Pain-Related Behavior and Potential Confounds in the Study of Laboratory Animals

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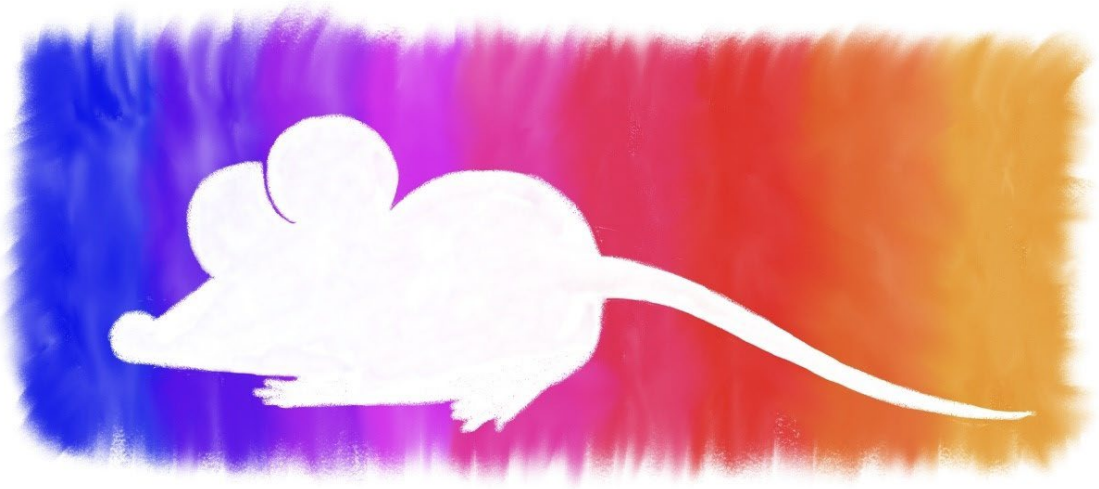


ABSTRACT

Just over 20% of American adults suffer from chronic pain, making chronic pain one of the most significant health concerns affecting individuals in the United States. For many chronic pain disorders, the prevalence is higher amongst women.

In order to elucidate the actions underlying the development and treatment of chronic pain, as well as associated behaviors, animal models are necessary. However, as we have not produced a novel treatment for chronic pain in some time, many researchers are calling for better behavioral measures, citing a lack of translational validity in our measures as the reason for this slow progress. Efforts to design novel behavioral assays increased, but this movement has not yet had its intended impact, and whether it ever will is questionable. This is because other major issues exist in the field that are being ignored: insufficient understanding of the patient population and individualized medicine, the omittance of negative data that would inform other researchers of what *not* to do, a lack of personal accountability amongst researchers, and an inappropriate perception of the goals of animal research are some of these issues.

It is not uncommon for chronic pain to be accompanied by multisensory hypersensitivity—an increased sensitivity to non-somatic sensory stimuli that exacerbate pain despite the fact that these stimuli are non-noxious. The most

common sensory hypersensitivity is to light—photosensitivity—which can result in photoavoidant behavior that increases the level of disability in patients experiencing it. The high prevalence and increased level of disability due to light exposure prompt many questions regarding the neurocircuitry linking photic input and pain exacerbation, as well as the associated behavior.

To examine this, I determined if naïve adult female Sprague Dawleys that were handled, habituated, and trained in one of four different Environmental Light levels (meant to mimic light exposure that *is not* part of an experiment) differed in mechanical sensitivity prior to exposure to Experimental Light (meant to mimic light exposure that *is* part of an experiment). I found that animals in the 2000 lux group, substantially brighter than the light level in their home cage, displayed decreased mechanical sensitivity as compared to the other three Environmental Light groups. In addition, I found that exposure to Experimental Light that was less bright than their Environmental Light group (100 lux vs. 2000 lux, respectively) reduced their mechanical thresholds back to the level of the other three Environmental Light groups. Thus, exposure to light, whether as part of a study or not, has the capacity to influence pain-related behavior in female rats.

Because light has the capacity to influence pain-related behavior prior to the experiment beginning, light is a confound in these studies. However, this is certainly not the only confound that researchers studying pain-related behavior will encounter. Confounds can arise while in transit to the laboratory, such as

weather-related temperature extremes, as well as while being housed, such as wet bedding, cage movement/vibration, and novel scents. Researchers must be cognizant of these issues while studying these behaviors in laboratory animals.

CHAPTER 1

Introduction

The International Association for the Study of Pain (IASP) defines “pain” as “an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage,” (2020). Acutely, this experience is useful, as pain serves a biological purpose that ultimately encourages us to exercise nocifensive behaviors that protect our bodies from additional tissue damage (Grichnik & Ferrante, 1991; Tracey, 2016). Thus, acute pain is considered both normal and necessary (Yong et al., 2022). However, chronic pain serves no biological purpose and oftentimes places burdens on those who are suffering (Grichnik & Ferrante, 1991; Yong et al., 2022).

Chronic Pain as a National Health Concern

Chronic pain negatively impacts just over 20% of the adult population in the United States (Yong et al., 2022), and millions of Americans are left to deal with the physical, psychological, and social burdens accompanying this pain. Financial difficulties also arise in the form of direct costs (involving monetary exchange) for medical expenses related to being diagnosed and treated for chronic pain (Dagenais et al., 2008). However, indirect costs—financial consequences without direct monetary exchange, which are often related to a loss of productivity at work and/or in the home—and intangible costs, such as a reduction in enjoyment of life, can be equally burdensome (Dagenais et al., 2008;

Goetzel et al., 2003; Orhurhu et al., 2019). Because of the prevalence of chronic pain, and the associated hardships placed on patients who are suffering, chronic pain is now considered one of the most significant health concerns affecting Americans (Institute of Medicine, 2011).

Chronic Pain and the Prevalence among Females

The prevalence of both chronic pain disorders are higher in females than in males for various types of pain (Johannes et al., 2010; Wang et al., 2022; Wilbarger & Cook, 2011), including, but not limited to, headache (Buse et al., 2013; Hardt et al., 2008), abdominal pain (Sandler et al., 2000), and fibromyalgia (Arout et al., 2018; Bennett et al., 2007). Many reasons for this increased prevalence of chronic pain among females have been revealed (for review, see Bartley & Fillingim, 2013; Fillingim et al., 2009; Samulowitz et al., 2018), and one of these factors is hormonal.

Hormonal contribution to the development of chronic pain has been documented in both clinical and experimental pain studies (for review, see Fillingim et al., 2009). In humans, for example, prepubertal girls have a similar prevalence rate of migraine as prepubertal boys, but this prevalence rate splits dramatically post-puberty, with migraines increasing to 18% for women and 6% for men (Lipton et al., 2001; Stewart et al., 1992). Temporomandibular disorders also have similar prevalence patterns, with post-pubertal woman diagnosed at higher rates than men (LeResche, 1997). Also indicative of hormonal contribution are the observed

alterations in self-reported pain severity across the menstrual cycle for multiple pain conditions (i.e., irritable bowel syndrome [Heitkemper et al., 2003], temporomandibular disorders [LeResche et al., 2003], headache [Keenan et al., 1992], and fibromyalgia [Alonso et al., 2004]. Studies of hormonal influences on experimental pain provide less consistent results (Fillingim et al., 2009). However, researchers have strongly suggested that this is due to inconsistencies in how cycle phases are defined, using differing pain modalities, and varying testing sites across studies (Riley et al., 1999; Sherman & LeResche, 2006) as opposed to a lack of hormonal influence.

For some time, the estrous cycle of female rodents and the unknown impact of normal cycling has been used as a justification to exclude female subjects from research entirely (Garcia-Sifuentes & Maney, 2021). This is why an analysis of rodent use in pain studies between the years 1980 and 2020 indicated that more studies than not utilized males only (Sadler et al., 2022). But again, chronic pain has a higher prevalence in females (Johannes et al., 2010; Wang et al., 2022; Wilbarger & Cook, 2011). Additional research has now revealed that data obtained from females is not more variable than males (Becker at al., 2016), and this has led more researchers to call for an increase in the heterogeneity of animal subjects in such a way that it models heterogeneity described in the population of patients with chronic pain (Sadler et al., 2022). For research investigating chronic pain, that means increasing the focus on females, among other groups (Mogil & Chanda, 2005; Sadler et al., 2022). The higher incidence

of both chronic pain and multisensory hypersensitivity in females justifies studies dedicated to determining the underpinnings of these experiences in female populations, specifically.

In addition to hormone-dependent sex differences influencing the prevalence of chronic pain disorders in females, a number of preclinical studies have revealed sex differences in immune responses to painful stimuli. Microglia, in particular, have received much attention. For example, in a neuropathic pain model, males exhibited a significantly greater microglial response and fully recovered 81 days post-injury. In contrast, females exhibited elevated immune cell activity and no recovery at 121 days post-injury (Bennett & Xie, 1988). Later studies indicated that microglia are required for the development of mechanical hypersensitivity in male mice, but females achieved the same level of mechanical hypersensitivity through activation of T-lymphocytes (Sorge et al., 2015; Vacca et al., 2014).

Sex differences in cellular responses to painful stimuli have also been observed. Dental tooth pulp stimulation with capsaicin and complete Freund's adjuvant applied to the trigeminal nerve both increased levels of calcitonin gene-related peptide, which has been proposed to be involved in pain transmission and inflammation (Bowles et al., 2011; Kuzawinska et al., 2014). In addition, the release of substance P from nociceptors, an indicator of peripheral pain signaling, led to internalization of neurokinin-1 (NK1) receptors, and this internalization was greater in females than males (Nazarian et al., 2014).

Differences in pain sensitivity also contribute to the higher prevalence of chronic pain disorders in females. In a cancer pain model, females displayed earlier development of pain as compared to males (Falk et al., 2013), as well as more fatigue-induced hyperalgesia (Gregory et al., 2013). In a complex regional pain syndrome model, females exhibited significantly more allodynia and swelling as compared to males (Tajerian et al., 2015). Additionally, systemic inflammation in human participants that was induced by lipopolysaccharide led to women reporting more pain and less pain inhibition as compared to men (Karshikoff et al., 2015).

In humans, studies of psychosocial factors have also revealed sex differences. Two important constructs—catastrophizing and self-efficacy—have emerged. Catastrophizing, a method of coping with pain that involves a negative hyperfocus on pain-related information and is more common amongst women (Forsythe et al., 2011; Sullivan et al., 2001), is associated with increased reports of pain and disability related to the reported pain (Keefe et al., 1989). Self-efficacy, a belief in one's ability to successfully reach a goal (Bandura, 1977), which is reported more often by men, has an inverse association with pain levels and symptomatology (Somers et al., 2012). Cold pressor pain sensitivity was lower in men who had greater self-efficacy (Jackson et al., 2002).

Importantly, sex differences in the response to analgesics have also been reported. In 2000, Kest et al. reviewed 50 analgesic assay comparisons in animal

subjects. 56% of assays (28/50) revealed a significantly greater analgesic response in males, while a mere 4% of assays (2/50) showed greater analgesic responses in females. More recently, in an animal pain model utilizing ligation of the masseter tendon, μ-opioid receptors were upregulated significantly more in males, and this correlated with response to μ-opioid drugs (Bai et al., 2015).

Direct evidence of sex differences in analgesic responses in humans came from experimentally-induced pain models. In a study of 10 healthy women and 10 healthy men utilizing electrical pain, women experienced greater analgesic potency, but the onset and offset of analgesia was slower, thus indicating sex differences in pharmacodynamics (Sarton et al., 2000). Women also report greater morphine-induced respiratory depression as compared to men (Dahan et al., 1998; Sarton et al., 1999). In addition, women report more negative side effects, including nausea and vomiting as compared to men (Fillingim et al., 2005; Lopes et al., 2021; Zacny, 2002).

Descending Pain Modulation Pathways

Investigations into descending pain-modulating pathways have been of great importance, because dysfunction in these pathways is believed to contribute to chronic pain states. Many structures are involved in the experience of pain (Fig. 1). One important structure in the descending pain-modulating system is the rostral ventromedial medulla (RVM). Dorsal horn-projecting neurons from the RVM allow for the facilitation or inhibition of nociceptive processing (Fields et al.,

1995; Heinricher & Ingram, 2008; Heinricher et al., 2009). Two cell types allow for RVM-mediated enhancement and suppression of nociceptive processing: pain-facilitating “ON-cells” and pain-inhibiting “OFF-cells” (Fields et al., 1983; Fields & Heinricher, 1985; Heinricher & Fields, 2013). With the activation of pain-facilitating neurons and the suppression of pain-inhibiting neurons, responses to subsequent input are facilitated.

Another important structure in the descending pain-modulating system is the periaqueductal gray (PAG). The PAG is a midbrain structure that, when stimulated electrically, produces analgesia while also inhibiting neurons at the level of the dorsal horn (for review, see Chen & Heinricher, 2019a; Heinricher & Fields, 2013). However, it does not have direct projections to the dorsal horn but rather relays through the RVM.

Both “bottom-up” and “top-down” processes are involved in the modulation of nociceptive transmission, from the perception of the pain sensation to subsequent behavioral responses. The RVM receives direct projections from both the parabrachial nucleus (PBN), which allows for the transmission of nociceptive information to the RVM (Chen et al., 2017; Roeder et al., 2016) while establishing an affective dimension of pain via projections to the central amygdala (Roeder et al., 2016), as well as the dorsomedial hypothalamus (DMH), which has been demonstrated to be involved in the production of stress-induced hyperalgesia (Martenson et al., 2009). In addition, the PAG receives

direct input from the medial prefrontal cortex, amygdala, and anterior cingulate cortex (ACC)—structures providing top-down information regarding the pain experience (Calejesan et al., 2000; Cheriyan & Sheets, 2018; Hardy, 1985). It has been suggested that the mPFC-amygdala-PAG pathway may also mediate fear-conditioned analgesia (Butler et al., 2011).

