VERIFICATION OF ANIMAL PAIN MODELS BY REVERSE TRANSLATION

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PUBLISHED IN: Frontiers in Pharmacology

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88971-801-6
DOI 10.3389/978-2-88971-801-6

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VERIFICATION OF ANIMAL PAIN MODELS BY REVERSE TRANSLATION

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Citation: Caudle, R. M., Romero-Sandoval, E. A., Smith, M. T., eds. (2021). Verification of Animal Pain Models by Reverse Translation. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88971-801-6

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frontiers in Pharmacology

EDITORIAL
published: 08 October 2021
doi: 10.3389/fphar.2021.778880

Editorial: Verification of Animal Pain Models by Reverse Translation

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Keywords: nociception, animal models, analgesic, behavioral testing, bedside to bench

Editorial on the Research Topic

Verification of Animal Pain Models by Reverse Translation

The validity of preclinical models in evaluating and developing potential analgesics has been an area of discussion for over a decade. The poor success of these models in predicting clinical efficacy (Woolf, 2010; Barrett, 2015; Burma et al., 2017) leads to the question as to whether the fault lies in the models not invoking the same pathology as the diseases being modeled, or if the failure is due to the differences in the physiology, anatomy, and pharmacology of the model species versus humans. An additional factor is that the design of proof-of-concept clinical trials of novel analgesic agents may have been sub-optimal (Hijma and Froeneveld, 2021). Ideally, the animal models being used for analgesic discovery would have the same pathophysiology as the human disease and would predict with 100 percent accuracy the efficacy of an agent in human trials. This ideal, however, is not realistic and compromises are necessary. However, repeated verification and optimization of the models can inform investigators of the limitations of the models, and by utilizing multiple models a realistic evaluation of the therapeutic potential of an agent can be realized. This research topic is devoted to outlining methods to increase confidence in the translatability of the data obtained in animal models of human pain conditions. An important aspect of this process is to use known information about human disease to improve the animal models, create new models, and to eliminate non-productive models. The papers in this research topic can be divided into three general categories of discussion (see **Figure 1**). The first category evaluates rodent models by reverse translation. Known therapies or known characteristics of the disease in humans are examined in established models to determine if there is a correlation between humans and rodents. The review by Fisher et al. evaluates neuropathic pain models and argues that matching quantifiable endpoints between the model and humans is critical to improving the translatability of the data obtained from the model. They further suggest, as do several of the authors in the topic, that pain suppressed behaviors may be superior to pain enhanced behaviors when determining therapeutic potential of an agent. The original research by Negus et al. addresses the affective/motivational aspect of pain when using a rodent model. In humans the distress induced by pain is often the most critically relevant feature of pain that determines the duration of disability or the quality of life for a patient. The authors present data indicating that rodents have a weak affective/motivational response to injury, suggesting that rodents may only model the sensory/discriminative aspect of pain; thus, making them less relevant as human models. This conclusion is countered by Cho et al. who suggest that a complete evaluation of the biopsychosocial model of pain in rodents is possible and can lead to a better understanding of pain and pain control in humans. They present methods of evaluating rodent behavior that provide insight into the psychological and social aspects of pain. The review by Pineda-Farias et al. evaluates models of cancer pain and provides hope that greater knowledge of how cancer and cancer therapy produce pain in humans will inform modeling in animals. The interesting review by Shen et al.

OPEN ACCESS

Edited and reviewed by:
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Specialty section:
This article was submitted to Translational Pharmacology, a section of the journal Frontiers in Pharmacology

Received: *17 September 2021*
Accepted: *28 September 2021*
Published: *08 October 2021*

Citation:
Caudle RM, Smith MT and Romero-Sandoval EA (2021) Editorial: Verification of Animal Pain Models by Reverse Translation. Front. Pharmacol. 12:778880. doi: 10.3389/fphar.2021.778880

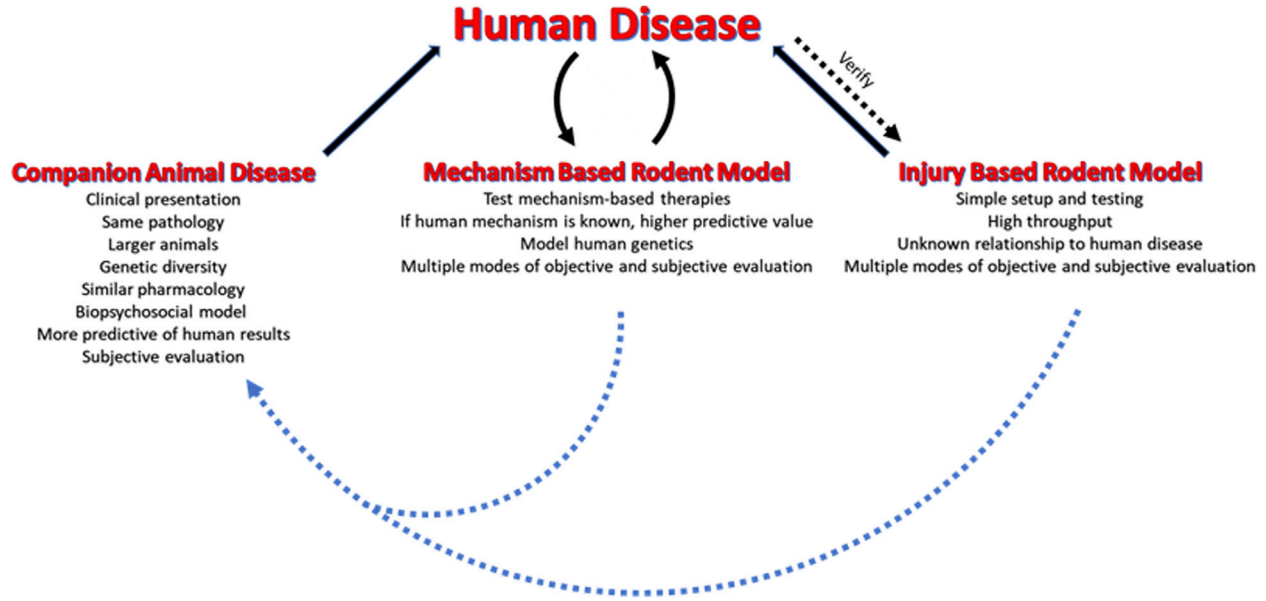

FIGURE 1 | Modeling human pain disorders in animals. Rodent injury models have been the standard for pain research and therapy evaluation. These models need to be compared to the human disease they model to ensure that they are predictive. The mechanism based rodent models are theoretically superior to the injury models due to their focus on specific mechanisms for producing pain. These models are developed based on known or suspected causes of the human disease. The companion animal models are most closely related to human disease and can bridge the gap between rodent studies and humans. If possible, it is recommended that companion animal clinical trials be conducted after the rodent studies and prior to initiating human trials.

examines complex multi tissue injuries associated with damaged bone. The authors argue that understanding traumatic injury in humans is necessary for developing animal models that are informative for therapy evaluation. Finally, the original research by Caudle et al. reexamines a model of migraine using an operant orofacial pain assay in rats to demonstrate that standard migraine rescue agents do not work in this model. Their findings indicate that the model may be inadequate for evaluating therapeutics for migraine headache. The second category of papers utilizes known facts about human disease to create novel more representative models of the disease. Castañeda-Corral et al. examine type 2 diabetes induced painful peripheral neuropathy in rodents by feeding a "cafeteria style" diet to rodents. The diet mimics a western diet, inducing weight gain, increasing blood sugar, and sensitizing the rodents to streptozotocin induced diabetes. The resulting diabetes produces a slow onset neuropathy that has many characteristics of human type 2 diabetes painful peripheral neuropathy. The work by Levine et al. creates a model of migraine headache using data from human studies suggesting that there is a dysregulation of the endocannabinoid system in migraineurs. Blocking diacylglycerol lipase, a key synthetic enzyme for generating 2-arachidonoylglycerol, produced facial mechanical allodynia and photophobia. Critically, there was no paw sensitivity produced by diacylglycerol lipase blockade indicating that the model was specific for trigeminal regions. The authors further demonstrated that the allodynia could be reversed with clinically used rescue agents. In Cho et al. the authors suggest that engineering models that genetically match humans with the disease and identifying ways to evaluate pain memory and affect are critical to improving rodent models. The final category in this topic describes using companion animals that have naturally contracted disease. Iadarola et al. provide an overview of their use of canine osteosarcoma and osteoarthritis patients to evaluate novel pain therapeutics following extensive testing in multiple rodent models. Similarly, Cho et al. consider companion animals as an intermediate step between rodents and humans. Both sets of authors indicate that these veterinary clinical trials provide strong verification of efficacy, dosing information, and toxicological information that significantly reduces the probability of failure in a human trial. Overall, the authors in this topic remain optimistic that the problems with animal models can be resolved with extensive feedback from human studies that inform the refinement of old models and the design of new models. It is also becoming clear that multiple rodent models and testing methods may be needed to capture the full complexity of human disease when assessing new agents, and routinely incorporating companion animal clinical trials, when possible, could further enhance the success rate of human trials.

AUTHOR CONTRIBUTIONS

RC wrote the manuscript. MS and ER edited the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

MINI REVIEW
published: 26 November 2020
doi: 10.3389/fphar.2020.610894

Animal Models of Cancer-Related Pain: Current Perspectives in Translation

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The incidence of pain in cancer patients during diagnosis and treatment is exceedingly high. Although advances in cancer detection and therapy have improved patient prognosis, cancer and its treatment-associated pain have gained clinical prominence. The biological mechanisms involved in cancer-related pain are multifactorial; different processes for pain may be responsible depending on the type and anatomic location of cancer. Animal models of cancer-related pain have provided mechanistic insights into the development and process of pain under a dynamic molecular environment. However, while cancer-evoked nociceptive responses in animals reflect some of the patients' symptoms, the current models have failed to address the complexity of interactions within the natural disease state. Although there has been a recent convergence of the investigation of carcinogenesis and pain neurobiology, identification of new targets for novel therapies to treat cancer-related pain requires standardization of methodologies within the cancer pain field as well as across disciplines. Limited success of translation from preclinical studies to the clinic may be due to our poor understanding of the crosstalk between cancer cells and their microenvironment (e.g., sensory neurons, infiltrating immune cells, stromal cells etc.). This relatively new line of inquiry also highlights the broader limitations in translatability and interpretation of basic cancer pain research. The goal of this review is to summarize recent findings in cancer pain based on preclinical animal models, discuss the translational benefit of these discoveries, and propose considerations for future translational models of cancer pain.

