miyahara et al., 2021; Klein et al., 2014; Klein et al., 2016; Sousa et al., 2018; Dagnino et al., 2019; Yao et al., 2020; Fischer et al. 2020; Zhang et al., 2020; Nazeri et al., 2014; Nazeri et al., 2016). However, reduced latencies for the reserpine model have been recorded up to 21-days post-injection (Fusco et al., 2019; Yao et al. 2020).

Importantly, animals randomized to the reserpine and subchronic swim combination condition (RES/SWIM) exhibited the most significantly reduced thresholds compared to control

animals (Day 8). This combination of swim stress and biogenic amine depletion exacerbated mechanical hyperalgesia among animals beyond that which was recorded for each of the conditions individually. These reduced thresholds were maintained for RES/SWIM animals treated with a saline control treatment (Day 9), although these thresholds were no longer statistically different from animals randomized to the sole swim stress condition. When administered duloxetine, mechanical thresholds among animals in the combination reserpine and swim stress condition increased significantly, such that there were no differences observed between this group and vehicle treated control or duloxetine treated control animals. Overall, these results imply that the combination of reserpine and swim stress exacerbated mechanical hyperalgesia beyond that of either of these conditions solely, offering effective back-translatability. Additionally, these reduced thresholds were significantly attenuated by the administration of duloxetine, reflective of predictive validity. However, these results were not as consistent in evaluations of thermal hyperalgesia. At pre-treatment, animals in the reserpine and subchronic swim combination model showed reduced latencies compared to controls, but these latencies were not different from baseline measures. These results pose a challenge for translatability of the combination condition, given that the combination of alterations in *both* mechanical and thermal hyperalgesia are associated with fibromyalgia clinical pain presentations of pain intensity, spatial summation, wind-up, and number of pain areas (Staud et al., 2012).

While many studies have been conducted investigating some aspects of affect or cognition in animal behavior, little work has been done evaluating these models from a multidimensional pain approach (Souza et al., 2013; Kaur et al., 2019; Singh et al., 2020; Kaur et al., 2020; Singh et al., 2021; Nazeri et al., 2014; Nazeri et al., 2016). Due to differences in experimental timelines, affective and cognitive paradigms employed, or simply confounds in reliability or validity, there

has been little consistency across studies in determining the ability of these models to replicate the FM experience from an affective and cognitive approach. Controlled comparative measures of affect within the current study included anxiety-like behavior (EPM; Thigmotaxis) and depression-like behavior (Splash test), while measures of cognition included learning and memory (Object-Location Memory; Olfactory Discrimination), as well as attention (Object-Location Memory).

Within measures of affect, there were no meaningful differences found in locomotive or thigmotaxic behavior between groups, either pre- or post-treatment. However, it is likely that these effects are a result of a test-retest effect, wherein animals became more familiar with the chambers as a result of repeated exposures — an effect that we have previously observed in analyses of open field behaviors (Argenbright et al., 2021). This is supported by the main effect of time observed in measures of distance and velocity, where animals generally traveled more and faster during habituation to the chamber than during subsequent exposures. Regardless, these effects were not expected particularly within the independent reserpine model, given previous studies reporting significant changes in thigmotaxic behaviors among animals (Fischer et al., 2020; Brum et al., 2020; Brusco et al., 2019). However, the most meaningful outcome observed was in total distance traveled after the administration of duloxetine, where all animals, regardless of pain condition, traveled significantly less compared to animals treated with a saline control. While these results do not imply the presence of anxiety-like behaviors as a result of the induction of the individual or combination pain models, the administration of duloxetine appears to have produced a significant decrease in overall locomotion, as was observed on test day. However, it has been reported clinically that duloxetine as a treatment for Major Depressive Disorder (MDD) is associated with treatment-emergent adverse events such as fatigue and

somnolence (Hudson et al., 2005), and little is known about the carry-over of these events in a fibromyalgia pain state as a result of duloxetine administration (Scholz, Hammonds & Boomershine, 2009).