Plasticity in Pain-Processing Circuits Contributes to Chronic Pain

Information about noxious stimuli is transmitted from a site of injury to the brain via ascending nociceptive pathways. Important details, such as the location of the damage, as well as specifics regarding temperature and pressure, are transmitted (Dubin & Patapoutian, 2010), and this information is useful in the acute stage of pain (Grichnik & Ferrante, 1999; Yong et al., 2022). However, changes in nociceptive transmission can occur at the molecular, cellular, and systemic levels, which then contribute to chronic pain and associated hypersensitivity (Sandkulher, 2009; Sandkulher, 2013).

At the level of primary afferent nociceptors, numerous changes have been demonstrated. One of these changes is increased membrane excitability. In addition, there are reports of enhanced presynaptic release and post-synaptic effect of various substances that are involved in the nociceptive response, such as substance P (Sandkulher, 2009). Furthermore, dorsal horn neurons show enhanced responsiveness to noxious stimulation (Sandkulher, 2009; Sandkulher, 2013). The combination of these alterations produce enhanced nociceptive

transmission, with the results being an increased response of nociceptive neurons to noxious stimuli and/or the development of responses to normally non-noxious stimuli. Therefore, pain transmission may be altered in chronic pain states. However, it is not the only process that undergoes changes.

Because the regulation of nociceptive transmission is accomplished not just by the ascending pain transmission system, dysfunction in the descending pain-modulating system can also result in chronic pain. Plasticity in the RVM is observed one day after an injection of complete Freund's adjuvant, with an upregulation of AMPA receptors (Guan et al., 2003). Descending inhibition is enhanced and hyperalgesia is attenuated when these receptors are activated. In addition, κ- and μ-opioid receptors in the RVM display enhanced descending inhibition (Schepers et al., 2008).

Plasticity in the PBN is also observed in chronic pain states (Chen & Heinricher, 2019b). Acutely, the contralateral PBN relays nociceptive information. However, when pain persists, the ipsilateral PBN is recruited to maintain both hyperalgesia and responsiveness of RVM neurons. Thus, plasticity in the PBN contributes to both the development and maintenance of chronic pain.

Modeling and Measuring Acute and Chronic Pain in Animals

Although studying humans in pain is necessary and valuable, studies investigating cellular-, molecular-, and circuit-level actions require the use of rodent models of pain-related conditions, such as migraine (Storer et al., 2015) and chronic inflammation (Ren & Dubner, 1999). A successful model will produce symptoms that are similar to those seen in humans with acute or chronic pain, and these symptoms should be reversed by standard treatments for the type of pain in question. It should be noted that, because the experience of pain is complex, no single model can recapitulate all aspects. Thus, models should not be evaluated solely by how many aspects of the pain experience they produce.

Mechanical nociceptive threshold, the threshold for withdrawal evoked by a von Frey fiber, is frequently assessed in studies of various types of acute pain. Fibers are applied to the hind paw (Chaplan et al., 1994), and a decrease in the amount of force it takes for an animal to withdraw its paw from baseline testing to post-treatment testing indicates hyperalgesia. Hypoalgesia and analgesia may also develop in response to treatments, and this would be indicated by an increase in mechanical threshold from baseline testing to post-treatment testing.

Thermal nociceptive threshold, the threshold for withdrawal evoked by a thermal stimulus, is also frequently assessed during investigations of acute pain. Tests of thermal sensitivity, such as Hargreave’s test and tail-flick, use stimulation of the skin to induce a withdrawal that is, to some extent, reflexive in nature

(Hargreaves et al., 1988; Le Bars et al., 2001). Latency to withdrawal is the variable of interest, and within-subject comparisons of these latencies before and after pain-inducing or -relieving stimuli are applied can indicate the presence of hyperalgesia, hypoalgesia, and/or analgesia. Much like von Frey, measures of thermal sensitivity require no learning and can be conducted in decerebrate animals.

Measures of acute pain-related behavior in animals have been critically important to our understanding of opioid analgesics (Le Bars et al., 2001). However, like any measure, there are some limitations in the study of animals in pain. First, experimenter differences in the performing of these tests can lead to completely different results (Chesler et al., 2002). This is due to the methods used in these measures. In tests of mechanical sensitivity, an experimenter applies a punctate stimulus to the hind paw (Chaplan et al., 1994). Although the von Frey fibers are intended to be of different forces, variations in force from the experimenter while applying the fibers may have a significant impact on results. Similarly, two methods for assessing tail-flick latency in acute pain states, application of heat to the tail and submersion of the tail in heated water, are also subject to experimenter differences (D'Amour & Smith, 1941; Le Bars et al., 2001).

While measures assessing withdrawal responses are commonly considered reflexive in nature and can be used with decerebrate animals, measures of withdrawal thresholds in acute pain states do possess a learning component in

spinally intact animals. For example, the tail-flick response is prone to habituation, and shortening the time between heat exposures and increasing the temperature increases this habituation (Carstens & Wilson, 1993; Groves & Thompson, 1970; Le Bars et al., 2001). Although these assays can be used in both spinally intact and decerebrate animals, top-down processes can be recruited in spinally intact animals that may produce different results than in decerebrate animals.

Another limitation in behavioral measures used to analyze acute pain-related behavior is related to the habituation mentioned previously: because learning occurs in spinally intact animals, these tests do not always reveal significant differences in animals in chronic pain states (Le Bars et al., 2001). In fact, reliance solely on withdrawal measures in subjects with chronic pain has been cited as a reason for why treatments for chronic pain are lacking (Mogil & Crager, 2004; Vierck & Yezierski, 2015).

While testing of mechanical and thermal nociceptive thresholds is common, particularly in acute pain states and while testing analgesic effects of drugs, more researchers are stating the importance of also assessing the affective and motivational components of pain in behavioral tests that are more relevant to human pain conditions (Mogil & Crager, 2004; Vierck & Yezierski, 2015). This is because pain is not defined by the presence of a nociceptive input alone, but rather by a full cascade of events that occur in the wake of tissue damage, either

real or potential (IASP, 2020)—in addition to potential changes in mechanical and thermal sensitivity, affective and motivational states are also involved in the perception and response to painful events.

At the heart of this call for better behavior is a belief that the slow progress in developing novel treatments for chronic pain is due to a lack of translational validity in the behavioral measures currently employed in studies of pain-related behavior (Mogil and Crager, 2004; Mogil, 2009; Sadler, Mogil, & Stucky, 2022; Vierck, Hansson, & Yezierski, 2008; Vierck & Yezierski, 2015). However, this argument implies that affective and motivational states are not relevant to acute pain, and that altered mechanical and thermal sensitivity are not relevant to chronic pain, but neither of these points is true. Affective and motivational states allow us to commit to memory details about the acute pain experience such as where and how the injury occurred, which then allows us to avoid further injury (Grichnik & Ferrante, 1991; Tracey, 2016). And although mechanical and thermal sensitivity are reported in individuals with chronic pain, the primary complaint tends to be ongoing pain, not stimulus-evoked pain (Backonja & Stacey, 2004). Despite the argument's inaccurate implications, it is not wrong to call for better novel behavioral measures in studies of pain-related behavior, as novel measures can tell us more about the ongoing pain experience than measures of mechanical and thermal sensitivity can.

A number of novel measures seek to address these concerns regarding translational validity, and tests of affective and motivational states in chronic pain conditions are becoming more common. The value of these tests is that they indicate not simply the presence or absence of stimulus-evoked pain, but also ongoing and pain that occurs in the complete absence of a stimulus. An example of a measure that seeks to address the affective and motivational elements of chronic pain states is operant responding for sucrose following an abdominal incision modeling post-operative pain (Martin et al., 2004). While data indicated a suppression of exploratory behavior for 1-2 days post-incision, operant responding for sucrose was found to be affected for substantially longer. In addition, although animals in the incision condition eventually returned to baseline in sucrose pellet accumulation, matching that of sham-treated animals, sham-treated subjects were substantially more efficient in collecting their pellets than incised subjects. Thus, while a simple measure of assessing the number of pellets would have revealed no significant differences by post-incision day four, an operant responding measure revealed that incised subjects were significantly slower in their approach to sucrose as compared to sham-treated animals. This is in line with human patients' complaints of disability associated with chronic pain—in addition to being unable to complete day-to-day tasks, chronic pain may also lead to debilitating inefficiency, and this may be the result of impaired cognitive functioning associated with chronic pain (McCracken & Iverson, 2001).

Another example of a novel operant measure is of orofacial pain-related behavior from the Neubert laboratory (2005). Animals were trained to place their face on a heated stimulus to receive a reward, thus presenting a conflict between exposure to noxious temperatures and a reward. Outcome measures included rewards received, stimulus facial contacts, facial contact duration, and three additional relevant variables, and the hyperalgesia produced was reduced by morphine. Although previous studies of orofacial pain revealed mechanical sensitivity (Vos et al., 1994), allowing animals control of the amount of nociceptive stimulation demonstrated that stimulus-response relationships were related to nociceptive processing.

A third example of a novel measure of chronic pain-related behavior is the cage-lid hanging behavior assessment, produced by Zhang et al. (2021). Cage-lid hanging is considered a species-specific (mice) elective behavior, meaning that it is not necessary for survival, and this has been proposed to be indicative of well-being because of the way poor health reduces this behavior (Boissy, et al., 2007; Jirkof et al., 2010). The human equivalent would be joining a club or team sport, for instance. Similar to the orofacial pain study mentioned previously, the cage-lid hanging behavior measure utilizes a conflict to better elucidate the affective and motivational components of chronic pain. The subjects want to engage in this behavior, but the presence of pain forces them to make a choice. Using spared nerve injury, complete Freund’s adjuvant, formalin, capsaicin, anterior cruciate ligament transection, cyclophosphamide cystitis, and systemic

lipopolysaccharide, Zhang et al. (2021) demonstrated that cage-lid hanging was reduced in all seven pain models. In addition, cage-lid hanging behavior could be restored with administration of analgesics.

Finally, the Grimace Scale (Langford et al., 2010) was developed to assess grimacing behavior resulting from ongoing pain in rodents. The scale is based on Charles Darwin's work documenting facial expressions of both humans and animals under various emotional conditions. His observations led him to assert that animals display similar facial expressions as humans, and that animals display grimaces, specifically, when in pain as humans do (Darwin, 1872). With this in mind, the Grimace Scale was designed to allow for analysis of pain-related (and pain-specific) grimacing. Because grimaces do not develop in animals that are stressed but not in pain, many laboratories studying pain have adopted these scales as a way of demonstrating the presence of pain in their animals. Researchers analyzed grimacing behavior in animal models of chronic pain without applying additional stimuli to test withdrawal responses, which led them to conclude that this is not indicative of stimulus-evoked pain but rather ongoing pain. However, use of this measure has been demonstrated unsuitable for pain lasting beyond 24 hours, as grimacing behavior habituates over time (Langford et al., 2010; Mogil et al., 2020; Sotocinal et al., 2011). In addition, it is not known if grimacing reflects an affective component of pain in humans, and it is not known if grimacing exists in rodents as a means of social communication of pain (Mogil et al., 2020).

A Barrier in the Study and Treatment of Chronic Pain

Despite the large number of people who are affected by chronic pain, and researchers' attempts to call for better behavioral models, progress in treating these disorders has been slow. Part of the problem may be the number of co-morbidities (physical, emotional, and cognitive) that may develop with chronic pain, which complicate treatment. One such co-morbidity is multisensory hypersensitivity, or an enhanced sensory sensitivity to innocuous, non-somatic stimuli (Harriott & Schwedt, 2014; Schwedt, 2013; Wang & Frey-Law, 2023; Wilbarger & Cook, 2011). Although any sensory system can be impacted, the visual and olfactory systems are more often affected (Harriott & Schwedt, 2014; Schwedt, 2013).

Multisensory hypersensitivity is not uncommon in patients with chronic pain (Schwedt, 2013), and, as with many chronic pain disorders, the prevalence is higher in females (Wilbarger & Cook, 2011). This co-morbidity is particularly problematic in that it may interfere with appropriate treatments for pain. For example, patients with chronic pain and co-morbid multisensory hypersensitivity are more likely to be prescribed sedatives, they are more likely to be misdiagnosed as mentally ill and/or undertreated (Hoffmann & Tarzian, 2001), and they are less likely to receive pain medications (Chen et al., 2008). Differences in the way patients with multisensory hypersensitivity are treated speak to the poor understanding of the neurological mechanisms underlying this co-morbidity in the medical community—while some are treated as though they

are truly in pain, others are treated as though they are simply suffering from a psychiatric disorder (Hoffmann & Tarzian, 2001). However, the lack of understanding regarding multisensory hypersensitivity in the medical community stems from a lack of understanding in the scientific community.

Photosensitivity resulting in photophobia is often reported as a comorbidity with chronic pain (Harriott & Schwedt, 2014). This photophobia is not induced by direct, light-induced activation of trigeminal nociceptive pathways, and it is not related to ophthalmic conditions (Noseda et al., 2010; Okamoto et al., 2010). Additionally, no development of enhanced sensory acuity or amplified processing in primary sensory pathways has been identified (Carrillo-de-la-Pena et al., 2006; Geisser et al., 2008; Lopez-Sola et al., 2014; Lotsch et al., 2012). Despite these facts, researchers have not come to an understanding of the extent to which non-somatic sensory stimuli, such as light, interact with pain-processing circuitry and influence pain-related behavior. A report that a subset of ON- and OFF-cells in the RVM respond to light (photic) stimuli (Martenson et al., 2016) began to address this, providing evidence from both naïve rodents and humans with functional pain disorders that normally non-noxious light has the potential to engage the descending pain-modulating system and produce hyperalgesia. Because the RVM is recruited by light, these data suggest that light is not transmitting pain in these patients but is rather modulating pain via the RVM.

The observation that photic stimuli can activate a subset of ON- and OFF-cells in the RVM led to the question of what particular circuitry would allow photic stimuli to get to this area of the brain. One way that light is detected is by non-image forming vision cells called intrinsically photosensitive retinal ganglion cells (ipRGCs). These cells target the olivary pretectal nucleus (OPt), which is a relay in the pupillary light reflex (Baver et al., 2008; Gooley & Saper, 2005; Trejo & Cicerone, 1984; Young & Lund, 1994). The OPt sends projections to the PBN, which sends projections to both the RVM and amygdala, and also projects to the PAG, which projects to the RVM (Chen et al., 2017; Martenson et al., 2016; Roeder et al., 2016). Structures involved in top-down processing of pain, such as the DMH and ACC, have the capacity to influence responses to light further via direct projections to the RVM and indirect projections via the PAG (Calejesan et al., 2000; Martenson et al., 2009). Thus, there does exist a pathway by which photic stimuli could modulate pain rather than transmit it (Fig. 2).