Keywords: cancer pain, animal model, cancer treatment, nociception, behavior

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Translational Pharmacology, a section of the journal Frontiers in Pharmacology

Received: *27 September 2020*
Accepted: *30 October 2020*
Published: *26 November 2020*

Citation:
Pineda-Farias JB, Saloman JL and Scheff NN (2020) Animal Models of Cancer-Related Pain: Current Perspectives in Translation. Front. Pharmacol. 11:610894. doi: 10.3389/fphar.2020.610894

INTRODUCTION

Cancer-related pain can occur at any time during the evolution of the disease (Caraceni and Shkodra, 2019). Many patients present with pain as the first sign of cancer, and 30–50% of all cancer patients will experience moderate to severe pain; frequency and intensity of pain can increase with cancer progression (Mercadante, 1997; Mercadante and Arcuri, 1998). Despite significant advances in cancer treatment as well as early detection, cancer-related pain treatment strategies remain limited. Opioids remain the current therapeutic regimen for cancer-related pain, based on the World Health Organization (WHO) analgesic ladder (Nersesyan and Slavin, 2007), despite debilitating side effects and inadequate efficacy (Mandala et al., 2006). The limited neurobiological understanding in

analgesia pharmacology has restricted novel therapeutic development; cancer pain treatments have relied largely on scientific advancements in other pain conditions.

Animal models of cancer-related pain have provided mechanistic insights into how cancer pain is generated and progresses under a constantly changing molecular architecture. *Cancer* pain is thought to result from processes involving crosstalk between neoplastic cells, the host's immune system, and peripheral and central nervous systems (Jimenez Andrade and Mantyh, 2010; Lozano-Ondoua et al., 2013; Schmidt, 2014). The field of cancer pain is just beginning to apply nociceptive behavioral assays to established rodent cancer models to reflect the symptoms experienced by patients. The goal of this review is to assess translational ability of the current animal models of cancer pain to the clinical presentation and provide a reference and direction for researchers studying cancer pain.

CLINICAL ASSESSMENT OF CANCER PAIN

Assessment of cancer-related pain is broken down into that arising directly from the tumor (85%), pain due to disease progression (9%), pain as a side effect of treatment (17%) (e.g., chemotherapy, surgical resection), and pain from other causes not related to malignancy (Grond et al., 1996). *Cancer* can affect any type of tissue, including viscera, bone, soft and nervous tissue. Pain can arise from the original site of the cancer (e.g., pancreas, head and neck) or from distant sites (e.g., bone), where common cancers metastasize (Coleman, 2006). *Cancer* patients often experience pain at multiple sites. Focal pain is experienced at a single site, usually in the region of the underlying lesion. Referred pain is denoted as progressive pain in a site lacking focal pathology (Caraceni and Portenoy, 1999; Portenoy and Ahmed, 2018). Physiological mechanisms in focal cancer pain are broadly described as nociceptive, inflammatory, or neuropathic (Falk and Dickenson, 2014). Nociceptive pain can be further classified into somatic and visceral. Somatic nociceptive pain is usually well localized and described as sharp, aching, throbbing, or pressure-like. When caused by obstruction, visceral nociceptive pain is often described as gnawing or crampy; when caused by involvement of organ capsules or mesentery, visceral pain may be aching, sharp, or throbbing. Neuropathic pain, defined as pain caused by a lesion or damage to the nervous system, is present in about 39% of patients including those with mixed pain (i.e., including both a nociceptive and neuropathic component) (Bennett et al., 2012); it is described as burning, tingling, or shock-like (lancinating) (Caraceni and Portenoy, 1999; Portenoy and Ahmed, 2018).

Due to disease progression and often resulting tissue damage, the temporal variation of cancer-related pain is classified as acute, chronic, or intermittent. Acute pain is defined by recent onset and brevity. Chronic cancer pain persists for three or more months, often increases with disease progression, and may regress with tumor shrinkage. Chronic pain may be associated with affective disturbances (e.g., anxiety, depression) as well as vegetative symptoms (e.g., anorexia, sleep disturbances) (Bennett et al., 2019). In the cancer population, a brief increase of intense pain in the presence of cancer pain successfully managed with opioid drugs is common and has been defined as "breakthrough" pain; it is estimated that more than one in two patients with cancer pain will also experience breakthrough pain (Portenoy and Hagen, 1990; Deandrea et al., 2014).

Advances in cancer treatment have significantly prolonged survival, and thus the appearance of pain as a sequela is becoming more prominent (Bennett et al., 2012; Liu et al., 2017). *Cancer* treatment-related pain may include bone pain immediately after radiotherapy (Loblaw et al., 2007; Hird et al., 2009) and post-surgical pain due to mastectomy, neck lymph node dissection, laparotomy, and thoracotomy or due to nerve sacrifice during surgery, such as post-thoracotomy pain syndrome (Brown et al., 2014; Liu et al., 2017). Mixed pain is common when caused by cancer treatments (Urch and Dickenson, 2008; Fallon, 2013); however, pain resulting from chemotherapy, termed chemotherapy-induced peripheral neuropathy (CIPN), is the most common and purely neuropathic in nature (Lema et al., 2010). CIPN usually exhibits dose-dependence and distribution through the upper and lower extremities. CIPN can persist for several months to years, even after discontinuation of chemotherapy. Symptoms can include sensory loss, paresthesia, dysesthesia, and pain (Loprinzi et al., 2007; Grisold et al., 2012; Sisignano et al., 2014).

ANIMAL MODELS OF CANCER-RELATED PAIN

To study cancer pain in the laboratory setting, several animal models have been developed over the last 2 decades to assess pain related to cancers in somatic and visceral tissues as well as cancer treatment related pain. These models seek to reflect the complex pain state observed clinically by measuring mechanical, thermal, and spontaneous pain-related behaviors.

Cancer-Related Pain Models

The transplantation model is the most popular model in focal and metastatic cancer pain research, and investigators have utilized several permutations. The two broad categories of transplant models are xenograft and allograft (i.e., syngeneic). Xenografts utilize commercially available human tumor cell lines; allografts use tumor cell lines derived from species with the same genetic background (e.g., mouse, rat). A variety of cell lines have been used, all of which have provided insights into the similarities and differences by which different tumors drive cancer pain; a comprehensive list is compiled in **Table 1**. The major benefits of transplantation models are easy replication, high modeling success rate, and high stability to facilitate the generation of abundant experimental mice. Orthotopic tumors provide a more comprehensive assessment of nociceptive behavior in response to tumor growth within an experimental environment more like the origin site (i.e. anatomical structure and sensory fiber innervation). Immunodeficiency is required for xenograft models, which is an important component when considering the multifaceted cancer pain phenotype. While immune cells are not absent in the cancer microenvironment (Chodroff et al.,

TABLE 1 | Transplantation cancer pain models.

Cancer type	Cell line	Injection site	References
		Xenograft transplant models	
Prostate	ACE-1, PC3, PPC-1	Bone, hindpaw	(Halvorson et al., 2005b; King et al., 2008; Thudi et al., 2008; Jimenez-Andrade et al., 2010)
Pancreas	SW 1990, Panc1, AsPc-1, Capan1	Pancreas, bone, sciatic, flank	(Wang et al., 2017; Miura et al., 2018; Han et al., 2020)
Melanoma	WM-127	Hindpaw	(Pickering et al., 2008)
Oral	HSC3, HSC2	Hindpaw, tongue, floor of mouth	(Pickering et al., 2008; Ye et al., 2011; Ye et al., 2012; Chodroff et al., 2016; Lam, 2016; Scheff et al., 2017)
Breast	MDA-MB-231-BO	Bone, mammary pad	(Hiraga et al., 2001; Bloom et al., 2011; Ungard et al., 2014; Zinonos et al., 2014)
		Allograft transplant models	
Pancreas	PANC02, KPC K4242	Pancreas	(Selvaraj et al., 2017)
Prostate	AT3B-1, AT-3.1, AT3B, MATLyLu	Bone	(King et al., 2008; Jimenez-Andrade et al., 2010; Jimenez-Andrade et al., 2011; Kolosov et al., 2011; Kolosov et al., 2012; Muralidharan et al., 2013; Gdowski et al., 2017)
Colon	Colon-26	Bone	(Sabino et al., 2003)
Breast	4T1, 66.1, MADB-106, MRMT-1, walker 256, MAT B III, EO771, erlich	Hindpaw, vibrissal pad, bone, mammary pad	(Medhurst et al., 2002; Zhang et al., 2005; Liu et al., 2010; Yin et al., 2010; Wang et al., 2012; Calixto-Campos et al., 2013; Lofgren et al., 2018; Appel et al., 2019)
Lung	Lewis lung, ETCC clone 1,642	Bone, hindpaw, back, sciatic nerve	(Constantin et al., 2008; Isono et al., 2011; Maeda et al., 2016; Xiao et al., 2016; Wakabayashi et al., 2018)
Oral	SCC-158	Gingiva, hindpaw	(Nagamine et al., 2006; Shinoda et al., 2008; Hironaka et al., 2014)
Melanoma	B16-F10, B16-BL6	Bone, hindpaw	(Kuraishi et al., 2003; Fujita et al., 2010; Gao et al., 2015)
Sarcoma	MC57G, NCTC2472, MethA	Bone, sciatic nerve	(Schwei et al., 1999; Honore et al., 2000a; Honore et al., 2000b; Cain et al., 2001; Luger et al., 2001; Wacnik et al., 2001; Wacnik et al., 2003; King et al., 2007; Hald et al., 2009a; Hald et al., 2009b), (Halvorson et al., 2005a; Sevcik et al., 2005a; Wacnik et al., 2005a; Sevcik et al., 2005b; Wacnik et al., 2005b; Mouedden and Meert, 2005; Shimoyama et al., 2005), (Sabino et al., 2003)
Bladder	MBT-2	Bladder	(Roughan et al., 2004b; Roughan et al., 2014)
Myeloma	5TGM1-GFP	Systemic, bone	(Diaz-delCastillo et al., 2020)

2016), the loss of important signaling lymphocytes may lead to clinically irrelevant infiltration and neuro-immune communication.

To date, genetic cancer models have only been utilized to study pancreatic cancer pain [e.g., SV40 (Saenz Robles and Pipas, 2009), KPC (Biankin et al., 2012; Singhi et al., 2019)]. Genetically engineered models allow for the study of pain and neuroplasticity throughout the initiation and transformation of normal cells to malignancy as well as natural dissemination and metastasis. Typically, genetic models closely recapitulate human disease because they are based on specific genetic mutations that have been documented in patient populations. One of the greatest benefits of genetically engineered models is that the natural microenvironment remains intact, which permits modeling of complex processes that require interactions between multiple cells types (e.g., axonogenesis, metastasis, immune regulation). However, maintaining genetic models presents practical challenges. Several models have an average age of onset between 40 weeks and 20 months (Ding et al., 2016). Furthermore, as the number of transgenic alleles in a model increases, the cost-effectiveness and breeding efficiency decrease, making them expensive and time-consuming. Thus, while genetic models can play an important part in understanding biological mechanisms and pharmacology, they may not be ideal for high throughput testing (Webster et al., 2020).

Chemical-induced carcinogenesis has been utilized to study oral cancer pain (e.g., 4-nitroquinoline 1-oxide (4NQO) (Lam et al., 2012; Scheff et al., 2017; Scheff et al., 2018)) and colon cancer pain [e.g., azoxymethane (AOM) and dextran sulfate sodium (DSS) (Chartier et al., 2020)]. Like genetic models, chemical models allow for the study of pain development during the multistage dynamic carcinogenicity from initiation and progression. While some studies have employed local exposure (Bersch et al., 2009), a major benefit of these models is that the chemical is typically given systemically (e.g., drinking water) and can be used across multiple species. However, to date, cancer-related pain behavior has only been assessed in mouse and rat models. Additionally, due to uncontrolled exposure to the chemical, not all animals develop the same lesion at the same time, and a variety of lesions can be seen in a single rodent. While this can be clinically relevant as multiple primaries do occur in patients, high variability in the site and number of lesions between animals can also severely limit interpretations of pharmacology and behavioral studies. Unintended esophageal and gastrointestinal lesions are also possible and may confound results using spontaneous pain behavior assays.