Within further assessment of potential anxiety-like behavior, results from the EPM test found that the time spent in the closed arms of the chamber by animals induced into the reserpine model or the subchronic swim model were no different from controls. This was also the case with time spent in the open arms of the chamber, where subchronic swim animals and reserpine animals behaved no differently from controls. While some studies have reported differences in anxiety-like behavior within this paradigm as a result of these models (Wu et al., 2017; Kaur et al., 2019; Sachdeva et al., 2011; Nazeri et al., 2018; Xue et al., 2020), the current study supports the majority of literature reporting a failure to elucidate potential anxiety-like behavior in an EPM/EZM paradigm within both the reserpine (Wu et al., 2015; Dagnino et al., 2019; Martins et al., 2022; Souza et al., 2013) and subchronic swim model (Dhir & Kulkarni, 2008; Trivedi & Sharma, 2011; Li et al., 2017; Bagues et al., 2022). Interestingly, when both individual pain model and control animals were treated with duloxetine, these groups spent significantly less time in the open arms of the chamber and significantly more time in the closed arms of the chamber compared to their control-treated counterparts. We believe this is likely a result of the reduced locomotion that was also observed in measures of distance traveled and thigmotaxis, especially due to the fact that, generally, groups of animals administered duloxetine served as the driving force behind the main effect for differences in open arm entries. More succinctly, animals that were treated with duloxetine entered the open arms of the chamber less than control treated animals, potentially as a result of the reduced locomotion that was observed in open field measures. However, animals subjected to the combination of both subchronic swim and

reserpine injection spent significantly more time in the closed arms, and significantly less time in the open arms of the chamber compared to the individual model groups as well as controls, indicating an expected increase in anxiety-like behavior. When these combination model animals were treated with duloxetine, animals did not yield any changes in chamber crossings or time spent in either the open or closed arms of the chamber, implying that either duloxetine was not effective for alleviating increased anxiety-like behavior in this combination model, or that the impact of duloxetine on anxiety-like behaviors was thwarted by fatigue effects.

In additional measures of depression-like behavior, a trending main effect was observed as a result of pain and treatment condition. Subchronic swim animals treated with a saline control treatment had a near-significantly increased latency to begin grooming than reserpine animals, implicative of increased depression-like behaviors as a result of the model, which was not significantly improved by duloxetine treatment. The latencies observed among animals in the independent reserpine model being similar to control animals was not a surprising outcome in this study, given the mixed results previously reported for this model in the Splash Test (Martins et al., 2022; Roversi et al., 2019; Oliveira et al., 2016). However, animals induced into the novel reserpine and subchronic swim combination model also displayed an increased latency to begin grooming compared to reserpine animals, which was not significantly alleviated by the administration of duloxetine. While there were differences between models in their latency to begin grooming, no statistically significant differences were observed as a result of pain or treatment when compared to control animals— only when compared to animals in the independent reserpine model. Regardless, animals induced into the subchronic swim model showed a trending increase in depression-like behaviors compared to controls that which remained unalleviated by duloxetine administration. These results are novel given that, to the

author's knowledge, the effects of the subchronic swim model have never been investigated within the Splash Test paradigm before (Argenbright et al., *submitted*). Further evaluation of depression-like behavior in the Splash Test revealed no significant differences between groups in total grooming time or number of grooming sessions, regardless of pain or treatment condition.

In evaluations of cognitive function within the Olfactory Discrimination paradigm, there were no significant differences found in the percentage of time that animals spent in the familiar or the unfamiliar compartments of the chamber. While these results were unexpected, this implies that the individual reserpine model, subchronic swim stress model, or their combination, were not able to produce changes in cognitive functioning that have been previously reported (Souza et al., 2013). However, when evaluating the total number of crosses between the compartments of the chambers, it was found that animals treated with duloxetine crossed between the compartments of the chamber significantly less than animals administered a saline control treatment. This unexpected main effect of treatment supports the previously discussed results that the administration of duloxetine was associated with reductions in locomotive activity among animals.

Further evaluation of spatial memory and attention within the Object-Location Memory task revealed no differences between groups as a result of pain or treatment condition. Animals displayed no differences in discrimination index, or time spent exploring the objects on test day, implying that these pain models, both individually and in combination, did not disrupt cognitive function in a manner that was able to be detected within this paradigm. During the training phase of this paradigm, the amount of time that animals spent exploring the objects was dependent on the number of objects animals were introduced to (either two or four). However, we believe this is likely a result of the mere fact that there were more objects to explore in the four-object

condition, and therefore, animals utilized more time to adequately explore the objects. This is reinforced conceptually by the object exploration time on test day, where animals displayed no differences in attending to the objects, regardless of the presentation of two or four objects, as the objects themselves were no longer novel. To the author's knowledge, this is the first investigation of the effect of these models in an Object-Location Memory task. However, these results were not expected, given that previous studies have reported changes in memory and learning associated with the reserpine model (Kaur et al., 2019; Singh et al., 2020; Kaur et al., 2020; Singh et al., 2021) and the subchronic swim model (Nazeri et al., 2014; Nazeri et al., 2016).