It is of critical importance to elucidate the mechanisms underlying multisensory hypersensitivity, and particularly photophobia due to its prevalence, when attempting to treat chronic pain. Photophobia can be difficult to study in rodents though, because light is a stressor for nocturnal rodents (Bowen et al., 2012; Walker & Davis, 1997). Stressful conditions can produce hyperalgesia or analgesia, depending on multiple factors (Bardin et al., 2009; Maier, 1986). Thus, light has the capacity to not only impact, but to also confound studies of pain-related behavior in rodents.

Summary

While acute pain serves a biological purpose that encourages us to protect ourselves from additional injury, chronic pain can be debilitating (Grichnik & Ferrante, 1991; Yong et al., 2022). Chronic pain affects approximately 1 in 5 Americans, and it is often accompanied by physical, psychological, social, and economic/financial burdens (Yong et al., 2022). With this high prevalence rate, chronic pain is considered by many to be one of the most significant health concerns affecting Americans (Institute of Medicine, 2011).

Multiple processes contribute to the development of chronic pain. While altered plasticity at numerous points in the ascending pain transmission system can certainly facilitate chronic pain (Dubin & Patapoutian, 2010; Sandkulher, 2009; Sandkulher, 2013), alterations in the descending pain-modulating system also contribute. The RVM, the greatest source of output of this system (Heinricher & Fields, 2013), contains pain-facilitating and -inhibiting cells (ON- and OFF-cells, respectively) that allow for enhancement and suppression of nociceptive processing (Fields et al., 1983; Fields & Heinricher, 1985; Heinricher & Fields, 2013; Heinricher & Ingram, 2008; Heinricher et al., 2009). When pain-facilitating neurons are activated and pain-inhibiting neurons are suppressed, facilitation of subsequent input occurs, and chronic pain may result (for review, see Chen & Heinricher, 2019a).

Because chronic pain is considered by many to be a national health concern (Institute of Medicine, 2011), studies of underlying mechanisms are imperative to the development of treatments for chronic pain. In order to study these mechanisms, animal models that mimic symptoms of the pain experience, from hyperalgesia to learned avoidance, must be employed (Mogil et al., 2010). In addition to developing models that have some translational relevance to the human population, pain-related behavior must be carefully tested, and all potential confounds must be noted. However, there is disagreement amongst researchers studying pain-related behavior about what constitutes a successful measure and what behaviors we should be testing for. Thus, critical discussion needs to occur regarding measures of pain-related behavior in studies of laboratory rodents.

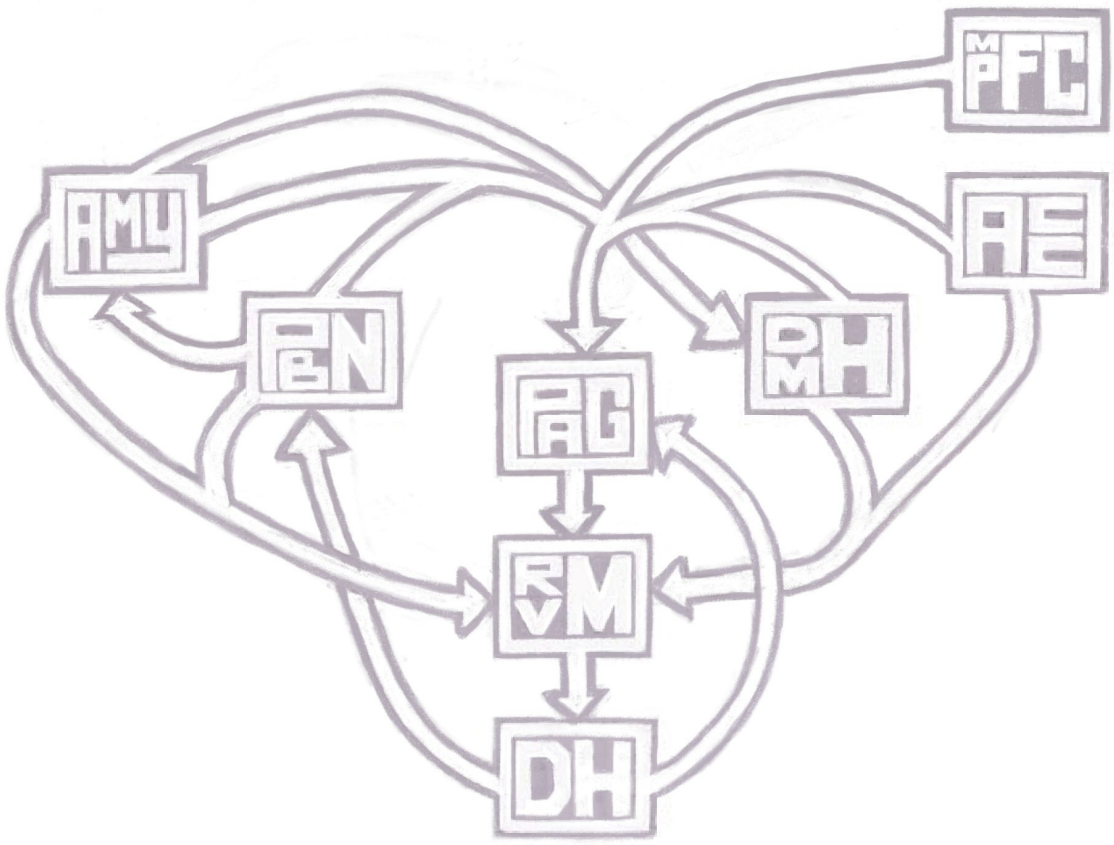


Figure 1. Descending pain modulation pathways. ACC = anterior cingulate cortex; AMY = amygdala; DH = dorsal horn; DMH = dorsomedial hypothalamus; mPFC = medial prefrontal cortex; PAG = periaqueductal gray; PBN = parabrachial nucleus; RVM = rostral ventromedial medulla.

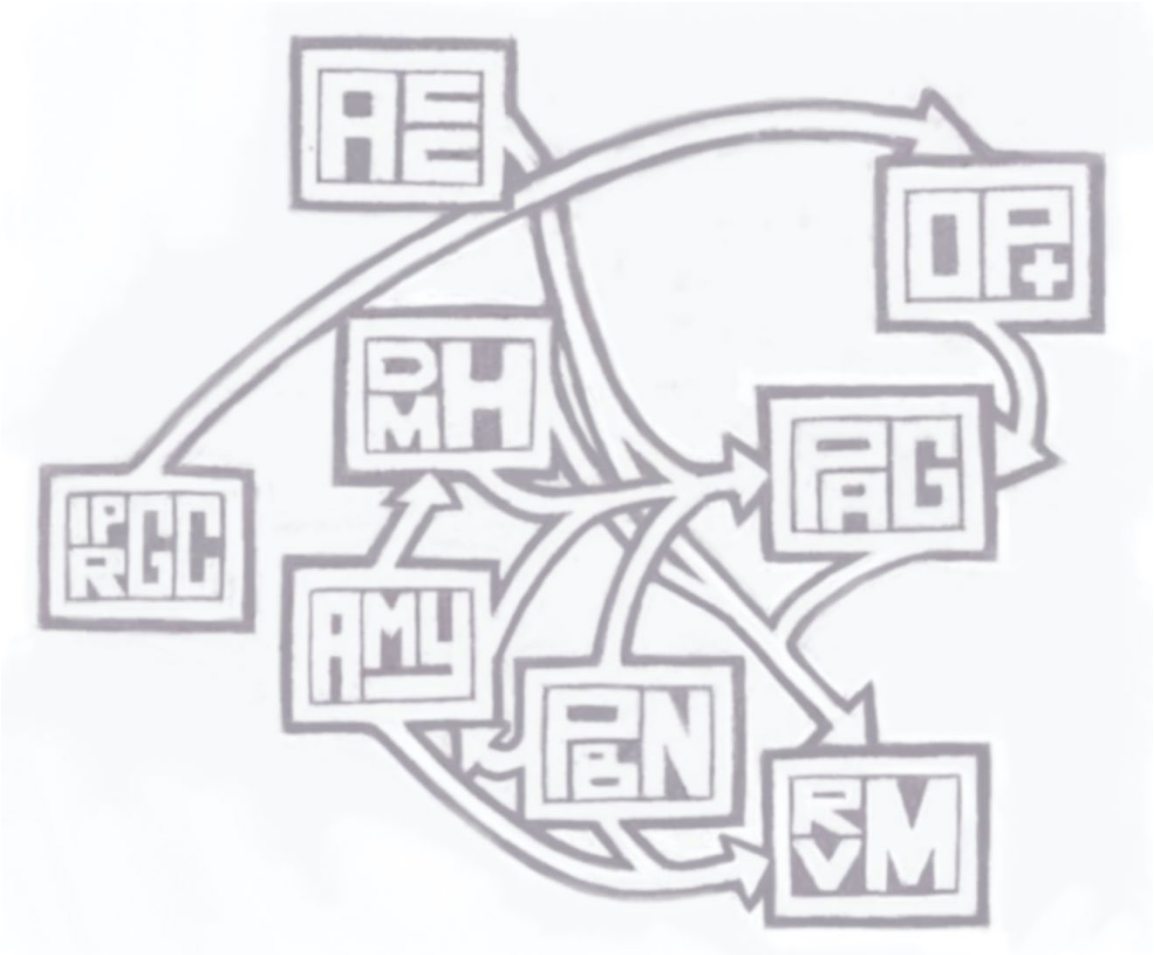


Figure 2. Diagram of circuitry potentially *modulating* responses to photic stimuli, detected by ipRCGs. ACC = anterior cingulate cortex; AMY = amygdala; DMH = dorsomedial hypothalamus; ipRCG(s) = intrinsically photosensitive retinal ganglion cell(s); OPt = olivary pretectal tract; PAG = periaqueductal gray; PBN = parabrachial nucleus; RVM = rostral ventromedial medulla.

CHAPTER 2

Traditional versus Novel Measures of Pain-Related Behavior

Introduction

In the field of pain research, there is much that is still up for debate. But one thing that is not in question is that treatments for patients suffering from chronic pain disorders are lacking, and this is both a national and global health care concern (Rice et al., 2016). Because chronic pain can be debilitating and is linked to the development of other burdens (i.e., depression, anxiety, and reduced quality of life [for review, see Nicholas, 2007]), each with their own physical, psychological, social, and financial hardships, the development of novel, non-addictive treatments for chronic pain is imperative. This need for appropriate medications has prompted many scientists to speculate about what might be hindering progress in this area of research.

One proposal explaining this slow progress in finding treatments is that there is a lack of translational validity in traditional preclinical measures of chronic pain-related behavior; an often-cited solution is that we must measure affective and motivational elements of the pain experience with novel measures to increase translational validity (Mogil and Crager, 2004; Mogil, 2009; Sadler, Mogil, & Stucky, 2022; Vierck, Hansson, & Yezierski, 2008; Vierck & Yezierski, 2015). However, traditional measures were initially designed to assess behavior during acute pain and responses to analgesics, not chronic pain and its associated

behavior (Hargreaves et al., 1988; Hunskaar et al., 1986; Le Bars et al., 2001; Takagi & Iwamoto, 1952). Because they were designed for studies of acute pain, these traditional measures often focus on hypersensitive states, which is a common symptom of acute pain (Pedersen & Kehlet, 1998). Hypersensitivity is not typically the main concern for those dealing with chronic pain though; Backonja and Stacey (2004) reported that only 64% and 38% of patients with chronic neuropathic pain reported mechanical or thermal hypersensitivity, respectively. This same study also reported that 96% of patients reported on-going pain, which indicated that, although hypersensitivity is present, on-going pain is more problematic for humans than stimulus-evoked pain in chronic pain states such as neuropathic pain. Thus, the value of using traditional tests of pain-related behavior in order to better understand chronic pain *should* be in question.

Careful review of methodologies used to assess pain-related behavior is warranted when progress in finding treatments slows, and several questions arise:

1. *What are some traditional measures and novel measures of pain-related behavior?*
2. *What are some of the assumptions underlying both traditional and novel measures of pain-related behavior, and are they accurate?*
3. *What can novel measures tell us that traditional measures cannot?*

4. *Have we moved closer to a viable treatment for chronic pain because novel behavioral measures of pain-related behavior were used in the basic or preclinical phase of drug discovery/development?*
5. *What other issues might contribute to the slow movement toward viable treatments for chronic pain?*

In this review, we delve into tests of pain-related behavior with the ultimate goal of addressing the question of whether or not novel measures of pain-related behavior provide more information than traditional measures. We conclude with a list of additional factors that may also be hindering progress in treating patients suffering from chronic pain. (A comprehensive list of traditional and novel pain-related behavioral measures can be found in Table 1.)

1. Defining Traditional and Novel Measures of Pain-Related Behavior

Traditional measures of pain-related behavior tend to rely on simple, acute input-output methods and assessment of hypersensitive states (i.e., application of a stimulus and observation/recording of response directly after [for review, see LeBars, 2001]). The applied stimuli may be thermal, mechanical, electrical, or chemical in nature. The latency to respond to the applied stimulus is generally of particular interest, but the specific outcome measure depends on the type of stimulus used. For example, in tests of thermal sensitivity (i.e., tail-flick, hot plate, Hargreaves test), heat is applied to a part of the body (or the subject is placed on a heated surface), and so latency to respond is often the variable of interest

(Woolf & MacDonald, 1944). However, in the von Frey test, which is used to test mechanical sensitivity, it is more common to apply a series of punctate stimuli to a part of the body (most often the hind paw) in ascending order by force, and so latency to respond is related to the amount of force required to produce a withdrawal (Chaplan et al., 1994; Dixon, 1980).