The most common treatment-related cancer pain modeled in animals is CIPN, wherein chemotherapeutic agents (e.g., paclitaxel, oxaliplatin) are administered to animals resulting in dose-dependent damage to peripheral nervous system. The severity and temporal dynamics of neuropathy depend on the type of neurotoxic antineoplastic agent, dosage, and route of administration. CIPN rodent models have been extensively devised and studied in the last 30 years (Cavaletti et al., 2019). There is a wide variety of strains utilized, chemotherapeutic agents and routes of administration employed which have been systematically reviewed here (Gadgil et al., 2019). Many advantages are typical of these models: small size and prolific nature of the animals, ease of handling, along with the availability of reliable methods for peripheral nerve assessment. One of the most important benefits of this model is reproducibility within consistent methodology. Mouse CIPN models utilizing either paclitaxel or cisplatin have high efficacy in causing CIPN across sex and strains which is similar to clinical studies demonstrating a high incidence of CIPN in patients treated with these agents (Seretny et al., 2014; Molassiotis et al., 2019). However, one of the model's major limitations is the high degree of variability regarding the chemotherapeutic agents used as well as the dose and route of administration chosen. Inconsistency in the model characteristics makes pharmacological conclusions across publications impossible to interpret.

Cancer-Related Nociceptive Behavior Assays

Due to the variety of clinical presentations and comorbidities (e.g., affective components) in cancer-related pain, choosing among nociceptive assays remains challenging. A comprehensive list is compiled in **Table 2**. The most used behavioral assay for cancer pain is evoked mechanical sensitivity measured by quantification of responses to the application of standardized von Frey monofilaments to or near the site of inoculation (e.g., hindpaw, pancreas, vibrissal pad). One benefit of this assay is its common use in non-cancer pain literature. However, subjectivity limits interpretation of results. An animal's lack of response to a noxious stimulus could indicate analgesia, paralysis, sedation, or lack of motivation; additional tests must screen for side effects that might confound the behavioral result. The alignment of animal research and clinical practice requires the use of similar behavioral endpoints. Hence, function-based tests, which facilitate the identification of drugs that inhibit nociception in the absence of disruptive side effects, are growing in popularity. Operant and function-related assays [e.g., gnawing (Dolan et al., 2010), wheel running (Tang et al., 2016), grid climbing (Falk et al., 2017)] have been used as an index of cancer-related pain; many of these behaviors and their clinical relevance have been thoroughly reviewed here (Tappe-Theodor et al., 2019).

Spontaneous pain behavior is one of the most difficult components of cancer pain to manage and is therefore the most consistent end point against which the efficacy of a candidate drug is tested. The major limitation to using spontaneous pain as the end point is that the methodology differs substantially in the literature; spontaneous cancer-related pain has been measured indirectly using hunching (Lindsay et al., 2005a; Sevcik et al., 2006; Stopczynski et al., 2014; Wang et al., 2017; Kajiwara et al., 2020), open field activity (Stopczynski et al., 2014; Selvaraj et al., 2017; Hirth et al., 2020), home cage activity (Selvaraj et al., 2017; Hirth et al., 2020), voluntary wheel running (Selvaraj et al., 2017), vocalizations (Lindsay et al., 2005a; Sevcik et al., 2006), conditioned place preference (Selvaraj et al., 2017) as well as impressions of appearance [e.g., grimace scale (Kajiwara et al., 2020), coat condition (Roughan et al., 2004a)]. While many of these

TABLE 2 | Cancer-related nociceptive behavior assays.

Pain type	Behavior	Pain assay	References
CIBP	Mechanical	Von frey	(King et al., 2007; King et al., 2008; Hald et al., 2009b; Ungard et al., 2014; Gdowski et al., 2017; Remeniuk et al., 2018)
	Thermal	Hargreaves, acetone, hotplate	(King et al., 2007; Miao et al., 2010)
	Spontaneous	CPP, open field activity, flinching, guarding, weight bearing, gait analysis, vocalizations, burrowing	(King et al., 2007; King et al., 2008; Ungard et al., 2014; Slosky et al., 2016; Gdowski et al., 2017; Remeniuk et al., 2018; Zhang et al., 2018; Buehlmann et al., 2019; Miladinovic et al., 2019; Sliepen et al., 2019)
	Function-induced	Forced limb use (limping, guarding), grip force, grid-climbing	—
	Breakthrough	Condition place aversion	(Havelin et al., 2017)
Oral cancer	Mechanical	Von frey	(Pickering et al., 2008; Ye et al., 2018; Salvo et al., 2020)
	Thermal	Hargreaves	(Ye et al., 2012; Ye et al., 2014; Salvo et al., 2020)
	Function-induced	Dolognawmeter	(Ye et al., 2011; Lam et al., 2012; Scheff et al., 2017; Scheff et al., 2018; Ye et al., 2018; Scheff et al., 2020)
	Spontaneous	CPP	(Chodroff et al., 2016; Scheff et al., 2019)
Pancreatic cancer	Mechanical	Von frey, visceromotor reflex	(Han et al., 2016; Wang et al., 2017)
	Spontaneous	Home cage activity, open field activity, hunching, vocalizations, grimace, wheel running, CPP	(Selvaraj et al., 2017; Wang et al., 2017)
CIPN	Mechanical	Von frey, randall-selitto	(Casals-Diaz et al., 2009; Han et al., 2018; Kyte et al., 2018; Laumet et al., 2019; Luo et al., 2019; Ma et al., 2019; Shahid et al., 2019; Bruna et al., 2020)
	Thermal	Hargreaves, hot plate, acetone, cold plantar test	(Chine et al., 2019; Luo et al., 2019; Shahid et al., 2019; Tonello et al., 2019; Bruna et al., 2020)
	Function-induced	Sensory and motor nerve conduction, functional autonomic tests, rota-rod, gait analysis	(Liu et al., 2018; Laumet et al., 2019; Luo et al., 2019; Bruna et al., 2020)
	Spontaneous	Burrowing, wheel running, CPP, adhesive recognition test	(Park et al., 2013; Flatters et al., 2017; Laumet et al., 2019; Toma et al., 2019; Bruna et al., 2020)
Colon cancer	Spontaneous	Burrowing, grimace	(Chartier et al., 2020)
Breast cancer	Mechanical	Von frey, hargreaves	(Lofgren et al., 2018)
	Spontaneous	Open field activity	(Lofgren et al., 2018)
Bladder cancer	Thermal	Hargreaves	(Roughan et al., 2014)
	Spontaneous	Open field, CPP, hunching, vocalization	(Roughan et al., 2004b; Roughan et al., 2014)

natural animal behaviors have minimal operator influence and can be translated to clinical representation of cancer-related pain, high variability of scoring criteria across studies and indirect output greatly limits the ability to assess the therapeutic impact. Additionally, distinguishing between spontaneous and "breakthrough" pain remains challenging, though recently an adaptation to the conditioned place aversion assay has been validated to measure movement-evoked breakthrough pain specifically (King et al., 2007; Havelin et al., 2017).

Pharmacology From Bench to Bedside

Nerve growth factor (NGF) has been considered the most potent pain inducer across multiple cancer models and currently holds the most promise for management of pain associated with cancer initiation and progression. Inhibition of NGF binding to the receptor TrkA in preclinical models strongly reduced mechanical, thermal and spontaneous facets of cancer-induced bone pain (Halvorson et al., 2005b; Jimenez-Andrade et al., 2011; Buehlmann et al., 2019), pancreatic cancer pain (Stopczynski et al., 2014; Amit et al., 2019), and oral cancer pain (Ye et al., 2011). A phase II clinical trial using anti-NGF antibody, fulranumab, as adjunctive therapy for cancer-related pain found no significant effect on pain intensity via visual analog scale; however, significant improvement on the Brief Pain Inventory subscales suggested improved quality of life (Slatkin et al., 2019). Additionally, there are two ongoing clinical trials, one testing the analgesic efficacy of a TrkA inhibitor on cancer patients with solid tumors or lymphoma (phase I, NCT03556228) and the other measuring the efficacy of anti-NGF monoclonal antibody tanezumab in the treatment of cancer pain due to bone metastasis in patients already taking background opioid therapy (phase III, NCT02609828).

For treatment-related cancer pain, accumulating evidence indicates that the initiation and progression of CIPN are tightly related with chemotherapeutic agent-induced impairment of intraepidermal nerve fibers (IENF) (Koskinen et al., 2011), oxidative stress (Butturini et al., 2013), abnormal spontaneous discharge, ion channel activation (Zhang and Dougherty, 2014), up-regulation of various pro-inflammatory cytokines, and activation of the neuro-immune system (Sisignano et al., 2014; Makker et al., 2017). A phase III clinical trial using duloxetine, a serotonin and norepinephrine reuptake inhibitor, for treatment of pain associated with CIPN found that the use of duloxetine compared with placebo for 5 weeks resulted in a greater reduction in pain (NCT00489411). Calmangafodipir, mimicking the mitochondrial enzyme manganese superoxide dismutase, is currently involved in two ongoing clinical trials to establish the efficacy in prevention of chronic CIPN induced by oxaliplatin (phase III, NCT03654729 and NCT04034355).

CONSIDERATIONS FOR REVERSE TRANSLATION

Despite the large amount of human and experimental studies, no effective prophylactic treatment exists for cancer-related pain, and treatments (e.g., opioids) remain flawed. Identification of new targets for novel therapies to treat cancer-related pain requires models that better recapitulate interactions between cancer and its microenvironment, along with standardization of such assays and methodologies. Since so many promising studies fail in clinical translation, one must question the inherent translatability of the models themselves. While cancer-evoked nociceptive responses in animals echo some of the patients' symptoms, the current models fail to address the complexity of interactions within the natural disease state. One of the major hypotheses for the etiology of focal cancer pain is cancer-secreted mediator-induced activation of the sensory nerve fibers innervating the cancer microenvironment (Jimenez Andrade and Mantyh, 2010; Schmidt, 2014; Lam, 2016). Therefore, anatomic site and neoplastic cell type should not be taken for granted when considering the translational relevance of the cancer pain model. Secreted mediators can differ depending on the cancer cell type (Sabino et al., 2003; Scheff et al., 2017; Scheff et al., 2020). Cancer-induced bone pain literature includes the most heterogeneity regarding cancer cell lines used (Currie et al., 2013). Substantial variability in pain behaviors and pharmacology may be attributed to the cancer cells selected to interact with peripheral nociceptive neurons; for this model multiple cancer cell lines might be required to determine if the findings are specific to one type of cancer or can be generalized to all bone metastasis. Similarly, variability in dosing and chemotherapeutic agent used in CIPN animal models could affect the consistency of findings (Gadgil et al., 2019).