With the introduction of two conditions into the Object-Location Memory task, wherein the test phase was conducted first among several behavioral paradigms on Test Day, we conducted secondary analyses to ensure that the number of objects animals were exposed to did not influence behavioral outcomes on subsequent behavioral tests. There were no meaningful effects of object-location condition on behaviors within the EPM, Olfactory Discrimination, or Splash Test. While there was a trending effect of object-location condition on latency to begin grooming in the Splash Test, we believe this to be a result of incongruent sample sizes related to the exploratory analyses of object numbers and find it likely that a more evenly distributed sample size among future analyses would absolve any undue influence.

Overall, the independent reserpine model proved efficacious in reducing mechanical thresholds compared to control animals. While TWLs reduced over time among animals randomized to the independent reserpine model, these thresholds were not significantly reduced compared to controls. However, this model did not alter overall locomotive function over time, which has been previously reported (Fernandes et al., 2008). In measures of anxiety-like

behavior using the EPM, animals in the independent reserpine model did not behave differently from controls in regard to time spent in the open arms or the closed arms of the chamber, and entrances into the open arms of the chamber. However, these animals did show a reduced number of entries into the closed arms of the chamber compared to controls, which could be implicative of less anxiety-like behavior. However, this lack of anxiogenic response was reinforced by the lack of thigmotaxic behavior observed among these animals, where data revealed these animals traveled in the center portion of the open field at a rate that was similar to controls. In measures of depression-like behavior, the independent reserpine model failed to produce changes in behaviors that were different from controls in total grooming time, latency to begin grooming, or number of grooming sessions. However, cognitive assessments showed that this model failed to produce any changes in attention, spatial memory, or olfactory discrimination compared to controls.

The independent subchronic swim model proved efficacious in reducing mechanical thresholds and TWLs compared to control animals. These animals also did not exhibit any significant changes in locomotion. While this model produced increases in mechanical and thermal sensitivity, data showed a failure in replicating anxiety-like behavior in both the EPM and in thigmotaxic behavior, where animals behaved similarly to controls. This same effect was observed in measures of depression-like behavior, where animals did not display any differences in grooming time, or number of grooming sessions compared to controls. However, there was a trending effect of this model in increasing latency to begin grooming, implying a potential depression-like component. In measures of cognition, no changes were produced in attention, spatial memory, or olfactory discrimination compared to controls.

The novel combination of reserpine injection and subchronic swim stress was effective in reducing mechanical thresholds beyond that which was observed in the models independently. Additionally, TWLs observed among these animals were significantly, yet time dependently, reduced compared to control animals. However, similarly to these models independently, their combination did not alter overall locomotion among animals. Contrary to as was observed in the models independently, animals randomized to a combination of reserpine and subchronic swim showed increases in anxiety-like behaviors within the EPM, as shown by an increased amount of time spent in the closed arms of the chamber and a decreased amount of time spent in the open arms of the chamber. However, these anxiety-like behaviors were not reflected in thigmotaxic behavior, as these animals did not behave differently from either controls or the models independently. In measures of depression-like behavior, combination model animals did not display any differences in total grooming time or number of grooming sessions when compared to controls. However, there was a trending effect observed in latency to begin grooming, wherein animals randomized to the reserpine and subchronic swim combination displayed an increased latency to begin grooming, implicative of more depression-like behavior. However, in measures of cognition, this novel combination model failed to produce any changes in attention, spatial memory, or olfactory discrimination compared to either the models independently or controls.

While there have been studies investigating the therapeutic effects of duloxetine in the reserpine model of FM-like pain (Tamano et al., 2016; Shibrya et al., 2017; Blasco-Serra et al., 2017; Nagakura et al., 2019), only one of these has identified efficacy for the pharmacologic in the sensory and affective realms of pain (Nagakura et al., 2019). To the authors' knowledge, there have been no investigations into the therapeutic role of duloxetine within the cognitive dimension of pain associated with the reserpine model, and no studies into the potential

therapeutic benefits of duloxetine for subchronic swim stress associated pain. Therefore, this study provided novel insight into the multidimensional intervention potential of duloxetine for preclinical FM. Within the current study, in measures of mechanical thresholds, duloxetine administration was most effective in reducing pain behaviors in animals induced into the independent reserpine model and the reserpine and subchronic swim combination model. However, the effects of duloxetine for reduced thermal latencies were difficult to conclude, given the time-dependent nature of the observed reductions, where significant differences in thresholds were alleviated prior to administration on test day among control-treated animals. In measures of locomotion, duloxetine administration significantly reduced traveling behaviors among all animals, which was unexpected and has not been previously reported, to the author's knowledge. However, these changes in locomotion may mimic nuances of clinical presentations of fibromyalgia treated with duloxetine (Scholz et al., 2009). In measures of anxiety-like behavior, the effects of duloxetine were not able to be assessed for thigmotaxic behavior, given the failure to detect changes in thigmotaxis across all models investigated. In anxiety-like measures of EPM, the administration of duloxetine significantly altered behavior among controls and the independent subchronic and reserpine models to be reflective of more anxiety-like behavior, which was not exhibited by animals treated with a saline control. Further, duloxetine treatment failed to alleviate the anxiety-like behaviors observed among animals within the reserpine and subchronic swim combination model, raising concerns for the predictive validity of this novel combination. In measures of depression-like behavior, duloxetine did not significantly alter total grooming time or number of grooming sessions for controls, or any models investigated. However, duloxetine treatment was effective for reducing the trending increase in latency to begin grooming among animals in the independent subchronic swim model, providing