These traditional measures are not typically considered learning-dependent, although the learning, or habituation, that many researchers have observed with repeated testing is routinely discussed (for review see Le Bars et al., 2001). One example of this came from Takagi and Iwamoto (1952), who demonstrated that animals placed on a hot plate (~21° C) had variable response latencies after the first exposure but significantly shortened their latencies to respond by the third exposure. In addition, simply exposing animals to the apparatus prior to hot plate testing is sufficient to reduce response latencies (Hunskaar et al., 1986). Although animals require habituation to the apparatus, testing room, and experimenter prior to testing, training is not considered necessary. Traditional measures often utilize a pre-/post-test procedure, as a comparison between behaviors both before and after a treatment is administered provides a better indication of the efficacy of the treatment. Thus, repeated testing, despite the behavioral impacts of prior exposure, is necessary (Sandkuhler et al., 1996).

While the number of times an animal is tested must be considered prior to beginning an experiment, repeated testing does not render the test results

invalid. Responses to both mechanical and thermal stimuli at various stages of the pain experience can provide important information to researchers, and an inability to habituate across repeated tests may indicate animal-related issues, particularly if more than one fails to habituate (see Chapter 4 for additional information).

Novel measures of pain-related behavior utilize more complex experimental designs that address affective and motivational elements of the on-going pain experience (Vierck et al., 2008; Yezierski & Vierck, 2010). For this reason, they are considered more appropriate in studies of chronic pain-related behavior. Because the tasks are more involved, the variables of interest are not as simple as latency to respond, and these tests tend to require time for training/conditioning procedures. These tests often include an opportunity for subjects to avoid and/or escape a stimulus, like traditional measures, but they also tend to include a learning-dependent task that provides an opportunity for subjects to make some decision related to the stimulus that is evoking pain. For example, a conditioned place preference test may be used to assess the motivational value of pain and/or a treatment that reduces it by pairing a pain-inducing or -relieving stimulus with one side of a test box and then testing the preference for each side (conditioning phase) before (preconditioning phase) and after (testing phase) the pairing (Sufka, 1994). Animals should not have a strong innate preference for either side of the chamber in the preconditioning phase. Preference develops during the conditioning phase when a pain-relieving

treatment (for example) is administered and paired with a specific side of the chamber. When tested, animals that have developed a preference for the treatment and the treatment-paired side spend significantly more time on that side. Therefore, the time spent on both sides during the preconditioning and testing phases, and the difference in time between these two phases, would be the most relevant variables.

2. Underlying Assumptions about Traditional and Novel Measures of Pain-Related Behavior

A number of assumptions exist regarding traditional and novel measures of pain-related behavior, many of which can be questioned. One assumption about traditional measures is that traditional measures are all reflexive measures—that is, obtaining a result depends on reflexive responses to pain-inducing stimuli, such as withdrawing a paw or tail from a heat source. However, this is not true of all traditional tests. The hot plate test, a traditional measure of thermal sensitivity, does not rely on reflexive behavior (LeBars, 2001; Woolf & MacDonald, 1944). Thus, although reflexive tests tend to fall into the category of traditional tests, traditional tests cannot be universally defined by the property of inducing a reflexive behavioral response.

Another assumption about traditional measures of pain-related behavior is that the reflexive behaviors elicited from certain tests (i.e., von Frey, tail-flick) lack a supraspinal component—in other words, reflexive behaviors occur in a way that

is not under the control of the subject. However, there is substantial evidence that top-down processes have the capacity to modulate the expression of pain-related reflexive behavior (Langford et al., 2006; Langford et al., 2011; Ossipov et al., 2010). Although these behaviors may be elicited in spinal animals (Borszcz et al., 1992; Cleland & Bauer, 2002; Herrero & Headley, 1991), supraspinal processing and resulting states, such as stress, can allow for the enhanced expression or near-complete suppression of reflexive behaviors (Bardin et al., 2009), it cannot be said that these behaviors are entirely spinally mediated. Therefore, changes in expression or suppression of reflexive behavior may very well be the result of any number of variables processed at the supraspinal level, from the interactions between the experimenter and subject to the subject's general stress level.

A third assumption about traditional measures of pain-related behavior is that a "good" measure of pain-related behavior must reveal affective and motivational aspects of the pain experience. However, individual measures of pain provide critical information about distinct elements of the pain experience, such as intensity and location to the affective and motivational values of the pain experience, and no single measure can address all of these. It is important to understand what our test data are actually telling us and choose a test, or battery of tests, that address our specific questions.

Just as assumptions are made about traditional measures, many assumptions are also made about novel measures of pain-related behavior. One of these is that novel measures of pain-related behavior do not rely on reflexive behavior, but this is arguable. For example, studies using the more-recently-developed Grimace Scale (Sotocinal et al., 2011) are utilizing a novel behavioral measure of facial expressions, and these expressions are indicative of underlying emotions. Fridlund (1991) described this view of faces and their expressions:

> "Thus, the most frequent classical emotions view of faces is essentially a "two-factor" model that posits two basic kinds of faces: innate reflex-like faces that read out ongoing emotion (Darwin's "facial expressions of emotion"), and learned instrumental faces connoting emotion that is not occurring (i.e., dissimulative "social" faces)" (p. 29).

Because the proposed Grimace Scale (Sotocinal et al., 2011) is based on Darwin's work (1872), and because we currently have no way to differentiate reflexive from "learned instrumental faces" in animals, it is reasonable to consider the Grimace Scale as at least "reflex-like." The head orientation measure, although novel, is also reflexive measures (Sokolov, 2001)

A second assumption made about novel measures of pain-related behavior is that using novel measures instead of traditional measures would be beneficial for drug development. This argument often cites the perceived failure of NK1 receptor antagonists in treating chronic pain, and a number of researchers have pointed out that treatment potential appeared high in basic and preclinical studies

of the drug, but it was unsuccessful in clinical studies. The behavioral measures used in preclinical studies were mechanical and thermal sensitivity (for review see Hill, 2000), and researchers of these agents were concerned that these measures were not translationally valid. Reliance on traditional measures came under fire, with many researchers claiming that behavioral methods in basic and preclinical studies had to be updated or reformed in order to gain translational validity. However, a number of studies that followed these claims provided a different explanation of why NK1 receptor antagonists were not successful in clinical trials: the dose was too low (Rupniak & Kramer, 2017). In higher doses than were initially tested, researchers observed benefits of the NK1 receptor antagonist Aprepitant in the treatment of neuropathic and inflammatory pain (Latorre et al., 2022; Yang et al., 2022). These data indicate that there were other factors at play that slowed the success of NK1 antagonists as treatments for pain. The study design influenced the success of the drug in clinical trials, not the pain measures themselves. Rupniak and Kramer (2017) suggested that a better understanding of NK1 receptor occupancy in the brain and the clinical response could have prevented this validated concept from being abandoned.

Another heavily-relied-upon assumption about novel tests of pain-related behavior is that novel measures of pain have provided new information about pain that traditional measures have not. The problem with this assumption can be seen in the way novel behavioral measures of pain are validated. In order to validate a novel measure, researchers often provide evidence that the novel

measure is providing data that is in line with traditional measures of pain-related behavior (Langford et al., 2010; Zhang et al., 2021). There is circular logic in this method—the claim is that traditional measures are not translationally valid and novel measures are needed, but these novel measures provide the same data as traditional measures in the validation process. Thus, while novel measures may provide new approaches to testing pain-related behavior, they are not producing new insights. With this in mind, one could certainly question whether novel behavioral tests will be the solution for the lack of new treatments that more and more researchers are pointing out.

3. What Novel Measures of Pain-Related Behavior can Tell us that Traditional Measures Cannot

The International Association for the Study of Pain defines pain as “an unpleasant sensory and emotional experience with, or resembling that associated with, actual or potential tissue damage” (2021). What this tells us is that there is more to the pain experience than reflexive responses—a variety of top-down processes are engaged in an attempt to not only simply feel pain, but to also understand its meaning and emotional value while placing it in an appropriate context so that a proper response can be generated. Thus, the pain experience involves cognitive functions, such as learning and memory, as well as motivation and emotionality, in addition to motor responses, such as reflexive withdrawals (for review, see Villemure & Bushnell, 2002). Novel measures are generally intended to move beyond the sensory-discriminative domain and into

the affective and motivational domains, testing learned reactions, such as escape, avoidance, and approach to pain-inducing and -relieving stimuli (Vierck, Hansson, & Yezierski, 2008; Vierck & Yezierski, 2015).

One advantage of using novel measures of pain-related behavior is that novel measures may have fewer dichotomies. Traditional measures often rely on simple yes/no behavioral questions (i.e., did the subject withdraw at a particular force?) (for review, see LeBars, 2001). However, novel tests often utilize methods that allow more complex questions to be asked. For example, the cage-lid hanging test (Zhang et al., 2021) examines time spent hanging from a cage lid as an elective behavior in mice when exposed to a pain-inducing stimulus. Although we can ask the simple question of whether or not subjects in different treatment conditions hang from the cage lids, we can also ask more complex questions. Some of these questions include, but are not limited to: are subjects engaging in this behavior differentially as a function of their treatment group? Do they have more difficulty with hanging from a cage lid depending on the type of pain model used (i.e., do subjects with complete Freund's adjuvant hang from lids more than subjects with migraine-like headaches—does the part/portion of the body injured matter for this behavior)? And are elective behaviors restored at different rates depending on the type of pain model used?

Investigations into the affective and motivational values of the pain experience are important because of the way chronic pain, as a disorder, is defined. The

Centers for Disease Control and Prevention define chronic pain as “having pain most days or every day in the past three months that limited life or work activities” (2021). Therefore, the goals of treatment are to both reduce pain and restore activity, not simply alter reflexive behavior. However, as stated earlier, there are novel reflexive tests of pain-related behavior (i.e., Grimace Scale, head-orientation assay). While some have attempted to use novelty as a justification for why these measures have greater translational validity, they are still reflex-dependent behaviors. As such, they are subject to the same limitations as other reflex-based tests (i.e., von Frey, tail-flick) and do not offer the same opportunity to ask more complex behavioral questions that other novel measures of pain-related behavior afford.

4. Novel Measures of Pain-Related Behavior and Movement toward Viable Treatments for Chronic Pain

The belief that novel measures of pain-related behavior have more translational validity because they (generally) allow for the investigation of more complex behavioral questions has guided arguments in favor of abandoning traditional measures. However, is there evidence that using novel measures of pain-related behavior in basic and preclinical studies has assisted in drug discovery/development in the clinical phase? At this point in time, there is little to no evidence to suggest that using novel measures has led us closer to viable treatments for chronic pain.

5. Additional Issues Slowing the Search for Viable Treatments for Chronic Pain

Although speculation abounds regarding the reasons we have been slow in producing viable treatments for chronic pain, and much blame is cast on the measures themselves, there are additional issues that may be in play.

a. The missing patient

It is well-known that the experience of pain is different for every individual. However, we do not study individuals. Researchers report measures of central tendency, which means we have pooled data of various individuals and are describing a sample from a population. But doctors do not treat a pool of individuals. Essentially, we design treatments that treat the average person, but there is no “average” person. It is possible that more careful parsing and categorization of history, symptoms, and disability in both animal and human subjects when studying pain-related behavior could result in more efficacious, disease-specific treatments for chronic pain. It is also possible that fewer potential medications would be abandoned in clinical trials. Just because a treatment works on average, that does not mean it will work in individuals. And just because a treatment does not work on average, that does not mean it does not work at all for anyone. In line with this belief is the continued call for individualized, mechanism-based treatments for customized chronic pain care (Vardeh et al., 2016; Woolf et al., 1998).

b. The missing data

The almost exclusive publication of data showing statistically significant results could have a substantial impact on drug discovery/development. This is because funding can be poured into repeating drug studies that other laboratories already know will fail. Because funding is a finite source, money spent on repeating studies cannot be recouped to spend on novel studies. Thus, the negative data missing from the literature leads to a waste of resources that could have been avoided, had the non-significant data been published as well. In addition to the waste of funding given to repeating studies, the loss of animal lives should also be concerning. Therefore, the insistence on not publishing negative data is not only slowing progress in the search for treatments, but it is also unethical, as it violates one of the three Rs of animal research (Reduction) by requiring researchers to use more animals to repeat studies (Russell & Burch, 1959).

c. The missing researcher

Oftentimes, when a measure appears to be failing, researchers will remove themselves from the problem completely, citing a problem with the measure itself. However, a measure does not have the capacity to misuse itself. It is in these times that we, as researchers, must take some responsibility for our failures. What must also be understood is that failure is a natural part of the discovery process, and we cannot avoid this in the search for treatments for chronic pain.

d. The missing step

One might say that our measures lack translational validity because we cannot extrapolate from animal behavior to human behavior. We must ask ourselves though: is that the true goal, or are we missing a step? Perhaps, our goal as scientists in search of novel drugs for the treatment of chronic pain should be to first understand the behavior in the context of the species as a whole and then consider the relevance to human behavior. For example, the Grimace Scale was proposed with the idea that humans and animals both produce facial expressions that change as a result of being in pain, and this was based on Darwin's work (1872) in identifying facial expressions in animals commonly seen on farms (i.e., cows, horses, dogs, cats, etc.). Rodents were not observed (likely because they are nocturnal), and it is not actually known if grimacing serves a purpose in rodent communication (Mogil et al., 2020). Thus, researchers have attributed human meaning to animal behavior—grimacing—despite a lack of evidence that this is true.

In future studies of pain-related behavior in animal subjects, we must first understand what, if anything, these behaviors mean to animal subjects before we start testing them. Thus, we must abandon the practice of allowing anthropomorphism to justify behavioral studies, no matter how novel, because these attempts to increase translational validity in this way are substantially decreasing both eco- and ethological validity. We must

also ask ourselves how decreasing eco- and ethological validity has the capacity to address the lack of progress in the search for viable treatments for chronic pain that has been so commonly cited. Furthermore, we must not be so easily taken in by novelty—"new" is not synonymous with "better".

Ideal measures of pain-related behavior will not sacrifice one type of validity for another, as each of these validities is related to each other. For example, if researchers elicit behaviors in animal subjects in a laboratory study that are not normally part of their behavioral repertoire (an issue with ethological validity), then we cannot say that test performance predicts behavior in nature (an issue with ecological validity). If the measures are lacking in both types of validity from the beginning, then how should these results be translated, and what is the value of translation?