To replicate symptoms observed in patients, reverse translation requires characterization of the pain associated with cancer or cancer treatment as either nociceptive, neuropathic, or mixed. Measures like numbness, tingling and ongoing pain rely on verbal report from the patient and often occur spontaneously. Fortunately, investigations into novel measures of ongoing pain in rodents are emerging (Tappe-Theodor and Kuner, 2014). A combination of pain assays including spontaneous pain should be used to demonstrate the translation of the cancer pain model to the clinical representation. However, consistency in criteria to score spontaneous pain across models is needed. Additionally, the stage of cancer progression at which pain develops in the animal model should align with the clinical representation. For example, oral squamous cell carcinoma pain in patients is thought to develop during the transition from precancerous lesion to malignancy (Lam and Schmidt, 2011). The 4NQO carcinogen model appears to be clinically representative with function-induced nociceptive behavior initiating at early stages in tumorigenesis (Scheff et al., 2017; Scheff et al., 2018); however, nociceptive behavior in an orthotopic xenograft mouse model does not develop for up to 14 days after inoculation (Ye et al., 2018; Scheff et al., 2019) suggesting that this model is more appropriate for pharmacological approaches to treat cancer-related pain at later stages in the disease when tumor burden is an active component. Lastly, the impact of age (Fujii, 1991; Lindsay et al., 2005b; Oh et al., 2018), sex (Scheff et al., 2018; Scheff et al., 2019; Rubin et al., 2020) and rodent strain (Vermeirsch and Meert, 2004; Zhang and Lao, 2012; Ono et al., 2015) can greatly

impact both tumor development and nociceptive behavior and should be taken into consideration when designing a study. The cancer-related pain field needs to work together to standardize the methodology regarding both animal models and pain assays to increase the potential for reproducibility and clinical translation.

The cancer biology field is rapidly growing, and advances in cancer detection and therapy have improved patient prognosis. Preclinical models of several cancers [e.g., oral (Li et al., 2020), pancreas (Yin et al., 2015; Bisht and Feldmann, 2019), breast (Whittle et al., 2015; Holen et al., 2017)] have been extensively studied to determine a suitable research animal model that reflects the intricacies of cancer biology. In order to fully match the achievements in the cancer field broadly and understand the pain that may develop prior to detection or in response to treatments, we need to integrate the animal models most commonly used in cancer biology into the cancer-related pain field. Assimilation and standardization across both fields will allow for better translational findings across preclinical and clinical modalities. For example, patient-derived xenograft models (Aparicio et al., 2015) present a potential opportunity for patient-reported pain to be recapitulated in a transplantation mouse model with preservation of the genotypic and phenotypic diversity of the original tumor tissue. It is also imperative to consider facets of tumor biology beyond the nociceptive system (i.e., tumor growth, immune response) in pharmacological studies related to cancer pain; novel cancer pain therapies should not exacerbate cancer progression and interpretation of analgesia should be considered along with tumor size. Lastly, reverse translation could also be improved through the inclusion of large animal models [i.e., porcine (Robertson et al., 2020), canine (Kamano et al., 1988)]. There have been significant advances in veterinary oncology as well as validation of pain scales for companion animals (Brown et al., 2015; Lascelles et al., 2019) which provides an opportunity to study spontaneous cancer pain in larger species (Brown et al., 2015; Brown, 2016; Monteiro et al., 2018). To date, the cancer-related pain field has yet to integrate standardized pain assessment instruments for large animals (Henze and Urban, 2010; Viscardi et al., 2017; Lascelles et al., 2019; Luna et al., 2020).

CONCLUSION

All cancer-related pain models have advantages and disadvantages, and there is no ideal cancer-related pain model that will perfectly recapitulate the human experience. The appropriate model depends on the cancer-related pain condition and the specific methods used. Despite limitations, the field has begun to provide insight into the mechanisms that generate and maintain cancer-related pain while discovering potential therapeutic strategies to treat it. Additionally, there has been a recent surge in data suggesting that manipulation of neuronal activity by cancer cells may be a central mechanism for cancer progression (Faulkner et al., 2019; Zahalka and Frenette, 2020). Thus, targeting sensory neurons in the cancer microenvironment may be a potentially actionable therapeutic strategy to stop cancer pain as well as slow cancer growth. As this new field of cancer neurobiology emerges, full collaboration between cancer biologists, neurobiologists and immunologists is pivotal for success.

AUTHOR CONTRIBUTIONS

All authors, JP-F, JS, NS, drafted the work, contributed to work design, revised it, and approved the final version to be published and are accountable for all aspects of the work.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 03 December 2020
doi: 10.3389/fphar.2020.527495

Pharmacological Characterization of Orofacial Nociception in Female Rats Following Nitroglycerin Administration

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Rodent models of human disease can be valuable for understanding the mechanisms of a disease and for identifying novel therapies. However, it is critical that these models be vetted prior to committing resources to developing novel therapeutics. Failure to confirm the model can lead to significant losses in time and resources. One model used for migraine headache is to administer nitroglycerin to rodents. Nitroglycerin is known to produce migraine-like pain in humans and is presumed to do the same in rodents. It is not known, however, if the mechanism for nitroglycerin headaches involves the same pathological processes as migraine. In the absence of known mechanisms, it becomes imperative that the model not only translates into successful clinical trials but also successfully reverse translates by demonstrating efficacy of current therapeutics. In this study female rats were given nitroglycerin and nociception was evaluated in OPADs. Estrous was not monitored. Based on the ED_{50} of nitroglycerin a dose of 10 mg/kg was used for experiments. Sumatriptan, caffeine, buprenorphine and morphine were administered to evaluate the reverse translatability of the model. We found that nitroglycerin did not produce mechanical allodynia in the face of the rats, which is reported to be a consequence of migraine in humans. Nitroglycerin reduced the animals' participation in the assay. The reduced activity was verified using an assay to measure exploratory behavior. Furthermore, the effects of nitroglycerin were not reversed or prevented by agents that are effective acute therapies for migraine. Two interesting findings from this study, however, were that morphine and nitroglycerin interact to increase the rats' tolerance of mechanical stimuli on their faces, and they work in concert to slow down the central motor pattern generator for licking on the reward bottle. These interactions suggest that nitroglycerin generated nitric oxide and mu opioid receptors interact with the same neuronal circuits in an additive manner. The interaction of nitroglycerin and morphine on sensory and motor circuits deserves additional examination. In conclusion, based on the results of this study the use of nitroglycerin at these doses in naïve female rats is not recommended as a model for migraine headaches.

Keywords: nitroglycerin, orofacial pain, operant assay, migraine, rodent, female

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Edited by:
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Specialty section:
This article was submitted to Translational Pharmacology, a section of the journal Frontiers in Pharmacology

Received: *16 January 2020*
Accepted: *19 October 2020*
Published: *03 December 2020*

Citation:
Caudle RM, Caudle SL, Flenor ND, Rohrs EL and Neubert JK (2020) Pharmacological Characterization of Orofacial Nociception in Female Rats Following Nitroglycerin Administration. Front. Pharmacol. 11:527495. doi: 10.3389/fphar.2020.527495

INTRODUCTION

Migraine headaches are one of the most common debilitating chronic pain conditions, affecting more than ten percent of the global population (Woldeamanuel and Cowan, 2017). Triptans, opioids, NSAIDs and caffeine are commonly used to abort migraine headaches and are relatively effective, but prevention of migraines remains problematic. Recently, botulinum toxin, calcitonin gene-related peptide (CGRP) antagonists and antibodies to CGRP and CGRP receptors have been developed for long term control of migraine headaches (Matak et al., 2011; Dodick et al., 2014a; Dodick et al., 2014b; Lupi et al., 2019). However, these therapies typically reduce the incidence of migraine by just a few attacks per month (Dodick et al., 2019; Urits et al., 2019), indicating that extended therapy for chronic migraine is still not satisfactory. Given the debilitating nature of migraine headaches and the large number of people who suffer from the disease novel therapies are needed.

One common model for migraine headache drug discovery programs is to give rodents a nitric oxide donor such as nitroglycerin to produce headaches. Nitroglycerin induces the activation of guanylate cyclase, stimulating cGMP formation, which in turn leads to a vasodilatory effect (Seiler et al., 2013). Nitroglycerin is usually prescribed for angina patients (Bray et al., 1991), but headaches are a frequent side effect (Schwartz, 1946). This side effect led to nitroglycerin being used to model migraine headaches. However, because the pathologic mechanisms for migraine headache are not well established and nitroglycerin produces global pain in the head rather than focal pain like migraines it is not clear that nitroglycerin is truly modeling the disease. Furthermore, in a recent study triptans did not reverse nitroglycerin induced headaches in humans even though they are a highly effective abortive therapy for the majority of migraine sufferers (Tvedskov et al., 2010). A previous study by the same researchers, however, did indicated some degree of headache prevention when sumatriptan was given prior to nitroglycerin in healthy volunteers (Iversen and Olesen, 1996). These observations suggest that nitroglycerin in humans may not be an accurate model of migraine headache.

Despite the differences between nitroglycerin induced headache and migraine headache a significant number of migraine studies have utilized rodent nitroglycerin models. These studies often demonstrate that triptans reverse nitroglycerin induced hypersensitivity in rodents using reflex based thermal and mechanical sensitivity on the limbs or tail rather than examining sensitivity in trigeminal nerve territories, which would be more appropriate for migraine (Tassorelli et al., 2003; Bates et al., 2010; Tipton et al., 2016; Moye et al., 2019). Other studies utilized von Frey mechanical assays in the periorbital region of rodents (Di et al., 2015; Kopruszinski et al., 2017; Marone et al., 2018). These experiments rely on the suppression of a pain induced enhancement of a behavioral response, such as a shortened paw or head withdrawal latency from a heat or mechanical stimulus. The animal's response to the stimulus must be interpreted by an observer leading to the possibility that any sedation or change in motor function is interpreted as an anti-nociceptive response in these models. Furthermore, it is difficult to blind the investigators running the assay to the rodents' treatment due to several characteristic behaviors displayed by the rodents when they are treated with nitroglycerin, e.g., eye squinting, stretching, and reduced motor activity. These behaviors may bias the investigator during the testing process and have little to do with the desired behavioral outcome. Another issue with the use of investigator evoked responses in rodents is that nitroglycerin may produce a state of hypervigilance in which the animals respond to the detection of the stimulus rather than responding only to nociceptive stimuli. The hypervigilance may lead to the misinterpretation of the rodent's experience with the stimulus and any agent that reduces the animal's anxiety may appear to be analgesic in these assays.