strength for the model's predictive validity for depression-like behavior. Unfortunately, this same alleviation was not observed for animals in the novel combination model. In further measures of cognition, the effects of duloxetine for the treatment of cognitive deficits were not able to be evaluated due to the failure to produce changes in attention, memory, or discrimination as a result of the models independently or in combination.

This study sought to offer controlled comparative insight into each of these models independently, across the same experimental timeline and behavioral paradigms. The current study contributes to the mixed evidence for both independent models in producing anxiety-like behavior (Dhir & Kulkarni, 2008; Sachdeva et al., 2010; Trivedi & Sharma, 2011; Saha, 2011; Li et al., 2017; Xue et al., 2020; Bagues et al., 2022; Wu et al., 2015; Wu et al., 2017; Dagnino et al., 2017; Martins et al., 2022; Kaur et al., 2019), as well as depression-like behavior (Oliveira et al., 2016; Roversi et al., 2019; Martins et al., 2022; Li et al., 2017; Chen et al., 2018; Zhang et al., 2020; Xue et al., 2020). Additionally, the results from the current study contrast previous findings of cognitive deficits associated with these models independently (Nazeri et al., 2014; Nazeri et al., 2016; Souza et al., 2013; Kaur et al., 2019; Singh et al., 2020; Kaur et al., 2020; Singh et al., 2021). Due to this collective body of evidence, future research should focus on parsing apart the contexts within which these models independently replicate the multidimensional fibromyalgia pain experience. However, this current study offered controlled comparative analyses between these models independently and revealed the reserpine model's failure to produce anxiety- or depression-like behaviors, alongside the subchronic swim stress model's failure to produce anxiety-like behaviors while offering potential trending effects of depression-like behavior. Further, the combination of these models showed advantages in replicating the mechanical, and potentially thermal, experiences in fibromyalgia, alongside

significant anxiety-like behavior and trending effects towards depression-like behavior, which may prove to be more reliable in evaluating potential management profiles for fibromyalgia. However, all the models investigated failed to produce any cognitive changes as are often reported in clinical fibromyalgia presentations. Future preclinical research should seek to evaluate the validity of cognitive deficits in these models, given that the cognitive deficits associated with fibromyalgia are well reported, and are believed to not be mediated by reported mood disturbances (Kravitz & Katz, 2015; Sarzi-Puttini et al., 2020; Bennett et al., 2007; Mease et al., 2009). Additionally, future research evaluating the specific biological underpinnings that contribute to the sensory presentations and affective presentations associated with the combination of reserpine and subchronic swim may aid in future work at reliably replicating the fibromyalgia pain experience within the realms of sensation, affect, and cognition.

Although we may not have a clear etiological understanding of FM as a disorder, the development of a preclinical FM model that has been validated across all dimensions of clinical representations and diagnostic criteria could prove extremely beneficial in developing a robust treatment methodology for the disorder. While FM is a disorder characterized by many challenges to patients, clinicians, and researchers, having a preclinical representation of FM that is reliable in its' affective, cognitive, and sensory replication of the disorder can aid in the current challenge of developing a robust, long-term pain management strategy.

5. Conclusion

This study sought to offer a controlled comparative insight of two primary models of fibromyalgia-like pain, the reserpine model and the subchronic swim stress model, in the wake of contrasting evidence for these models in reproducing the multidimensional pain experience. The reserpine model was effective in producing mechanical hyperalgesia, and potentially time-

dependent thermal hyperalgesia, but ineffective in replicating anxiety- and depression-like behavior. The subchronic swim stress model was effective in producing mechanical hyperalgesia, and time-dependent thermal sensitivity, as well as effects of depression-like behavior, but no changes in anxiety-like behavior. The combination of these models produced mechanical sensitivity, and potentially time-dependent thermal sensitivity, alongside anxiety-like behaviors and trending depression-like behaviors. However, all models failed to produce any changes in cognitive function. The administration of duloxetine selectively alleviated effects within mechanical sensitivity and depression-like behaviors but may have offered adverse effects in measures of anxiety-like behavior and overall locomotion.

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