Conversations amongst researchers studying pain-related behavior in laboratory animals must be approached with an openness to considering alternative perspectives on issues in the field and the impact on patients suffering from chronic pain. Although novelty is a solution to the slow progress in drug discovery/development, it is certainly not the only one, and one could argue that it is not even the best one. Missing an "average" patient, negative data, the researchers themselves, and/or an entire step not only *has* impacted studies and

the search for treatments, but it will also *continue* to impact these studies if these issues are ignored in favor of more popular ideas.

Conclusion

Many people around the globe suffer from chronic pain, and lacking treatments are not just a serious concern for medical professionals working with patients; researchers are increasingly concerned about this as well. Although it is becoming more common for pain researchers to point to a lack of translational validity in measures of pain-related behavior as a reason for why we do not have more viable treatments, with use of novel measures of pain-related behavior cited as a potential solution, there are other factors and solutions that are deserving of consideration. We must consider the underlying assumptions that this argument is based on, and careful attention must be paid to the accuracy of these assumptions. In addition, it will be critical to continue discussions about the many reasons why treatments are lacking in lieu of simply focusing on a single issue. Finally, the state of the field requires that we stop allowing researchers to justify the use of animal lives based on concepts like novelty and anthropomorphism—there is no scientific basis for these rationales, and many studies based on these concepts are lacking in multiple types of validity, which only move us further away from discovering viable treatments for individuals who are suffering from chronic pain.

Table 1. Traditional and novel measures of pain-related behavior

Traditional Measures of Pain-Related Behavior		
Measure	**Variable(s) of Interest**	**Reference**
Tail-flick (thermal)		
Application of heat to tail	Latency to withdraw	Hardy, 1953
Tail immersion in heated water	Latency to withdraw	Ben-Bassat et al, 1959
Tail-flick (cold)	Latency to withdraw	Pizziketti et al., 1985
Hargreave's test (thermal)	Latency to withdraw	Hargreave's et al., 1988
Randall-Selitto test (mechanical)	Latency to withdraw	Randall, 1957
von Frey test (mechanical)	Force at withdrawal	Chaplan et al., 1994
	Latency to withdraw	Moller et al., 1998
Hot plate test	Latency to withdraw	Woolfe & MacDonald, 1944
Writhing test	Number of writhes	Blumberg et al., 1965

Novel Measures of Pain-Related Behavior		
Measure	**Variable(s) of Interest**	**Reference**
Cage-lid hanging behavior (mice)	Pre- and post-pain testing Difference in hanging	Zhang et al., 2021
Conditioned place aversion/avoidance	Pre- and post-pain testing Aversion to mechanical stimulation-paired compartment	LaBuda & Fuchs, 2000
Conditioned place preference	Pre- and post-pain testing Preference for analgesic-paired compartment	Sufka, 1994
Grimace score (multiple species)	Pre- and post-pain testing Difference in grimacing	Langford et al., 2010; Sotocinal et al., 2011
Home cage wheel running	Pre- and post-pain testing Difference in running	Kandasamy et al., 2016
Locomotor activity	Pre- and post-pain testing Difference in locomotor activity	Alsalem et al., 2020
Mechanical conflict-avoidance assay (mic	Pre- and post-pain testing Difference score	Gaffney et al., 2022
Mechanical conflict-avoidance assay (rats	Pre- and post-pain testing Difference score	Harte et al., 2016
Nest building	Pre- and post-pain testing Difference in nest score	Gaskill et al., 2013
Social interaction	Pre- and post-pain testing Difference in lever presses for social interaction	Baldwin et al., 2022
Sucrose preference	Pre- and post-pain testing Difference in lever presses for sucrose	Martin et al., 2004
Thermal conflict-avoidance assay	Pre- and post-pain testing Difference in heat tolerance for reward	Neubert et al., 2005

CHAPTER 3

The Influence of Light on Pain-Related Behavior in Female Rats

Introduction

Multisensory hypersensitivity—a condition in which discomfort and pain exacerbation are induced by innocuous non-somatic stimuli, such as light, sound, or smell—is oftentimes reported by patients with chronic pain disorders (de Tommaso et al., 2002; Harriott & Schwedt, 2014; Schwedt, 2013). However, patients experiencing multisensory hypersensitivity do not display enhanced sensory acuity or amplified processing in primary sensory pathways (Carrillo-de-la-Pena et al., 2006; Geisser et al., 2008; Lopez-Sola et al., 2014; Lotsch et al., 2012). This strongly suggests that multisensory hypersensitivity is due to integration of non-somatic sensory information with nociceptive processes.

One potential mechanism through which multisensory hypersensitivity may occur is via engagement of the descending pain-modulation system. Through pain-facilitating neurons (ON-cells) and pain-inhibiting neurons (OFF-cells) in the rostral ventromedial medulla, descending control systems modulate pain via outputs that facilitate or suppress excitability of nociceptive neurons at the level of the dorsal horn, producing hyperalgesia or analgesia, respectively (Fields & Heinricher, 1985; Heinricher, Barbaro, & Fields, 1989; Heinricher & Fields, 2013; Kincaid et al., 2006; Mo et al., 2022). Although activation of ON-cells has been demonstrated to contribute to hyperalgesia in various pain models (i.e.,

inflammatory pain [Cleary & Heinricher, 2013; Kincaid et al., 2006], neuropathic pain [Porreca et al., 2001], migraine-like pain [Edelmayer et al., 2009]), studies of ON-cell recruitment have relied on somatic stimuli to influence pain-related behavior (e.g, noxious heat stimuli such as complete Freund's adjuvant [Cleary & Heinricher, 2013]) as opposed to using non-somatic sensory stimuli. However, a report that photic, or light, stimuli can activate at least a subset of pain-facilitating ON-cells and produce hyperalgesia in anesthetized rats (Martenson et al., 2016) addressed this gap by revealing that non-somatic stimuli can engage the descending pain modulation system to modulate pain in a way similar to noxious somatic stimuli. These findings identified a previously unknown mechanism for photosensitivity.

In preclinical studies of photosensitivity in nocturnal rodents, a potential confound comes into play. This arises from three facts: 1) light is a known stressor for nocturnal rodents (Bowen et al., 2012; Walker & Davis, 1997), 2) stressful conditions can lead to the development of hyperalgesia or analgesia, depending on the intensity of stress and other factors, including controllability (Bardin et al., 2009; Maier, 1986), and 3) experimenters may be using a specific experimental light level, but they may not take into consideration the impact of environmental light (i.e., that used by the experimenter for testing). Therefore, it is not only necessary to differentiate the effects of experimental light (for use *in an experiment*), which is a controlled variable, from environmental light (for use *by an experimenter*), which may be less controlled and less often reported, but also

to understand the impact of perceived controllability of the light stimulus on pain-related behavior. Exploring the impact of both experimental and environmental light on pain-related behavior may be particularly important in females, as chronic pain and comorbid photosensitivity are more prevalent in females (Bolay et al., 2015; Buse et al., 2013; Cho et al., 2017; Johannes et al., 2010).

The primary goal of the present study was to investigate the effects of both experimental and environmental light exposure on mechanical nociception in female rats. Additionally, this study sought to examine the influence of experimental light *controllability* on mechanical nociception of female rats.

Methods

Subjects/Housing Conditions

Upon arrival at the colony, adult female Sprague Dawley rats (150-250 g; Charles River, Hollister, CA) were pair-housed, randomly assigned to an environmental lighting condition, and acclimated for 7 d. Food and water were available *ad libitum,* and a 12/12-h light/dark cycle was used (lights on = 0600). Light level in the colony room, measured in racked cages, was 1000 lux. Animals were transported from the colony room to the testing room via a cart covered in blackout material to eliminate exposure to additional light sources.

Light Conditions

Four Environmental Light levels were utilized in the testing room: 10 lux, 100 lux, 1000 lux, and 2000 lux (see Table 2 for lux level equivalents; taken from Hawks, 2012). These light levels are lower than that used by Walker and Davis (1997), which was approximately 2,400 lux.

Measures of Anxiety-Like Behavior

Because between-group differences in baseline anxiety-like states have the potential to influence mechanical threshold, baseline anxiety-like behavior was assessed with the open-field test. Subjects were tested in a 5-min trial prior to beginning handling/habituation. The apparatus was 40 x 40 x 40 cm^3 and was made of clear Plexiglas. Testing started between 0700 and 0900. The testing chamber was located outside of the colony and away from conspecifics. All testing was conducted in 1000 lux by a single experimenter. Variables recorded were total time spent in the corners, total entries into the corners, total time spent in the center, total entries into the center, and total distance moved.

As additional measures of anxiety-like behavior, body weight and total fecal count during testing were recorded. Body weight was taken prior to testing, and total fecal count during testing was obtained by summing the number of fecal boli throughout all behavioral testing.

Mechanical Nociceptive Threshold

Mechanical nociceptive threshold was determined by testing with von Frey filaments (1 g to 180 g force). Thresholds were assessed by applying filaments in ascending order a maximum of 3 times per filament until a withdrawal rate of 67% was achieved (withdrawing 2 out of 3 times). Rats were handled (5 min each) and habituated (testing room and von Frey apparatus—15 min each) for a total of 5 d following baseline open field testing in their assigned environmental lighting condition. Following the completion of handling, animals underwent a habituation procedure (3 d, 15 min each) on the von Frey apparatus. Additional habituation to the room and apparatus (15 min each) was used on test days. All habituation and testing occurred during the light cycle, and a start time between 0700 and 0900 h was used. Testing was conducted during the early hours of the light cycle in order to evaluate the impact of exposure to photic stimuli specifically during light hours. The apparatus consisted of 6 individual chambers (28 x 12 x 15 cm^3) made of clear Plexiglas and a metal grid (1/4 x 1/4 in squares) used as flooring. The testing rack was located outside of the colony room and away from conspecifics. All testing was conducted by a single experimenter.

Light Switch-Off Box (LSOB)

To test the impact of light stress controllability, a light switch-off box (LSOB) was used as a light delivery apparatus (100 lux, all conditions) and was considered "Experimental Light." Animals were tested in the LSOB in the same environmental lighting condition as previously assigned (10, 100, 1000, or 2000

lux). This box consisted of a two-chambered box made of black Plexiglas; each chamber was 35 x 35 x 35 cm^3. Linking the two chambers was an opening (8 x 10 cm^2) that allowed the animal to pass from one side to another. The LSOB sat atop a weight-sensing holder that permitted the animal to be tracked within the apparatus. The testing chamber was located outside of the colony and away from conspecifics. Subjects underwent 10 min of habituation to the chamber (the side of the chamber animals were first placed in was counterbalanced), and testing begun immediately following habituation (5 min). Dependent variables were number of center line crosses (an indicator of light- or photo-avoidance), total time spent in the light across light trials, and average time spent in the light per light trial (total time spent in the light divided by the number of center line crosses). All testing was conducted by a single experimenter.

Statistical Analyses

Total fecal count, open field behaviors, body weight, and LSOB behaviors were analyzed with one-way ANOVA. Mechanical nociceptive threshold was analyzed with repeated-measures ANOVA. Tukey's tests were used for post-hoc analyses. SPSS Statistics (v. 29; IBM, Armonk, NY) was used to perform analyses, and GraphPad Prism (v. 7; GraphPad Software, Boston, MA) was used to produce figures.

Results

Environmental light significantly influenced mechanical nociceptive threshold

A significant difference in mechanical sensitivity was found between Environmental Light conditions ($F_{(3,37)} = 5.32$, $p = 0.004$; n = 10-11 per group). The thresholds in the 2000 lux group were significantly higher than in other light level groups (Fig. 3). There were no significant differences among the groups in total fecal count, open field (anxiety-like) behaviors, or body weight (data not shown).

Exposure to light stressor significantly influenced LSOB behavior and mechanical nociceptive threshold

The higher mechanical thresholds of rats exposed to an uncontrolled Environmental Light level of 2000 lux prompted the question of whether or not perceived controllability of light played a role. Light level in the LSOB, the Experimental Light level, was the same for all Environmental Light level conditions. There was a significant difference in the total number of center line crosses as a function of Environmental Light level condition ($F_{(3,37)} = 5.12$, $p = 0.005$), and animals in the 10 lux Environmental Light group crossed the center line significantly more than animals in the 100, 1000, and 2000 lux conditions (Fig. 4A). Although there was no significant difference in the total time spent in the light between light level conditions (Fig. 4B), there was a trend in the average time spent in the light per light trial ($p = 0.059$; Fig. 4C).

Mechanical nociception was tested on the von Frey apparatus immediately following LSOB exposure, and a significant difference between pre- and post-

LSOB exposure mechanical nociception was observed in the 2000 lux group ($F_{(3, 37)} = 11.84$, $p < 0.001$; Fig. 5), with mechanical thresholds in this group decreasing significantly more than animals in the other three light exposure conditions.

Discussion

The goal of this study was to examine the effect of Environmental Light on nociception, as well as the effects of exposure to a controllable light on subsequent nociceptive testing. We first determined that female rats that were exposed to an Environmental Light level of 2000 lux displayed increased mechanical nociceptive thresholds. This light level is well above the subjects' standard colony room light level.

We next determined that, in the process of being exposed to Experimental Light of 100 lux in the LSOB, subjects in the 10 lux Environmental Light group displayed increased photoavoidant behavior as compared to the other light conditions. These animals were exposed to a light level in the LSOB that was ten times their normal handling, habituation, and testing light level. Interestingly, although there was a significant difference in center line crosses and a trend in average time spent in the light per light trial, subjects did not spend significantly less time in the light overall. Thus, exposure to Experimental Light that is brighter than Environmental Light can produce photoavoidance; animals in the 10 lux condition moved more to avoid it.

The LSOB was also used to examine an element that has been well-documented to influence pain- and stress-related behavior: controllability of the stressor (Maier, 1986). While Environmental Light exposure was beyond the subjects' control, exposure to Experimental Light in the LSOB significantly reduced mechanical nociceptive thresholds in the 2000 lux group. This suggests that Environmental Light, a stimulus that is rarely reported, has the capacity to alter pain-related behavior, which is likely due to the stress-inducing nature of light exposure in nocturnal animals.