To address the adequacy of nitroglycerin in rats as an acute model of human migraine headache this study evaluated the model pharmacologically in an operant assay using Orofacial Pain Assessment Devices (OPAD, Stoelting, Co.). The hypothesis tested was that agents that can abort human migraine headaches would disrupt nitroglycerin induced headaches in rats. The advantages of the OPAD testing system are that 1) it assesses nociception in trigeminal nerve territories, which is more relevant to migraine than limb sensitivity; 2) data collection is automated so that investigator bias is reduced; 3) the assay utilizes a behavior that is suppressed by pain so that effective analgesics restore the behavior; thus, sedation or motor impairment do not register as analgesic effects; 4) the assay utilizes a rodent initiated behavior rather than an investigator evoked behavior which reduces the impact of rodent hypervigilance; and 5) the assay is an operant reward/conflict type of assay that quantifies the full experience of the pain including the nociceptive, and cognitive/emotional elements of the experience rather than measuring a simple reflex arc (Neubert et al., 2005; Neubert et al., 2006; Rossi et al., 2006; Neubert et al., 2007; Neubert et al., 2008; Rossi and Neubert, 2008; Rossi et al., 2009; Caudle et al., 2010; Kumada et al., 2012; Nolan et al., 2011; Nolan et al., 2012; Ramirez and Neubert, 2012; Rossi et al., 2012; Anderson et al., 2013; Mustafa et al., 2013; Anderson et al., 2014; Ramirez et al., 2015; Bowden et al., 2017; Caudle et al., 2017; Sapio et al., 2018). By evaluating agents that are effective in the acute clinical management of migraine headache in humans in this rat nitroglycerin model the validity of the model was tested. The results of this reverse translation study indicate that the treatment of rats with nitroglycerin is not likely to be a valid model of human migraine headache.

MATERIALS AND METHODS

Animals

Female Sprague Dawley rats (200–350 g, Charles Rivers) were utilized for the experiments. The animals were fed standard rodent chow and water ad libitum throughout the study and were housed in pairs at 22°C with 30% humidity. The rooms were

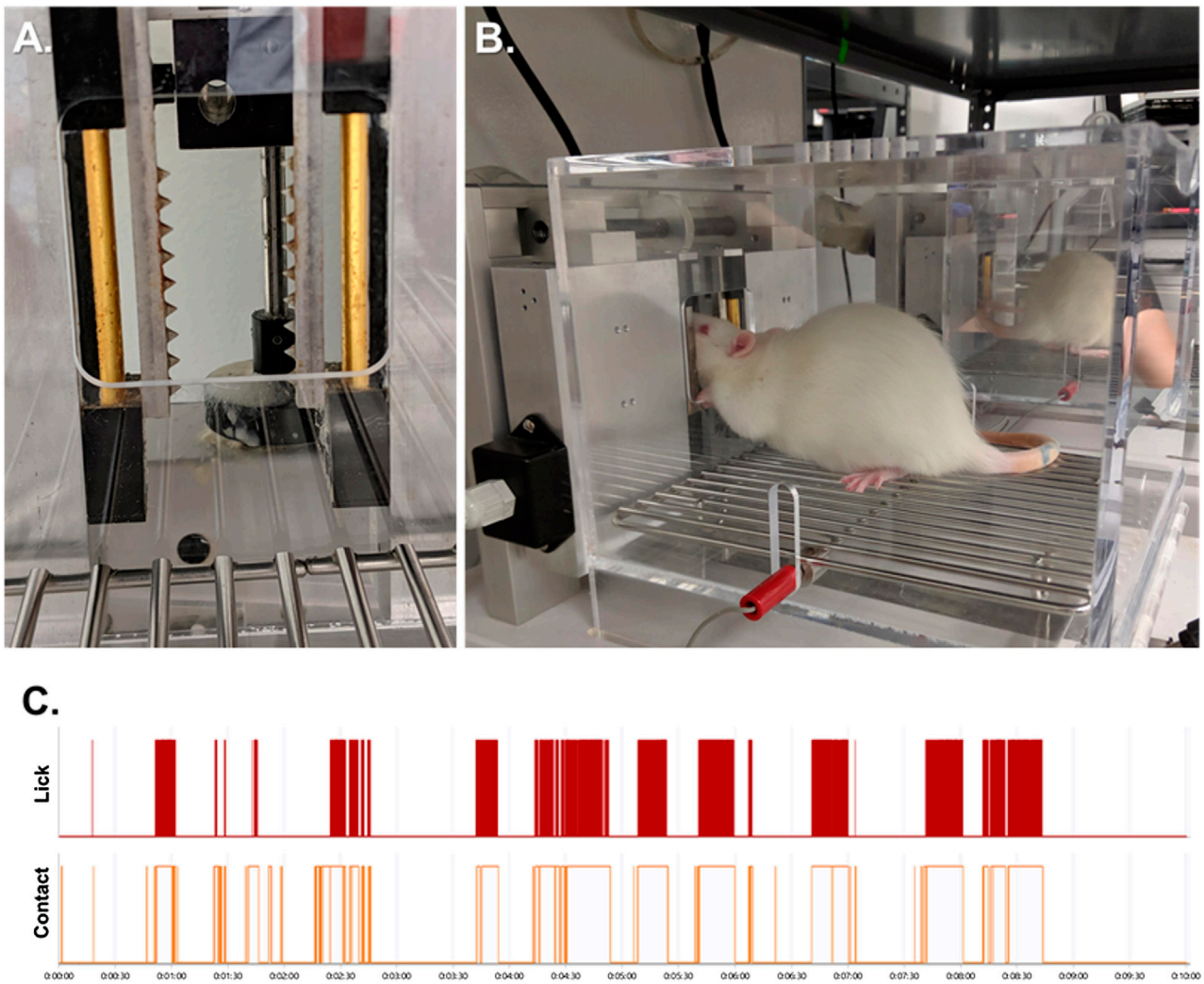

FIGURE 1 | The Orofacial Pain Assessment Device (OPAD) for Mechanical Nociception. **(A)** Spiked bars that the rats must press their faces against to obtain a reward solution. **(B)** Example of a rat performing the OPAD assay. **(C)** Examples of raw licking and stimulus contact data collected in the OPAD during a 10-min testing session.

on a 12-h light/dark cycle (7 AM–7 PM lights on). The animals were not food or water restricted prior to training or testing and estrus cycles were not monitored. All experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals and were reviewed and approved by the University of Florida Institutional Animal Care and Use Committee.

Orofacial Pain Assessment Device Behavioral Assay

Standard OPADs (Stoelting Co.) were fitted with spiked bars on each side of the reward solution access window (**Figure 1A**). The spikes on the bars were spaced 5 mm apart and extended 3 mm into the opening. The bars were positioned relative to the reward solution bottle so that to obtain the reward the rats had to press their faces firmly against the spikes (**Figure 1B**). The reward solution was sweetened condensed milk diluted with water (1 milk: 2 water) and the stimulus bars were kept at room temperature (~22–23°C).

Rats were trained daily in the OPADs between the hours of 10:00 to 12:00 Monday through Friday. Ten training sessions were completed prior to beginning experiments. Experiments were also conducted between 10:00 and 12:00 and each session was 10 min in duration. The rodents' licks on the reward bottle and their contact with the stimulus bars were recorded electronically using AnyMaze software (Stoelting Co.). An example of the data collected is presented in **Figure 1C**. The use of the OPAD assay has been described extensively in our previous publications (Neubert et al., 2005; Neubert et al., 2006; Neubert et al., 2007; Neubert et al., 2008; Rossi and Neubert, 2008; Rossi et al., 2009; Caudle et al., 2010; Kumada et al., 2012; Nolan et al., 2011; Anderson et al., 2012a; Anderson et al., 2012b; Nolan et al., 2012; Ramirez and Neubert, 2012; Rossi

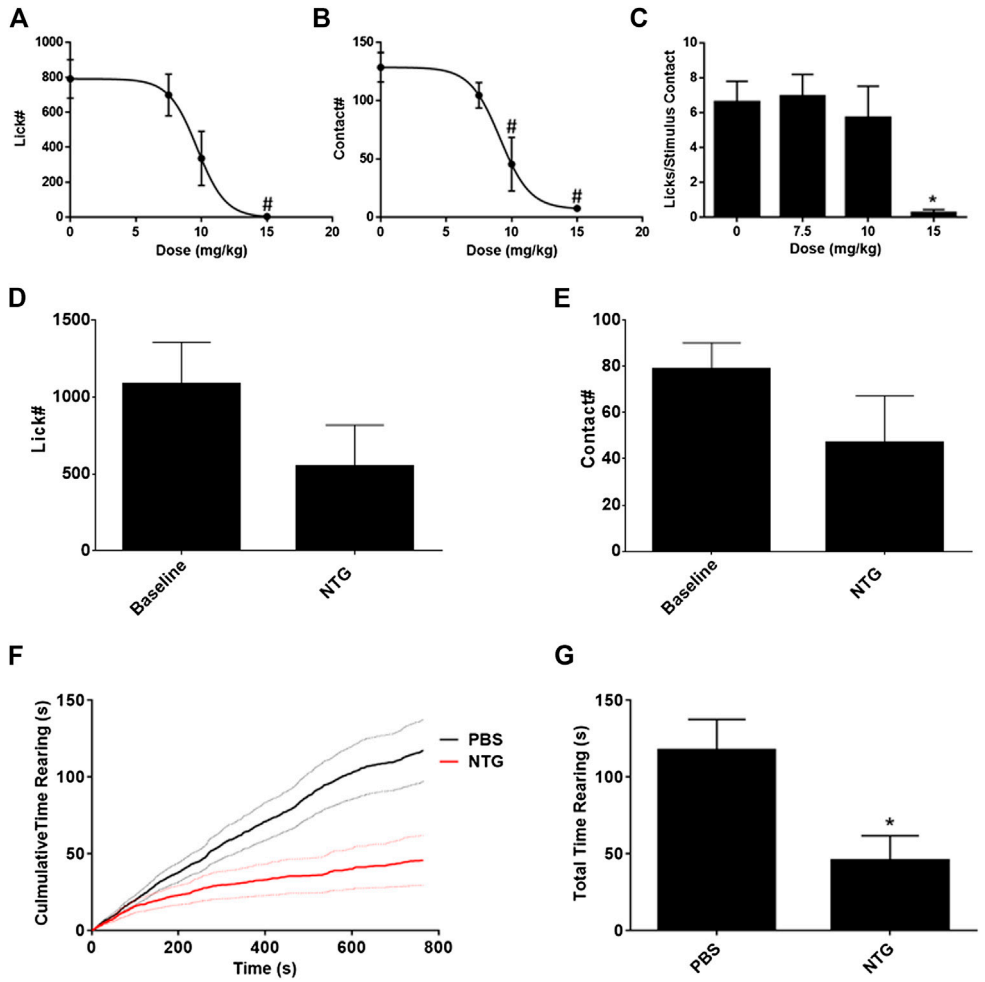

FIGURE 2 | Effect of nitroglycerin on rat behavior. **(A)** Nitroglycerin dose response relationship on reward bottle licking in the OPAD assay. ED50 = 9.68 ± 1.00 mg/kg, One-way ANOVA: F (3, 56) = 6.79, $p = 0.0006$. #$p < 0.05$ Dunnett's test when compared to 0 mg/kg. **(B)** Nitroglycerin dose response relationship on stimulus contacts in the OPAD assay. ED50 = 9.11 ± 0.85 mg/kg, One-way ANOVA: F (3, 56) = 12.99, $p < 0.0001$. #$p < 0.05$ Dunnett's test when compared to 0 mg/kg. **(C)** Nitroglycerin dose response relationship on the ratio of lick to stimulus contacts in the OPAD assay. One-way ANOVA: F (3, 54) = 3.490, $p = 0.0217$. *$p <$ 0.05 Holm-Sidak's multiple comparisons test when compared to 0 mg/kg. **(D)** Effect of nitroglycerin (10 mg/kg, i.p.) on licking in the OPAD assay 4 h following injection. ($N = 7$, Baseline vs NTG paired t-test: $t = 1.812$ df = 6, $p = 0.120$). **(E)** Effect of nitroglycerin on stimulus contacts in the OPAD assay 4 h following injection. ($N = 7$, Baseline vs. NTG paired t-test: $t = 1.580$ df = 6, $p = 0.165$). **(F)** Effect of nitroglycerin (10 mg/kg, i.p.) in the rearing behavioral assay ($N = 14$ nitroglycerin, $N = 15$ PBS). Dashed lines are standard errors for the curves. **(G)** Nitroglycerin reduces total time rearing. * t-test: t = 2.758 df = 27, $p = 0.010$.

et al., 2012; Anderson et al., 2013; Mustafa et al., 2013; Anderson et al., 2014; Anderson et al., 2015; Ramirez et al., 2015; Bowden et al., 2017; Caudle et al., 2017).

Rearing Activity Assay

Our previously described rearing test was used as an assessment of motor activity and exploratory behavior (Neubert et al., 2007; Rossi and Neubert, 2008). Briefly, an acrylic cylinder (19.5 cm diameter × 40.5 cm height) was constructed with aluminum sheets placed both on the floor and 13.5-cm above the floor. The metal siding was connected to a DC power supply (12 V) and, in series, to a multi-channel data acquisition module (DATAQ Instruments, Inc.). The floor of the cylinder served as the ground for the circuit. Unrestrained animals were placed into separate cylinders and the data acquisition software (WinDaq, DATAQ Instruments, Inc.) was activated. When an animal reared its front paws would contact the metal side of the cylinder completing an electrical circuit with the grounded floor. The closed circuit was registered on the computer. Each session was 12.75 min in duration.

Drug Treatments

Nitroglycerin (7.5, 10, or 15 mg/kg, i.p., Henry Schein) was administered 20 min prior to the initiation of a 10-min OPAD session for acute experiments. For the repeated exposure experiments nitroglycerin (10 mg/kg, i.p.) was administered 30 min after OPAD testing for five consecutive days.

Morphine sulphate (1.5 and 10 mg/kg, Patterson Veterinary Supply, Inc.), buprenorphine (0.03 mg/kg, Patterson Veterinary Supply, Inc.), and caffeine (2 mg/kg dissolved in PBS, Sigma-

TABLE 1 | Effect of anti-nociceptive agents on nociception in the OPAD assay.

Treatment	*N*	Licks	Contacts
		Mean ± SEM	Mean ± SEM
Nitroglycerin (10 mg/kg)			
PBS	65	340.8 ± 55.7	45.0 ± 7.8
Sumatriptan 0.3 mg/kg	10	350.0 ± 136.2	51.0 ± 11.4
Sumatriptan 1.0 mg/kg	10	159.3 ± 81.6	18.5 ± 6.7
Caffeine	10	356.5 ± 134.4	62.9 ± 18.5
Buprenorphine	10	166.8 ± 89.4	10.5 ± 4.4
Morphine 1.5 mg/kg	10	506.5 ± 136.8	36.5 ± 10.4
Morphine 10 mg/kg	10	203.6 ± 160.7	9.8 ± 3.3
No Nitroglycerin			
Naïve	60	768.9 ± 78.9	112.4 ± 12.3
PBS	50	838.3 ± 101.5	127.3 ± 21.2
Sumatriptan 0.3 mg/kg	10	630.2 ± 225.8	58.6 ± 20.9
Caffeine	10	418.4 ± 159.8	194.2 ± 71.0
Buprenorphine	10	650.7 ± 265.5	105.8 ± 42.1
Morphine 1.5 mg/kg	10	826.3 ± 168.7	95.3 ± 23.0
Morphine 10 mg/kg	10	627.7 ± 228.4	42.4 ± 11.1

Nitroglycerin: One-way ANOVA Licks: F (6, 118) = 0.94, p *= 0.47; One-way ANOVA Stimulus Contacts: F (6, 118) = 1.92,* p *= 0.08.*
No Nitroglycerin: One-way ANOVA Licks: F (7, 162) = 0.90, p *= 0.51; One-way ANOVA Stimulus Contacts: F (7, 162) = 1.66,* p *= 0.12.*

Aldrich) were administered i.p. 30 min prior to testing in the OPADs. Sumatriptan succinate (0.3 mg/kg dissolved in PBS, Sigma-Aldrich) was administered i.p. 10 min prior to testing in the OPADs as a rescue treatment and in a separate group of rats 1 mg/kg sumatriptan succinate was administered i.m. 30 min prior to testing. Phosphate buffered saline (PBS, pH 7.4) was used as a vehicle and injection control. Naïve rats received no injections.

Statistics

The licks on the reward bottle and the contacts with the stimulus bars were collected automatically with Stoelting's AnyMaze software. To evaluate the interval between licks 20 consecutive intervals were manually sampled from a single bout of licking for each animal. The data analysis feature of AnyMaze was used to measure the interval from the offset of a lick to the initiation of the following lick.

Data was exported from AnyMaze to Excel and PRISM version 6.07 (GraphPad Software, Inc.) for statistical analyses. Non-linear regressions were used to fit dose response curves and determine ED50s. T-tests, One-way ANOVAs followed by Dunnett's or Holm–Sidak's multiple comparisons tests were used when appropriate. Data are presented as Mean ± SEM. Alpha was set to $p \leq 0.05$.

RESULTS

Behavioral Characterization of Nitroglycerin

Nitroglycerin presumably produces a headache in rats like that experienced by humans. To determine an appropriate dose for testing against acute migraine headache therapies a dose response relationship was performed. As demonstrated in **Figures 2A,B** the ED50s for suppression of licks on the reward bottle and suppression of contacts with the stimulus bars were 9.68 ± 1.00 mg/kg and 9.11 ± 0.85 mg/kg respectively. The ratio of the licks to the stimulus contacts, which is utilized as an index of hypersensitivity (Neubert et al., 2005; Neubert et al., 2006; Neubert et al., 2007; Neubert et al., 2008; Caudle et al., 2010; Nolan et al., 2011; Ramirez and Neubert, 2012; Rossi et al., 2012; Mustafa et al., 2013; Ramirez et al., 2015; Sapio et al., 2018), was not altered until the highest dose of 15 mg/kg (**Figure 2C**). Because the total number of licks and the total number of stimulus contacts were very low at 15 mg/kg, and an ED50 could not be calculated from the curve, the suppression of the lick to stimulus contact ratio was not considered an indication of mechanical hypersensitivity. Instead, the data indicate that nitroglycerin reduced overall activity in the animals rather than producing mechanical hypersensitivity. This lack of hypersensitivity contrasts with previous reports on

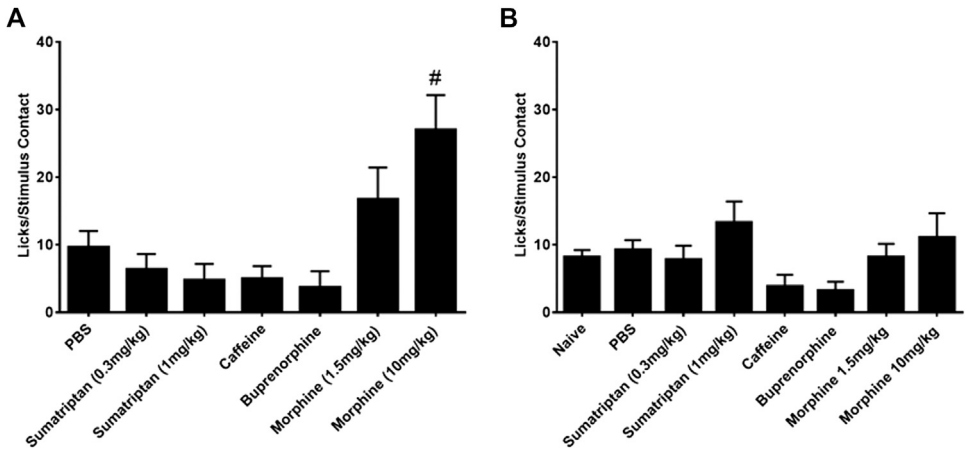

FIGURE 3 | Influence of anti-nociceptive agents on the ratio of licks on the reward bottle to stimulus contacts. **(A)** Agents tested in the presence of 10 mg/kg nitroglycerin (i.p.). The low dose of sumatriptan (0.3 mg/kg) was given as a rescue. All other drugs were given preemptively. One-way ANOVA: F (6, 107) = 3.25, p = 0.0056, #p < 0.05 Dunnett's test when compared to PBS. **(B)** Agents tested in the absence of nitroglycerin. One-way ANOVA: F (7, 150) = 1.55, p = 0.15.

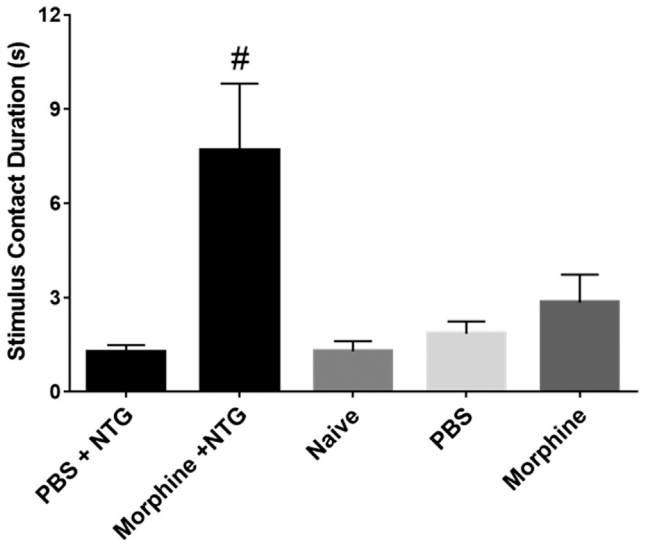

FIGURE 4 | Effect of 10 mg/kg morphine (i.p.) and nitroglycerin on average stimulus contact duration in the OPAD assay (Mean ± SEM). One-way ANOVA: F (4, 45) = 6.297, $p = 0.0004$, #$p < 0.05$ Dunnett's test when compared to PBS + NTG.

nitroglycerin in rodents (Di et al., 2016; Tipton et al., 2016; Farajdokht et al., 2018; Qin et al., 2018).

Based on the dose response data the dose of 10 mg/kg was utilized for subsequent studies, which is consistent with the work of others (Costa et al., 2005; Bates et al., 2010; Bobade et al., 2015; Farajdokht et al., 2018; Lai et al., 2019). The timing of the nitroglycerin administration relative to OPAD testing (20 min) was determined in preliminary studies based on the peak effect of nitroglycerin in the assay. Previous studies using various assays indicated that nitroglycerin induced hypersensitivity at time points as late as 4 h following injection (Guo et al., 2017; Lai et al., 2019). **Figures 2D,E** demonstrate that overall performance in the OPAD assay was slightly reduced at 4 h following 10 mg/kg nitroglycerin, but this effect did not reach statistical significance.