It should be noted that, although 2000 lux is a high light condition, substantially greater exposure is required to induce retinal light damage (e.g., 24 hours of continuous exposure [Costa et al., 2008; Gupta et al., 2020]). This is important, as it points to the possibility that the decrease, and then increase, in mechanical sensitivity that was elicited in the 2000 lux group was more related to exposure to a general stressor than to exposure to a noxious stimulus, specifically. This is very much in line with studies indicating that non-noxious stressors (i.e., restraint, foot shock, social defeat) can impact pain-related behaviors, such as mechanical nociceptive threshold and thermal/cold nociceptive threshold (Bardin et al., 2009; Wu et al., 2021; Yomogida et al., 2020). With the absence of a noxious stimulus, these results highlight the importance of perceived controllability of a stressor in subsequent pain-related behavior testing.

There are a substantial number of elements in any given environment that may elicit stress and result in an increase in anxiety-like behavior for any species (e.g., predator stress [Burgado et al., 2014], social defeat stress [Patki et al., 2013], maternal separation [Kalinichev et al., 2002], restraint stress [Gameiro et al., 2006]). For rodents, these stressors can then affect results of nociceptive testing (Maier, 1986). Because we were specifically interested in photoavoidant behavior as a result of light exposure and the subsequent impact on mechanical nociceptive thresholds, as opposed to a more generalized anxiety-like state that could have developed prior to beginning our experiments, initial assessments of baseline anxiety-like behavior were necessary. Baseline open-field testing indicated that there were no significant differences in these behaviors among groups. Importantly, this allows us to conclude that pre-existing generalized anxiety-like states were not likely the driving force behind the results of mechanical nociceptive testing and photoavoidant behavior testing. Additionally, fecal count during testing and body weight, two indicators of anxiety-like states, revealed no significant differences between Environmental Light level groups, which further supports this conclusion.

One limitation that might be noted is the time of day that animals were tested, along with the fact that animals were tested in the light during the early hours of their light/dark cycle. However, the behavioral testing protocol was intended to correlate with the test times of other studies being conducted in the same laboratory in order to better understand the impact that photic stimuli, both

experimental and non-experimental, has on subjects under the specific conditions of this laboratory. Although testing nocturnal rodents during the lights-on portion of the light/dark cycle is common in many areas of neuroscience, future studies should examine responses to light during the lights-off portion of the cycle. In addition, elucidation of behavioral tendencies in different animal models of chronic pain (as opposed to naïve animals, as employed in this study) will be necessary for uncovering the neurological underpinnings of light-exacerbated pain in chronic pain states.

Taken together, these studies emphasize the necessity of careful control and reporting of all lighting conditions in pain-behavior testing, both environmental and experimental. Furthermore, experiencing low light levels (e.g. 10 lux) during handling, habituation, and training can increase photoavoidant behavior when testing is conducted at a higher light level, so attention must be paid to changing light conditions within an experiment. These data also provide evidence that the perception of control over a stressor can influence the overall results of pain behavior testing substantially. In the study of photosensitivity, it will be of critical importance not only to consider the ways in which environmental light may become a confound, but to also consider how the perception of control may affect responses to experimental light. However, conclusions from these studies are not limited to researchers investigating pain-related behavior; the impact of light exposure in the study of stress-related behavior in general should not only be considered but also reported.

Table 2. Lux level estimates with comparisons to everyday light.

Lux Level Estimate	Comparison
1	Twilight
10	Sunset
100	Very dark overcast day
1000	Overcast day
10000-25000	Full daylight

Source: Hawks, 2012

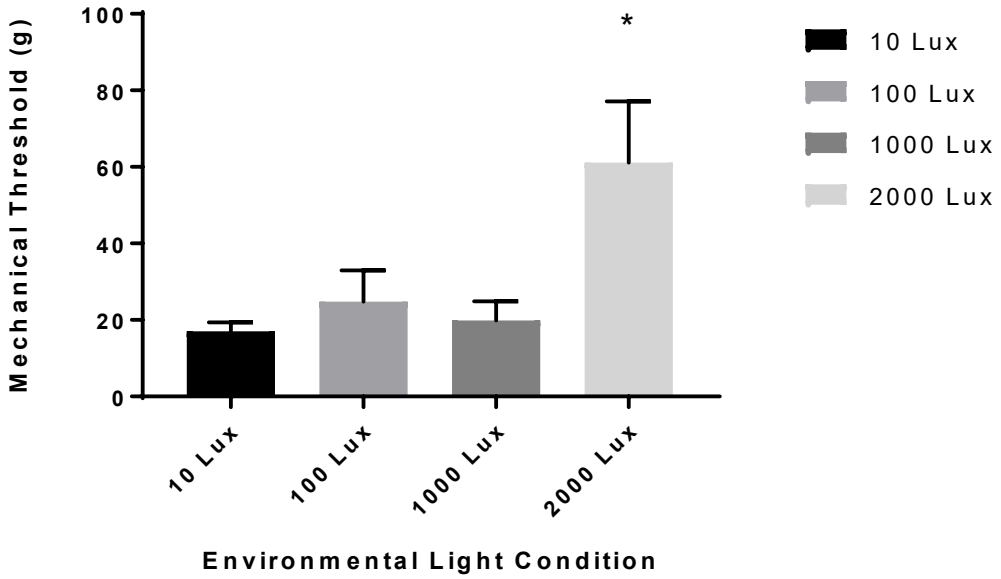
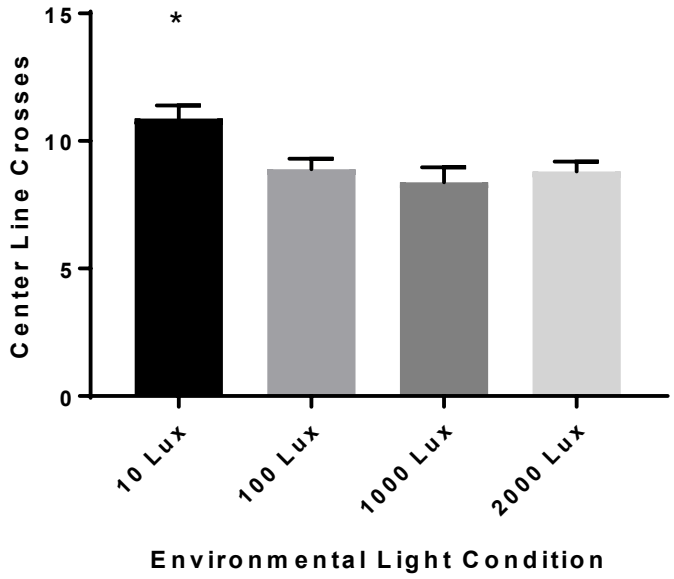


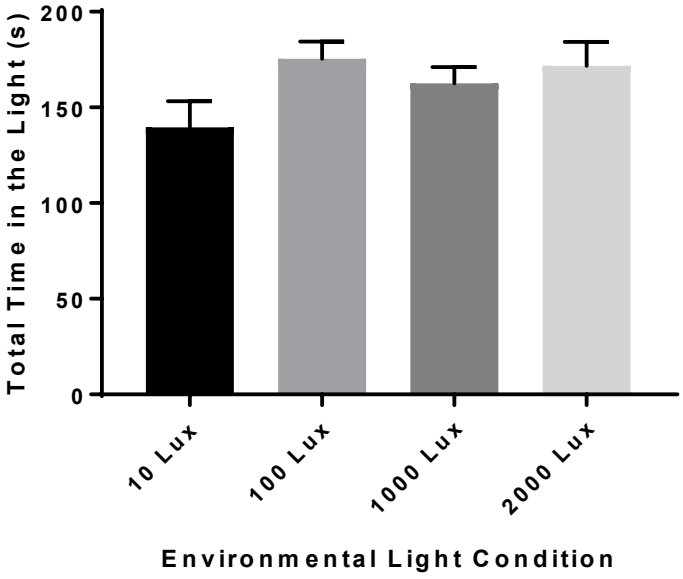
Fig. 3. Mechanical thresholds in female rats exposed to various Environmental Light conditions (pre-LSOB only).

There is a significant difference in pre-LSOB mechanical sensitivity between Environmental Light conditions. *p = 0.004 Tukey's post hoc test. Data presented as mean + SEM.

4A)


15
10
5
0
*
Center Line Crosses
10 Lux
100 Lux
1000 Lux
2000 Lux
Environmental Light Condition

4B)


200
150
100
50
0
Total Time in the Light (s)
10 Lux
100 Lux
1000 Lux
2000 Lux
Environmental Light Condition

4C)

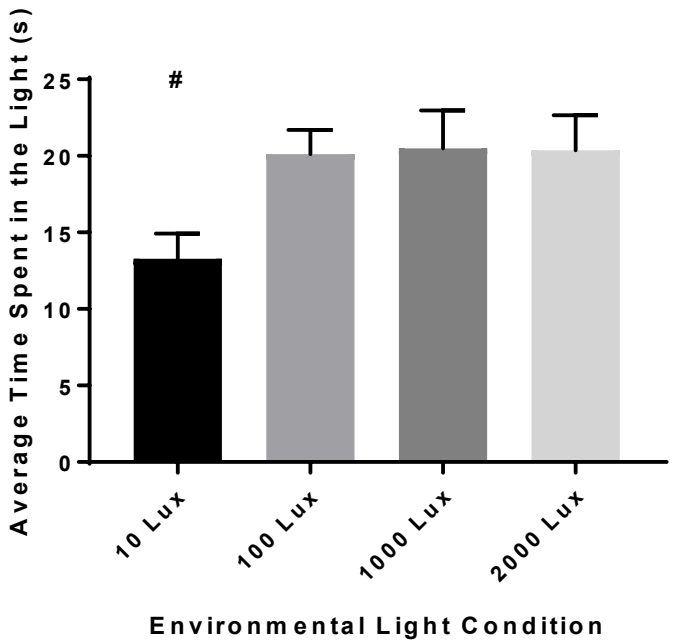


Fig. 4. Exposure to the light stressor significantly influenced LSOB behavior.

A) Center line crosses during LSOB testing by Environmental Light level condition, *p = 0.005 Tukey's post hoc test, B) total time spent in the light during LSOB testing (ns), and C) trend in average time spent in the light per light session during LSOB testing, #p = 0.059, one-way ANOVA (Tukey's post hoc test). Data presented as mean + SEM.

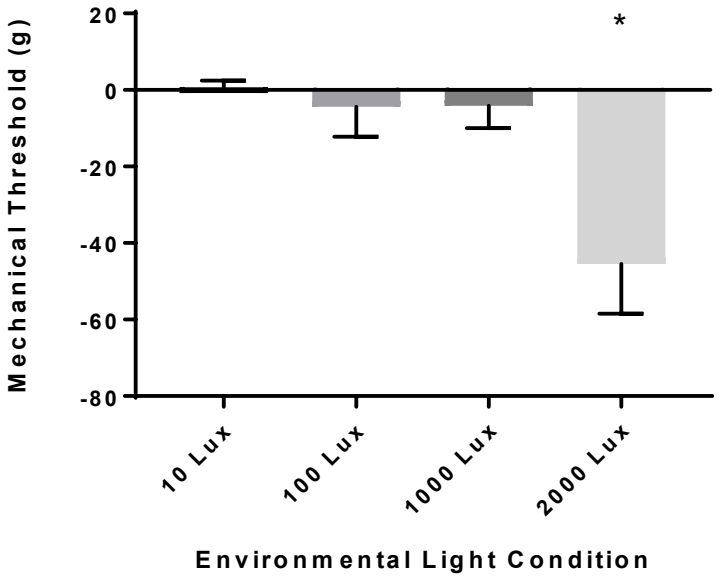


Fig 5. Difference scores in mechanical thresholds of female rats by Environmental Light condition

Post-LSOB von Frey score subtracted from pre-LSOB von Frey score, *p < 0.001 Tukey's post hoc test. Data presented as mean + SEM.

CHAPTER 4

Potential Confounds in the Study of Pain in Laboratory Rodents

Given the increasing number of people who develop and are diagnosed with chronic pain disorders, it is of critical importance that we create appropriate paradigms that reliably model elements of the human pain condition. However, a number of issues arise in the process of delivering, housing, and experimenting with animal subjects that may confound data. Increased within-group variability created by unknown and not considered interactions between the research subjects, their environments, their animal caretakers, and the researchers making use of them all need to be considered (Sare et al., 2021). These interactions are oftentimes stressful for rodents, in particular. Unfortunately, this increased variability leads to the abandonment of potential treatments and other novel ideas stemming from them that are actually quite viable, because significant data cannot be obtained.

Although we know that pain and stress are linked, they can be difficult to parse apart. This is because of the redundancy within each system, as well as their overlapping circuitry (Abdallah & Geha, 2017; Lewis et al., 1980; Martenson et al., 2009; Vachon-Presseau, 2018). These systems often work in tandem, and both are necessary for survival, meaning that they are not systems that can be shut down completely with ease. This is clear in studies where pain-related behavior is altered by a non-noxious stressor (i.e., restraint stress; Bardin et al.,

2009). Therefore, although we cannot investigate pain in the complete absence of stress, we should reduce the number of stressors our subjects are exposed to during the experimentation process so that our collected data is not distorted by confounds.

This review will discuss some environmental and experimental confounds that occur in the study of rodents in traditional laboratories, as well as behavioral and neurochemical impacts on the subjects.

Confounds in Transit to the Laboratory

Delivery of animal subjects by truck is unavoidable for laboratories that do not breed their own animals. A number of factors that have the potential to influence pain can arise in this period before the animals even arrive at the colony, but two issues can be especially problematic.

Weather-Related Temperature Extremes

The first issue is temperature extremes as a result of weather conditions. Thermoneutral zones (the temperature range within which thermoregulation can occur without the need to increase metabolic heat production) for mice and rats are between 26-34°C and 26-30°C, respectively (Gondor & Laber, 2007; The Guide for the Care and Use of Laboratory Animals, 2011). However, internal temperatures in delivery trucks for animal delivery companies, such as Charles River, are only controlled when transporting USDA-covered species, in which

case temperatures must not exceed ~29.4°C (Charles River, 2023). Standard laboratory mice and rats (of the genus *Mus* and *Rattus*, respectively) are not USDA-covered (APHIS, 2022). Thus, there is no set regulation for temperatures in trucks carrying these types of rodents for research purposes, despite research indicating behavioral, physiological, and morphological changes as a result of exposure to inappropriate temperatures (Gordon, 1990; Gordon, 1993; Pennycuik, 1967).

Within-Cage Aggression

Another confound that may occur while animals are in transit is within-cage aggression. Within-cage aggression is more likely to be seen in male subjects, although lactating mothers of many species have been reported to engage in physical aggression (Bosch, 2013; Luciano & Lore, 1975; Miczek et al., 2001; Svare, 1981). Within-cage aggression can be induced by external stressors (O'Kelly & Steckle, 1939), and movement in a delivery truck may be similar to cage-shaking stress—the continuous movement of a cage of animals as a chronic, unpredictable stressor (Lu et al., 2019). Cage-shaking stress is associated with altered dopaminergic and serotonergic function (Lu et al., 2019), and any resulting aggression could have an impact on subsequent data, particularly when dopaminergic and serotonergic function are in question or modulate the behavior of interest. For studies of pain-related behavior, within-cage aggression, especially that resulting in substantial injury to a subject (i.e., bites on ears/tail that remove skin and/or draw blood) is problematic. Injury and

resulting pain that is not associated with the experiment has the distinct capacity to alter pain-related behavior and neurochemistry in a significant, confounding way.

Both temperature extremes and within-cage aggression can confound data, but issues with temperature extremes do not necessarily have a long-term impact on behavior. Although Swoap et al. (2004) demonstrated that a drop in temperatures from 30°C to 18°C over several hours had a significant impact on cardiovascular parameters, which could further impact pain-related behavior, Crabbe et al. (1999) also demonstrated that a substantial acclimation procedure was sufficient to overcome shipping-induced stress (which included temperature extremes). The impact of acclimation on within-cage aggression, however, has not been established after substantial acclimation, and individual assessment of the animals and their behavior will be necessary when deciding whether to include these animals in studies of pain.

Utilizing behavioral tests relying on simpler methodologies of applying a stimulus and noting the response (i.e., withdrawing, licking touched area, etc.) can actually reveal problems with animals that developed prior to the experiment. Employing a multi-day habituation procedure where animals are exposed to the stimulus before the test day can help to determine the state of the animals, because animals tend to habituate across time to these tests and the stimuli used (for review, see Le Bars et al., 2001). Animals that are experiencing stress

that was produced before the experiment may develop more inconsistent results across habituation days that indicate that the animal does not appear to be habituating. Animals with inconsistent responses across days to a stimulus prior to experimental manipulation should be flagged for test day as potential outliers whose data may need to be removed.

Confounds While Being Housed

Bedding

Oftentimes, we think of bedding simply as padding that is designed to make cage life a bit more comfortable while limiting our subjects' contact with excreta. However, bedding has multiple purposes beyond comfort. These include: 1) to allow for nest-building, 2) to provide insulation to assist with thermoregulation, 3) to provide environmental enrichment, 4) to minimize growth of micro-organisms, and 5) to reduce within-cage accumulation of ammonia (Perkins & Lipman, 1995; Smith et al., 2004). Thus, reducing the amount of bedding within a cage has the capacity to alter thermoregulation in a way that requires subjects to increase metabolic heat production in order to maintain an optimal body temperature (Gordon, 2004). Because bedding can impact thermoregulation (Gordon, 2004), and body temperature can impact tests of pain-related thermal sensitivity (Le Bars et al., 2001), bedding has more of an impact on our data than we generally acknowledge.

Another potential confound is the type of bedding used. Bedding preferences amongst rodents are somewhat species-specific. For example, mice generally prefer large, fibrous materials that can be torn apart for nesting (Blom et al., 1996; Van de Weerk et al., 1997). Burrowing capability is also preferred by rodents (Gordon, 2004). Thus, corn cob bedding, although ideal in terms of absorbency and reducing ammonia in cages, is not preferred by mice or rats because it cannot be used for nest-building (Krohn & Hansen, 2008; Perkins & Lipman, 1995).

Wet bedding can also be a confounding factor for laboratory rodents in the study of pain-related behavior. Wet bedding is stressful for rodents, and indeed, it is used in studies of chronic, unpredictable stress (Kompagne et al., 2008; Matuszewich et al., 2007). One effect of wet-bedding stress is the development of a depression-like state, with anhedonia often observed (Kompagne et al., 2008; Matuszewich et al., 2007). Anhedonia could most certainly impact responses in tests of pain-related behavior.

It should be noted that chronic, unpredictable stress paradigms often apply multiple stressors of various types across multiple days. When studied individually, wet bedding was not the most potent stressor (Gorbunova et al., 2017). However, it can co-occur with a much stronger stressor: water deprivation, which produces notable alterations in hippocampal function (Gorbunova et al., 2017). In static cages using water bottles (as opposed to within-rack “lixit”

systems), the emptying of a water bottle into the cage leads to both wet bedding and water deprivation. Although wet bedding is not the strongest stressor when applied on its own, it may have a synergistic effect when combined with a stronger stressor such as water deprivation.

In sum, when providing bedding for newly arrived animals (or newly weaned animals), it is critically important to understand the impact of bedding on thermoregulation, as well as individual species' preferences for bedding, as these have the ability to influence pain-related behavior by altering stress levels. Rodents should have an amount of bedding that allows for burrowing, and mice require additional nesting materials (Blom et al., 1996; Gordon, 2004). It should be assumed that there will be no standard bedding type for all animals (The Guide, 2011). Furthermore, reporting of the specific type of bedding is encouraged in manuscripts for the sake of reproducibility.

Cage Movement/Vibration

Excessive movement and/or vibration of animal cages is related to cage-shaking stress described earlier. Although there is scant literature on the topic of laboratory rodent responses to excessive cage movement and/or vibration, there is enough to suggest that, not only are both human and animal behavior impacted by such movement, but also that rodents are more sensitive to this movement than humans (Norton et al., 2011; Seidel, 1993; Toraason et al., 1980). While vibration studies in rodents are limited, vibration studies in humans

working in industries where loud noise- and machinery-induced vibrations are a concern are much more common. Vibrations of this nature can lead to harmful effects on fetuses, as well as degenerative changes in the spine, among other issues (Seidel, 1993). Researchers working with animals should assume that vibrations in their home environments are at least as problematic for these animals as shown to be for humans.

Novel Scents

Every researcher brings in their own scent, and this cannot be avoided. Some laboratories have even suggested a difference in the way rodents respond to the scents of human males and females (Sorge et al., 2014). However, bringing additional scents into the laboratory environment, such as perfume, cologne, and/or essential oils, has the capacity to impact studies of pain-related behavior, and researchers are specifically warned about wearing these scents when working with animals (Deacon, 2006). For example, Kovacevic et al. (2006) demonstrated that mice that were exposed to perfume (undisclosed producer) on a daily basis had differences in seminiferous tubule diameter, indicating an effect of perfume exposure on postnatal reproductive organ development. Additionally, *Cymbopogon winterianus* Jowitt, an essential oil, has been shown to have an antinociceptive effect in rodents while also reducing locomotor activity (Leite et al., 2011). Because scents, particularly novel scents that subjects are not accustomed to, can impact pain-related behavior, it is advisable to keep scents

consistent across experiment days and avoid wearing highly scented perfumes, colognes, and essential oils.

Enrichment

The term “enrichment” has become important in both human and animal research. However, there is little agreement at this time regarding what constitutes “enrichment” for mice and rats, and researchers often make the mistake of thinking that enrichment is anything that can be added to a cage that does not interfere with the behavior(s) of interest. This is not the correct way to think about enrichment though.

Enrichment generally refers to improvements in captive animal enclosures (Newberry, 1995). The purpose of these improvements is to increase the expression of natural behaviors (i.e., burrowing, foraging, exploring, etc.), with this expression being an indicator of animal well-being (Bracke & Hopster, 2006). However, not all enrichment accomplishes this, and some enrichment has been shown to be detrimental to rodents. Importantly, Kimura et al. (2019) demonstrated that simple enrichment did not affect pain-related behavior but did reduce anxiety-like behavior, while more complex (“Improved”) environmental enrichment reduced pain-related behavior, as well as anxiety-like behavior. Importantly, these data indicates that certain types of environmental enrichment can act as modulators of pain.

Tests of the impact of environmental enrichment on various behaviors have been misleading (Newberry, 1995; Ratuski & Weary, 2022), and some have suggested that the way we use the term “enrichment” may be why. As Newberry (1995) pointed out, researchers often refer to enrichment as the type of environmental change used as opposed to the outcome. For example, additional item(s) for use in a cage are said to be enriching, and “enrichment” has come to be synonymous with the complexity of the cage and its contents, as opposed to what it should refer to: an outcome where the animals’ lives are improved because they are able to engage in more natural behaviors (Newberry, 1995; Ratuski & Weary, 2022).

Because of the differences in the use of the term “enrichment,” Newberry (1995) has argued that we need to make a distinction between the items being added and the outcome, which should be that the animal is “enriched.” Enrichment is “an improvement in the biological functioning of captive animals resulting from modifications to their environment,” (p. 230). Although there are many recommendations regarding appropriate enrichment, multiple factors should be considered when choosing what is to be done. In addition to finding an enrichment approach that actually results in improved biological functioning, enrichment should be provided on a species-specific basis. It is also highly important that enrichment does not interfere with study procedures (i.e., animals with head caps resulting from surgeries should not have tubes/tunnels that may bump and/or dislodge their head caps). Finally, we should be careful not to

assume that cage complexity is associated with healthy enrichment in the absence of evidence.

Housing Naïve with Treated Animals

Although many researchers have recognized this anecdotally, it is now firmly established in the literature that housing naïve rodents with pain-treated rodents can impact the behavior of naïve animals (Du et al., 2020; Langford et al., 2006; Li et al., 2014; Smith et al., 2016). Therefore, we must be careful not only to separate animals in different treatment conditions, but to also consider the order of handling and/or changing cages of treated and naïve animals. This is because scent is believed to be one of the primary sensory modalities that allows for the purported “social transfer of pain.” Odorants from pain-treated animals can attach to the experimenter’s gloves and lab coat, so handling and/or changing the cages of naïve animals without changing could substantially impact pain-related data. Therefore, being wary of introducing novel scents from perfumes, colognes, and essential oils is imperative, and researchers must be vigilant about the changing of gloves and lab coats between working with animals in different treatment conditions.

Summary of Recommendations

A number of potential confounds can arise when animals are being housed. In order to avoid these, appropriate bedding must be provided on a species-by-species basis. Although both rats and mice tend to dislike corn cob bedding and

both prefer burrowing capability, mice should be given large, fibrous materials that are suitable for breaking down into nesting (Blom et al., 1996; Gordon, 2004; Krohn & Hansen, 2008; Perkins & Lipman, 1995; Van de Weerk et al., 1997). Researchers must also be careful to ensure that bedding is dry, as wet bedding is a stressor (Kompagne et al., 2008; Matuszewich et al., 2007). In addition, researchers should reduce excessive movement and monitor housing racks for vibrations. Novel scents should be avoided, particularly when in the middle of conducting behavioral experiments. Enrichment should be provided with the goal being that the subjects are enriched, not just that they have more to do within their cage (Newberry, 1995). And finally, in studies of pain-related behavior, it is especially critical to house naïve animals with naïve animals, while housing treated animals with animals that are in the same treatment condition.

Confounds Arising during Experiments

Light

Although many still believe the myth that rodents do not rely heavily on vision, the perception of light is a critical entrainment cue for circadian rhythm in many species (for review, see Bahdra et al., 2017). While it is necessary to perceive light, light is also a known stressor for nocturnal rodents (Bowen et al., 2012; Walker & Davis, 1997), and this stressor has the capacity to influence pain-related behavior outside of experimental manipulations. This was demonstrated by Martenson et al. (2016), who provided evidence that light can activate at least a subset of pain-facilitating ON-cells in the rostral ventromedial medulla, which

produced hyperalgesia in otherwise naïve animals. Because light can produce stress (Bowen et al., 2012; Walker & Davis, 1997), and because stress can influence pain-related behavior (Bardin et al., 2009; Maier, 1986), alterations in light levels, changes in the time of day that the light comes on/off, or inconsistent lighting (i.e., flickering light), can become confounding. Routine checks of colony rooms at random times throughout the day can reveal instability in lighting conditions that may interfere with studies of pain-related behavior. Furthermore, researchers should be careful to provide light levels in methods sections, both from environmental light (i.e., light in the colony) as well as from any experimental light used, for the sake of reproducibility.

Keeping Track of Animal Welfare Across Experiments

Keeping track of animal issues, particularly when subjects are kept for longer experiments, can be difficult. However, taking care when noting these problems can help substantially when the decision to remove outliers arises. In order to keep track of animal issues, an “Animal Welfare Checklist” has been designed (Table 3) for use on test days that allows researchers to note likely behavioral results of confounds encountered in laboratories (i.e., over- or undergrooming, pica, etc.). These sheets can be referred to in the event that data analyses reveal outliers that may have resulted from these issues as opposed to an experimental manipulation.

Communicating Animal Needs with Animal Care Technicians

As studies within a laboratory expand and change, animal subjects' needs might also expand and change. Frequent communication with animal care technicians during these times is critical. However, both researchers and animal care technicians may find regular communication difficult due to demands on time. In order to assist with communications, a customizable "Animal Care Technician Handout" has also been provided (Table 4). This sheet was designed to be filled in with laboratory-specific information and posted in the colony room so that animal care technicians have easy access. It allows for easy sharing of critical information about the studies occurring in the laboratory, and categories can be adjusted by laboratory as needed.