Because the OPAD data indicated that the rats' total activity was suppressed by the nitroglycerin treatment a rearing assay was used to evaluate the motor and exploratory behavior of the rats when treated with nitroglycerin. The nitroglycerin was administered 20 min prior to testing. As indicated in **Figures 2F,G**, 10 mg/kg nitroglycerin significantly suppressed motor and exploratory activity in the rearing assay indicating that the effects of nitroglycerin disrupted their general activity levels, which may have contributed to the rats' not seeking the reward solution in the OPAD assay.

Effects of Therapeutic Agents in the Nitroglycerin Model

Two doses of sumatriptan that were demonstrated to be anti-nociceptive in previous rodent studies were tested against nitroglycerin (Tomić et al., 2015; Farkas et al., 2016). The low dose of sumatriptan (0.3 mg/kg, i.p.) was given as a rescue agent 10 min following the nitroglycerin injections and the high dose of sumatriptan (1 mg/kg, i.m.) was given as a preventative therapy. As demonstrated in **Table 1** neither dose significantly influenced the effect of the nitroglycerin in the OPAD assay. Similarly, doses of morphine and caffeine that were previously shown to be effective anti-migraine agents and the mixed opioid agonist/antagonist buprenorphine did not inhibit nitroglycerin's effects on the number of licks on the reward bottle or the number of contacts with the mechanical stimulus (**Table 1**) (Caudle and Isaac, 1987; Brasseur, 1997; Neubert et al., 2005; Neubert et al., 2006; Neubert et al., 2007; Neubert et al., 2008; Lobmaier et al., 2010; Nolan et al., 2012; Bird and Lambert, 2015; Ramirez et al., 2015; Tomić et al., 2015; Lipton et al., 2017; Walsh and Babalonis, 2017). The four agents also did not significantly alter the number of licks or stimulus contacts in the OPAD in the absence of nitroglycerin, indicating that they did not produce sedative effects that could have masked their antinociceptive actions.

In contrast to the lack of effect of all the therapeutic agents on the number of licks and stimulus contacts, the combination of morphine and nitroglycerin produced a significant morphine dose dependent increase in the ratio of licks on the reward bottle to contacts with the stimulus bars (**Figure 3A**). No other agent produced this increase in the ratio. Morphine and the other agents had no effect on this ratio in the absence of nitroglycerin (**Figure 3B**). This finding was further evaluated by examining the average duration of the rodents' contacts with the stimulus bars. The duration of the stimulus contacts in the presence of both nitroglycerin and 10 mg/kg morphine was significantly increased (**Figure 4**) which would provide more time for licking on the reward bottle during each bout. This finding suggests that although overall activity in the rats was reduced by the nitroglycerin treatment, the combination of nitroglycerin and morphine increased the rats' tolerance of the mechanical stimulus when they attempted to obtain the reward solution.

Effects of Nitroglycerin and Morphine on the Licking Motor Pattern Generator

In analyzing the OPAD data it was observed that the frequency of the licking on the reward bottle was reduced by 10 mg/kg morphine and by nitroglycerin (**Figure 5A**). This was analyzed by averaging a sampling of 20 consecutive intervals between licks. As **Figure 5B** demonstrates both morphine and nitroglycerin significantly increase the lick interval and their effects appear to be additive when they are administered in combination. No other agents in the presence or absence of nitroglycerin produced a similar increase in the intervals between licks (**Table 2**). As illustrated by the frequency distribution of the intervals (**Figure 5C**), morphine, nitroglycerin and the combination of morphine and nitroglycerin also increased the variability of the intervals suggesting that these agents disrupt the rat tongue motor pattern generator.

Repeated Nitroglycerin Injections

The overall reduction in motor activity produced by nitroglycerin suggested that the acute nociceptive, cardiovascular, or other

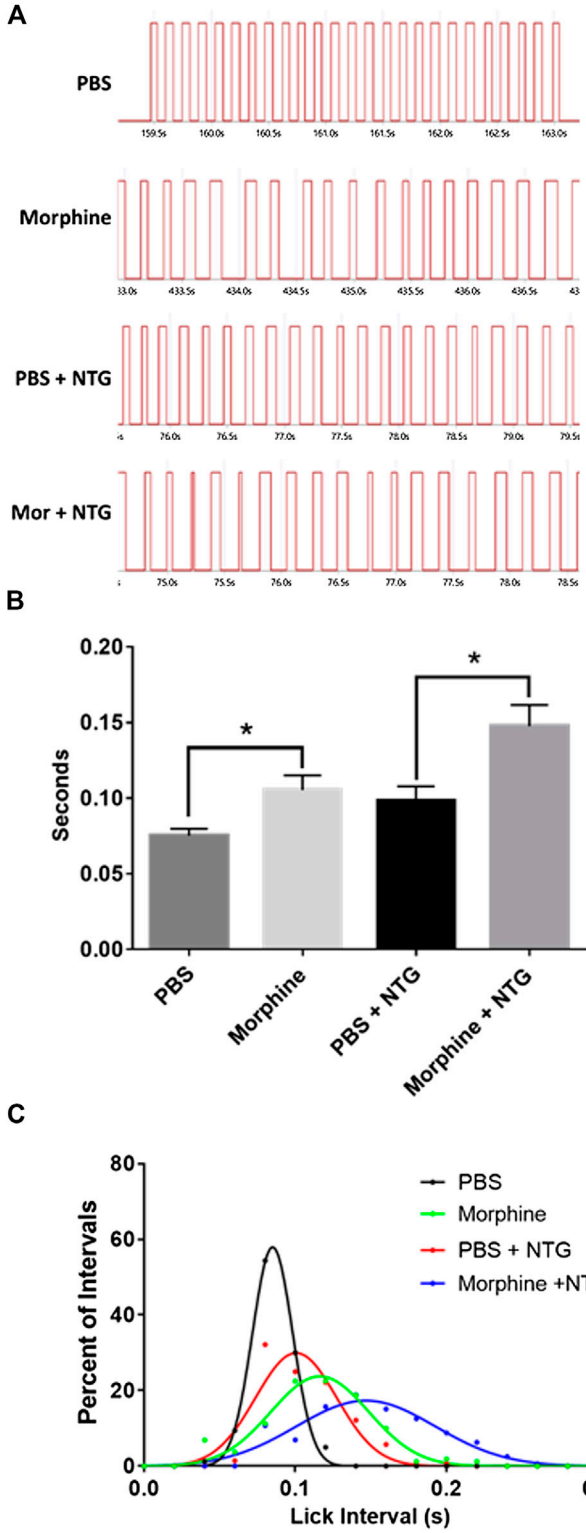

FIGURE 5 | Nitroglycerin and morphine increase the interval between licks. **(A)** Representative licking bouts from animals treated with PBS, morphine (10 mg/kg), PBS + nitroglycerin (10 mg/kg), or morphine (10 mg/kg) + nitroglycerin (10 mg/kg) i.p. **(B)** Averaged interval between licks (Mean ± SEM). T-test PBS vs. morphine: $t = 2.807$ df = 14, $p = 0.014$. T-test PBS + NTG vs morphine + NTG: $t = 2.819$ df = 13, $p = 0.015$. $N = 7$ to 8 rats per treatment group. **(C)** Frequency distribution of intervals between licks. The data demonstrate the increased variability of the intervals in the presence of morphine and/or nitroglycerin. Data was fitted with Gaussian curves using PRISM statistical software.

effects of nitroglycerin may interfere with the assay and mask nitroglycerin induced hypersensitivity. Previous studies have reported that rodents develop allodynia when they receive multiple nitroglycerin injections over the course of several days (Farajdokht et al., 2018; Jeong et al., 2018). To determine if repeated injections of nitroglycerin produce hypersensitivity, we injected 10 mg/kg daily for five days immediately after testing in the OPAD. This design let the animals fully recover from the acute effects of nitroglycerin and allowed testing for the development of chronic or sustained allodynia. As **Figure 6** illustrates, the treatments did not alter licking (6A), stimulus contacts (6B) or the ratio of licks to stimulus contacts (6C) indicating that chronic mechanical hypersensitivity was not produced by the treatment.

DISCUSSION

Nitroglycerin in Female Rats Is Not a Model of Migraine Headache

In this reverse translation project, the rat nitroglycerin model of migraine headache was evaluated in an operant orofacial nociception assay. The hypothesis tested was that drugs that are used to reverse migraine headache would significantly reverse or prevent the effects of nitroglycerin in the rodent model. The OPAD assay evaluates nociception in regions innervated by trigeminal sensory neurons. The assay also collects all data electronically which significantly reduces investigator bias. Furthermore, OPADs utilize a pain suppressed behavior rather than pain enhanced behaviors like the von Frey mechanical assay. This prevents sedative or motor effects of a drug from being interpreted as anti-nociceptive. In the current study the data indicates that the nitroglycerin model in the current context does not meet the criteria that would indicate it is a model of human disease. 1) The mechanisms for nitroglycerin's effects and for migraine headache are not established. Thus, it cannot be determined if nitroglycerin invokes the same mechanism of pathology as migraine. 2) The lack of trigeminal hypersensitivity suggests that the nitroglycerin is not producing the signs and symptoms of the disease. The one caveat to the assay is that the OPAD primarily measures nociception in the maxillary branch of the trigeminal nerve, whereas most hypersensitivity in human migraine is measured in the ophthalmic branch. However, many rodent studies report paw sensitivity following nitroglycerin injection, which is substantially more removed from the ophthalmic branch of the trigeminal nerve than the maxillary branch (Costa et al., 2005; Farkas et al., 2016; Farajdokht et al., 2018). 3) Although, the OPAD assay

TABLE 2 | Effect of sumatriptan, caffeine, buprenorphine and low dose morphine on Lick interval.

Treatment	*N*	Lick interval (ms) Mean ± SEM	*p* Dunnett's test vs. (PBS + NTG)
Nitroglycerin (10 mg/kg)			
PBS + NTG	7	98.1 ± 9.7	—
Sumatriptan 0.3 mg/kg	5	96.6 ± 9.2	>0.05
Sumatriptan 1.0 mg/kg	4	126.6 ± 9.7	>0.05
Caffeine	5	90.7 ± 7.1	>0.05
Buprenorphine	4	128.5 ± 7.2	>0.05
Morphine 1.5 mg/kg	9	109.3 ± 7.1	>0.05
No Nitroglycerin			
Naïve	19	71.9 ± 6.8	—
PBS	15	86.1 ± 3.7	—
Sumatriptan 0.3 mg/kg	6	89.1 ± 4.8	—
Sumatriptan 1.0 mg/kg	8	87.4 ± 6.5	—
Caffeine	6	88.6 ± 3.9	—
Buprenorphine	4	78.1 ± 8.4	—
Morphine 1.5 mg/kg	8	88.2 ± 5.3	—

Nitroglycerin: One-way ANOVA: F (5, 28) = 2.71, p = *0.04.*
No Nitroglycerin: One-way ANOVA: F (6, 59) = 1.337, p = *0.26.*
Animals that did not have 20 consecutive licks on the reward bottle were excluded from the analysis.

measures a clear and objective difference between the normal and disease state it is not evident that these measures are meaningful for the human disease. 4) The model did not reverse translate using effective therapies for the human disease as sumatriptan, morphine, buprenorphine, and caffeine did not impact the effects of nitroglycerin on nociceptive measures in the OPAD assay. Thus, we conclude that nitroglycerin in the current context is not a valid model of human migraine.