Table 3: Animal Welfare Checklist

Experimenter:

Date:

Subject ID:

Signs of Distress and/or Pain

- ❒ Undergrooming
  Dirty/oily/stained fur

- ❒ Overgrooming
  Patches of fur thin and/or completely missing

- ❒ Pica, abnormal feeding behavior
  Chewing and swallowing of non-nutritive substances

- ❒ Excessive urination
  Continued urination that is not attributed to initial reactions to new environment

- ❒ Excessive defecation/watery stool
  Continued defecation that is not attributed to initial reactions to new environment

- ❒ Aggression toward experimenter, other rats in cage
  Rat attempting to bite aggressively, *hard biting*
  Not little nips of curiosity

- ❒ Panicked running
  When cage lid is opened, when hand reaches in, when put in weigh boat
  Not simple exploratory behavior

- ❒ Escape attempts
  When cage lid is opened, when hand reaches in, when put in weigh boat
  Not simple exploratory behavior

- ❒ Porphyrin stains—eyes, nose
  Red, watery substance when recent
  Reddish-brown, dry/crusty substance when older (mark "undergrooming" if seen)

- ❒ Additional Notes?
  _____
  _____

-----OR-----

- ❒ No significant signs of distress and/or pain observed

Table 4: Communicating animal needs with animal care technicians (page 1)

General Information

Principal investigator:
Office:
Ext:
Lab research focus: Mechanisms involved in pain modulation
Lab:
Extension:
Animal rooms:

Current lab members:

Animal: Male and female rats (Sprague Dawley)

Animal Conditions (By Room)

	0568 - Front Room	0571 - Back Room	0545B
Housing	Animals treated with inflammatory agent Animals that have undergone surgeries	All new arrivals Naïve (untreated) animals Saline-treated animals	Animals for specific project--will be moved from 0568 by experimenter
Food/water	No restrictions	No restrictions	No restrictions
Enrichment	White nyla bones (no green) Bedding material	White nyla bones (no green) Bedding material	White nyla bones (no green) Red enrichment square
Cage changes	Animal Care Techs	Animal Care Techs	Experimenter only
Study types	Typically chronic (~3-5 weeks)	Acute and chronic (~1-5 weeks)	Chronic (~3 weeks)
Most busy time	8:00 - 10:30 am	8:00 - 10:30 am	7:00 - 10:00 am

Table 4: Communicating animal needs with animal care technicians (page 2)

Things You May See in Our Animals (By Procedure)

Procedure	Animal's Typical Appearance	Animal's Typical Behavior
Nitroglycerin injection	General: Unchanged Potential: Small scar from injection	General: A little more skittishness Potential: Blood in stool
CFA injection (hindpaw)	General: Severe, long-lasting swelling of hindpaw Potential: Secondary injury from biting around injection site	General: Initially slow, behavior returns to normal Potential: Sluggish, hesitant on inflamed hindpaw
Stereotaxic viral injection	General: Scar, suture along skull, shaved scalp Potential: Scabbing, inflammation along suture	General: Initially slow, behavior returns to normal Potential: Sluggish, scratching at suture

General Concerns

1. In order to allow our animals to habituate fully, cages need to be returned to the same rack space they were taken from. We can mark the rack space on the cage cards if this will help.
2. Food should not be put into the holder on cage lids while the lid is on and the animals are still in the cage—the sudden loud noise can lead to stress.
3. Excess food that does not fit into the holder should be removed—it can raise the filter top, causing the entire cage to get jammed in the rack.
4. Experimenters will typically pick up their animals before 10:30 am, and many will not return to the housing area. Waiting until after that time to change cages will reduce the stress of being picked up multiple times in the same day as well as decrease the number of cages that actually need to be changed.
5. There is concern that animals in pain are negatively affecting the behavior of animals that are not in pain, and the bedding seems to contain these cues (Smith et al., 2016), so it is important to change gloves between working in the front and back rooms to avoid cross-contamination. Similarly, please try to keep the door between the rooms closed as much as possible.
6. The experimenter using 0545B is responsible for changing cages and is typically here 7 days a week. The project requires careful handling, removal, and storage of bedding, so if a cage needs to be changed immediately, please contact the lab. If unable to get in contact with the lab, please change the cage and let the lab know.

CHAPTER 5

Discussion

The study of pain-related behavior in laboratory rodents is necessary for the elucidation of neurological mechanisms underlying the development of chronic pain, and for drug discovery that may alleviate these conditions. However, a number of factors can influence pain-related behavior, some of which may confound results in the process, which may mask potentially viable treatments. Thus, it is equally necessary that these confounds be elucidated.

The Importance of Modeling and Measuring Pain in Laboratory Animals

In Chapter 2, I discussed how, in order to study cellular-, molecular-, and circuit-level neurobiological processes underlying chronic pain, animal subjects must be used as models. These models include, but are certainly not limited to, migraine (Storer et al., 2015) and chronic inflammation (Ren & Dubner, 1999). In developing animal models of various chronic pain disorders, it is not enough to simply apply a pain-inducing stimulus to a subject and note resulting behavior—models must appropriately create an experience for an animal that resembles that which a human in pain would experience.

In addition to appropriately modeling pain in laboratory animals, it is also necessary to appropriately measure pain-related behavior. While many well-established methods of behavioral analysis rely on reflexive behavior (i.e., von

Frey, tail-flick [Le Bars et al., 2001]), more researchers are calling for measures that address the affective and motivational elements of the pain experience, citing a lack of translational validity in behavior analyses of reflexive behavior in chronic pain states (Mogil and Crager, 2004; Mogil, 2009; Sadler, Mogil, & Stucky, 2022; Vierck, Hansson, & Yezierski, 2008; Vierck & Yezierski, 2015). However, established methods were originally designed for the assessment of behavior in acute pain states (and acute responses to analgesics) as opposed to chronic pain states (Le Bars et al., 2001). Therefore, the argument that these measurement methods are not ideal for studies of chronic pain states is valid, but it is not the only potential barrier to novel drug discovery and development for the treatment of chronic pain.

While the questioning of behavioral methodologies in the analysis of pain-related behavior in rodents is warranted, there are additional factors at work that may accelerate novel drug discovery and development if addressed. For example, designing individualized, mechanism-based treatments for chronic pain may reduce the number of novel treatments that are ultimately discarded after failing in the clinical phase. Publishing non-significant data, which provides a necessary context for significant data, should also be considered as a way of increasing the likelihood of finding novel treatments, as this would alleviate the need to repeat studies that other laboratories have already conducted. In addition, researchers must take responsibility for failures, as opposed to blaming the measures themselves. Finally, we must be clear about what the overall goal of our work is;

should we be attempting to extrapolate directly from animal behavior to human behavior, or are we missing a step?

The Confounding Nature of Light in Studies of Pain-Related Behavior

As discussed in Chapter 3, light is a documented stressor for the nocturnal rodents we routinely work with (Bowen et al., 2012; Walker & Davis, 1997), and stressors can produce hyper- or analgesia, depending on various factors (Bardin et al., 2009; Maier, 1986). Often times, light exposure is reported when light levels are manipulated as part of an experimental protocol. However, light exposure that occurs from non-experimental light (i.e., environmental light—light that an experimenter uses to conduct their experiment) is less often reported. Environmental light exposure is, therefore, a potential, under-reported confound in studies of pain-related behavior.

A study of the effect of environmental versus experimental light exposure on pain-related behavior in female adult Sprague Dawley rats indicated an impact of environmental light exposure. After being assigned to one of four light level groups (10, 100, 1000, or 2000 lux), otherwise naïve animals were handled, habituated, and tested in their assigned light condition for withdrawal thresholds on the von Frey apparatus. Environmental light exposure significantly influenced mechanical nociceptive threshold in baseline testing, prior to any exposure to experimental light (Fig. 3). Animals exposed to the highest environmental light level (2000 lux) had significantly higher thresholds than animals in the three other

environmental light level conditions, thus demonstrating the potentially confounding nature of non-experimental light exposure on baseline pain-related behavior.

The perception of controllability of a stressor can also lead to the development of hyper- or analgesia (Maier, 1986), and this raised the question of how subjects' baseline mechanical nociceptive thresholds might change in post-tests if animals were given a choice about being exposed to light. Subjects were then tested with a controllable experimental light source: the light switch-off box (LSOB), which provided an experimental light level of 100 lux. Animals exposed to an environmental light level of 2000 lux had significantly reduced mechanical thresholds following exposure to this controllable experimental light (Fig. 5). Therefore, perceived controllability of the light during light exposure can significantly impact tests of pain-related behavior. Additionally, animals exposed to the lowest environmental light level (10 lux) displayed significantly more photoavoidant behavior in the LSOB (Fig. 4), which indicated that handling, habituating, and testing rodents in an environmental light level that is lower than the planned experimental light level has the capacity to induce photoavoidant behavior. Taken together, this study demonstrates the potentially confounding nature of environmental light exposure (as opposed to experimental light exposure) in tests of pain-related behavior in female rats. Accurate and thorough reporting of all light levels that animals are exposed to during their time in the

laboratory, whether experimental light or not, is a necessity in reducing the number of confounds within data regarding pain-related behavior.

Non-Experimental Confounds in the Study of Pain-Related Behavior in Laboratory Animals

I discussed non-experimental confounds that can be introduced before animals even arrive at animal facilities in Chapter 4. For example, temperature extremes that non-FDA-covered species may be exposed to in transit can produce behavioral, physiological, and anatomical changes (Gordon, 1990; Gordon, 1993; Pennycuik, 1967) that may be misinterpreted as the result of experimental manipulations. Additionally, within-cage aggression resulting from exposure to stressful transit conditions (O'Kelly & Steckle, 1939) may induce long-lasting physical damage that may interfere with pain-related behavior testing. Crabbe et al. (1999) demonstrated that a substantial acclimation period could sufficiently overcome transit stress, but it is not clear if this is true for animals that have experienced within-cage aggression. Therefore, individual assessment of subjects exposed to this aggression is warranted prior to including them in experiments.

In the process of being housed, bedding-related problems can also confound data describing pain-related behavior. This is because bedding has multiple purposes beyond providing a more comfortable living space for subjects; it allows for nest-building, minimizes growth of micro-organisms, provides enrichment, and

more (Perkins & Lipman, 1995; Smith et al., 2004). Importantly, bedding can also assist with thermoregulation (Gordon, 2004), and behavior on tests of pain-related thermal sensitivity can be impacted by altered thermoregulatory ability (Le Bars et al., 2001). In addition to this confound, non-preferred bedding (i.e., corn cob) and/or wet bedding can also impact behavior, as these may both introduce stress (Krohn & Hansen, 2008; Perkins & Lipman, 1995). When providing bedding to newly arrived animals, it is important to not only understand the impact of bedding on thermoregulation, but to also understand and report any species-specific bedding requirements.

Appropriate, species-specific forms of enrichment are necessary in studies utilizing animal subjects (Newberry, 1995), but confounds can arise from enrichment. This may stem from the fact that there is disagreement about what constitutes “appropriate enrichment.” While some regard enrichment as additional items added to a cage that do not interfere with the behavior being tested, Newberry (1995) suggested that we retool our definition of “enrichment” to instead refer to the hoped-for outcome. Thus, enrichment is less about a specific item and more about the overall result for the subject—that their lives are enriched. Because there is not substantial agreement on what constitutes enrichment, items that are added to a cage may later be labeled as problematic. For example, Kimura et al. (2019) provided evidence that pain-related behavior was abolished in animals housed with complex (“Improved”) environmental enrichment. While this may be beneficial information for establishing appropriate

housing conditions for subjects in non-pain studies, complex environmental enrichment does have the capacity to interfere with studies of pain-related behavior.

Additional confounds in the study of pain-related behavior in laboratory animals include, but are not limited to, excessive cage movement/vibration (Seidel, 1993), exposure to novel scents (Deacon, 2006; Kovacevic et al., 2006; Leite et al., 2011), and housing naïve with pain-treated animals (Du et al., 2020; Langford et al., 2006; Li et al., 2014; Smith et al., 2016).

Future Directions

A popular, but unspoken, approach to research is to start with an assumption that the species being studied is fully understood, that behavior is consistent from subject to subject, and therefore, these animals are predictable. With that belief, the goal in working with these animals becomes to better understand humans and human conditions through use of the subjects, as opposed to understanding the subjects better and then considering relevance to humanity. However, if our animals were fully understood, then we would have no use for significance levels, confidence intervals, or error bars. If they were consistent, predictable, then there would be no outliers, no variance. We would be able to offer proof in the same way that a mathematician might, as opposed to needing a series of complicated statistics that ultimately amount to “probably.” We do have statistics, though, which clearly indicate that the subjects we work with are neither consistent nor

predictable. Thus, forward movement in the field of pain-related behavior and the eventual discovery/development of new treatments for chronic pain necessitates an acknowledgment that we do not know as much about the subjects we work with as we assume. We must also acknowledge that, had we understood these subjects better, novel behavioral tests such as the Grimace Scale might never have been touted as the solutions to the slow progress in discovering/developing treatments for chronic pain.

Our subjects cannot speak for themselves, but that does not mean that they have nothing to tell us. We can continue putting words into their mouths, justifying studies with anthropomorphism-driven rationales that bring us no closer to bringing relief to individuals who are suffering, or we can accept that we still have much to learn about (and from) these animals, then stop talking, and start paying attention. It is imperative that researchers in future studies either respect the unknowns of the species in question or design and implement studies that elucidate the unknowns prior to studying them.

Conclusion

Chronic pain has a negative impact on one in five adults in the United States, making chronic pain a national health concern (Institute of Medicine, 2011; Yong et al., 2022). In order to appropriately treat patients who are suffering, we must first develop novel treatments that treat not only the pain, but also associated symptoms, such as multisensory hypersensitivity (Schwedt, 2013). This has been

a difficult task though. Although many researchers have speculated about various reasons that treatments for chronic pain are lacking, it is more likely that the real reason is a combination of these suggestions—misunderstandings about what tests of pain-related behavior were initially designed for, less-considered factors such as where the responsibility for a measure not working actually lies, and confounds that may ultimately impact behavior before subjects officially enter into a study. Thus, each of us, at every level in the field of pain research, must exercise vigilance and diligence when attempting to elucidate the neurobiological mechanisms underlying these experiences in laboratory animals, being wary of both the potential barriers to translational validity and potential confounds that may arise outside of our control. Furthermore, we must accept responsibility where applicable for the state of the field and the resulting continued suffering of human patients, understanding that we are each accountable at the level we are at for moving the field forward.

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