We have previously utilized the ratio of the licks on the reward bottle to the contacts with the stimulus as a "pain index" (Neubert et al., 2005; Neubert et al., 2006; Neubert et al., 2008; Nolan et al., 2011; Ramirez and Neubert, 2012; Rossi et al., 2012; Ramirez et al., 2015). This ratio decreases when the animals are not able to tolerate contact with the stimulus and increases when a drug suppresses the nociception produced by the stimulus. Thus, this ratio is indicative of the degree of hypersensitivity the animals experience. In this study the ratio was not altered by nitroglycerin until the maximum dose was tested. However, the highest dose of nitroglycerin reduced attempts to access the reward to an average of only 7.6 ± 2.6 events per session, which is too few to be confident of the validity of the ratio. Because lower doses of nitroglycerin did not alter the ratio it was concluded that nitroglycerin did not produce hypersensitivity in the face of the rats. If nitroglycerin produced trigeminal hypersensitivity its ED50 for hypersensitivity was substantially higher than for decreases in licks on the reward bottle and contacts with the stimulus. These finding are in contrast to previous publications which had indicated that acute administration of nitroglycerin produced hypersensitivity in the face (Di et al., 2016; Qin et al., 2018) and limbs (Tassorelli et al., 2003; Costa et al., 2005; Greco et al., 2008; Bobade et al., 2015) of rats. It should be noted that these studies used assays that required the investigator to interpret the response of the rodent. Since hypersensitivity in the face was reported in humans with migraine headaches an accurate rodent model of migraine headache should also produce allodynia in trigeminal nerve territories (Burstein et al., 2000a; Burstein et al., 2000b; Yarnitsky et al., 2003). Thus, nitroglycerin induced trigeminal hypersensitivity could not be verified in this study.

Interaction Between Morphine and Nitroglycerin

Despite the model's failure to imitate migraine headache there were a few interesting findings in this study that warrant further investigation. One observation was that the combination of morphine and nitroglycerin significantly increased the ratio of licks to stimulus contacts (**Figure 3A**). This was also evident by the increase in the duration of the stimulus contacts in the combination treated animals (**Figure 4**). Morphine did not produce this effect in the absence of nitroglycerin (**Figures 3B** and **4**). This finding suggests that morphine and nitroglycerin work in concert to alter the rodent's tolerance of the mechanical stimulus. This is particularly interesting because the overall number of licks on the reward and number of contacts with the stimulus were not significantly altered by the combination (**Table 1**). Yu Xu and colleagues previously demonstrated that nitric oxide can potentiate the opioid peptide ß-endorphin's analgesic effects (Xu et al., 1995). Since nitroglycerin is a NO donor it is possible that the increase in the duration of the stimulus contact is due to mechanisms similar to those described by Yu Xu's group. Interestingly, buprenorphine, a mixed opioid agonist/antagonist (Brasseur, 1997), did not demonstrate any interaction with nitroglycerin in our study. This finding suggests that nitroglycerin's interaction with opioids may be limited to mu agonists.

Another interesting finding in our study was that both morphine and nitroglycerin increased the interval between licks, or decreased the frequency of licks, on the reward bottle to similar degrees. When the drugs were given

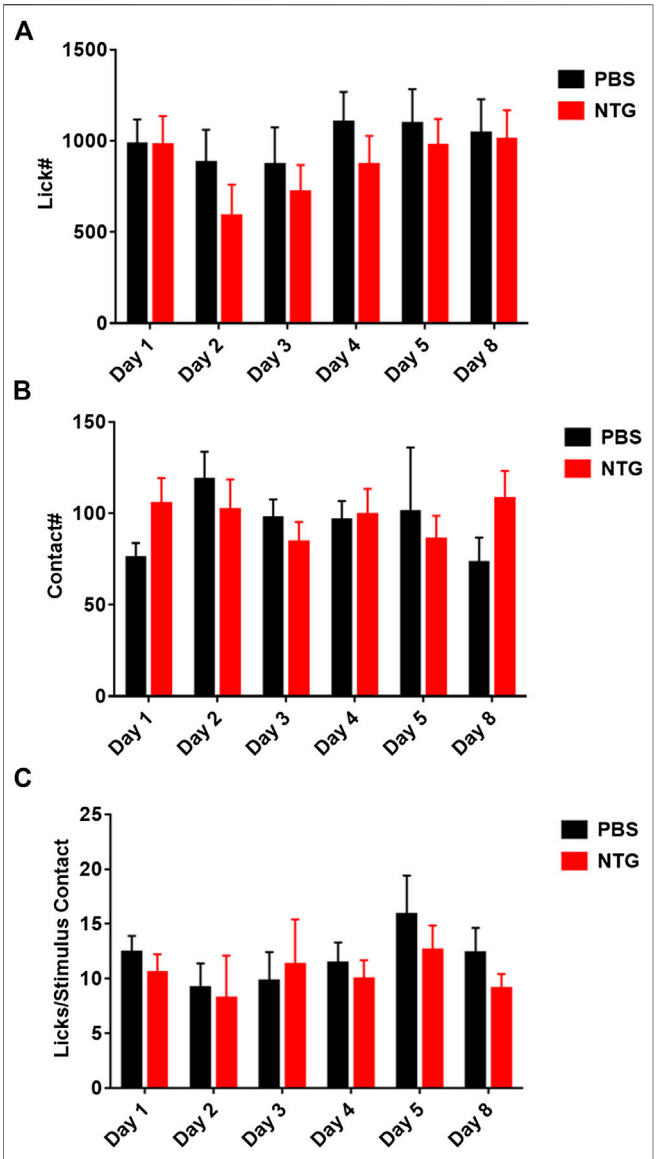

FIGURE 6 | Repeated administration of nitroglycerin does not induce mechanical allodynia in the OPAD assay. Nitroglycerin (10 mg/kg i.p.) was administered on days 1–5 immediately following the rats' session on the OPAD. **(A)** Effect of repeated nitroglycerin on licks on the reward bottle. One-way ANOVA: $F(1, 28) = 0.4614$, $p = 0.50$. **(B)** Effect of repeated nitroglycerin on the number of contacts with the mechanical stimulus. Two-way ANOVA: $F(1, 28) = 0.07417$, $p = 0.79$. **(C)** Effect of repeated nitroglycerin on the ratio of licks to stimulus contacts. Two-way ANOVA: $F(1, 28) = 0.2838$, $p = 0.60$. $N = 15$ rats per treatment group.

together, they produced what appeared to be an additive effect (**Figure 5**).

Previous work has indicated that nitric oxide plays a role in slowing down motor central pattern generators by enhancing inhibitory neuronal circuitry (Foster et al., 2014) and mu agonists such as morphine are well known for their inhibition of respiratory central pattern generating neuronal circuits (Kokka et al., 1965; Levitt et al., 2015). Thus, the effects of nitroglycerin and morphine on licking behavior in this study are not entirely surprising. The licking central pattern generator is likely regulated by NO and mu opioids. The interaction of NO and opioids in this circuit deserves further study.

CONCLUSION

Drug discovery is reliant on animal models of disease that ideally invoke the same pathological mechanisms as the human disease. It is also important that the model expresses the disease in an easily quantified manner.

In this project we reverse translated the rat nitroglycerin model of migraine headache to verify its validity. The results of our study indicate that this model is not suitable for studying migraine headache. Sumatriptan, morphine and caffeine, which are all used to treat migraine headaches did not alter the rats' discomfort with the nitroglycerin treatment. However, there are a few caveats with our study. The study utilized only female rats because women suffer from migraine headaches more frequently than men (Stewart et al., 1994; Hu et al., 1999; Bonafede et al., 2017) and female rats are more sensitive to nitroglycerin than male rats (**Supplementary Figure S1**). The enhanced sensitivity of female rats was also previously reported by Greco et al. (2013). We also did not monitor estrous in this project since the variance in the dose response data was not significantly different than that found with male rats (**Supplementary Figure S1**). Other studies have used mice and male rats which may, for unknown reasons, be better suited models. The validity of mice and male rats in the OPAD nitroglycerin model remains to be determined. This project also utilized a behavioral assay that considers multiple features of the rodent's experience, including motivation to access the reward solution. The therapeutic agents were clearly not able to block or reverse all the effects of nitroglycerin. But the agents may have had effects in the model that would be beneficial in treating migraine that the assay could not measure, such as a reduction in light sensitivity. Thus, further work is needed to completely rule out the use of nitroglycerin rodent models for migraine headache research.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/ **Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Florida Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

SC and NF performed all experimental procedures. JN, ER, and RC designed the experiments, analyzed the data and interpreted the results.

FUNDING

This project was funded by Velocity Laboratories, LLC, Alachua, Florida, United States. JN and RC are the founders of Velocity Laboratories, LLC.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.527495/full#supplementary-material.

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Conflict of Interest: JN and RC are co-founders of Velocity Laboratories, LLC, which provided funding for this project.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 12 January 2021
doi: 10.3389/fphar.2020.615028

DAGLα Inhibition as a Non-invasive and Translational Model of Episodic Headache

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Recent findings suggested that Clinical Endocannabinoid Deficiency underlies the pathophysiology of pain disorders, including migraine and headache. In models of medication overuse headache induced by sustained administration of sumatriptan or morphine, 2-AG levels were selectively depleted in the periaqueductal gray (PAG) and anandamide (AEA) increased in the cortex suggesting distinct regulation of the endocannabinoid system during headache pain. These results led to the hypothesis that blockade of DAGL, to reduce 2-AG levels would induce headache-like behaviors as a new, translationally relevant model of episodic headache. Our study investigated whether non-selective and selective blockade of DAGL, the main biosynthetic enzyme for 2-AG, induced periorbital and hind-paw allodynia, photophobia, anxiety-like behaviors, responsivity to abortive anti-migraine agents, and 2-AG/AEA levels. Injection of non-selective DAGL (DH376, 10 mg/kg, IP) and selective DAGLα (LEI106, 20 mg/kg, IP) inhibitors, but not DAGLβ agents, induced facial sensitivity in 100% and ~60% of female and male rats, respectively, without induction of peripheral sensitivity. Notably, male rats showed significantly less sensitivity than female rats after DAGLα inhibition, suggesting sexual dimorphism in this mechanism. Importantly, LEI106 induced periorbital allodynia was attenuated by administration of the clinically available abortive antimigraine agents, sumatriptan and olcegepant. Selective DAGLα inhibition induced significant photophobia as measured by the light-dark box, without anxiety like behaviors or changes in voluntary movement. Analysis of AEA and 2-AG levels at the time of peak pain sensitivity revealed reductions in 2-AG in the visual cortex and periaqueductal gray (PAG), without altering anandamide or significantly increasing diacylglycerol levels. These results provide foundational evidence for DAGL-2AG in the induction of headache-like pain and photophobia without extracephalic allodynia, thus modeling the clinical episodic migraine. Mechanistically, behavioral measures of headache sensitivity after DAGL inhibition suggests that reduced 2-AG signaling in the cortex and PAG, but not the trigeminal nucleus caudalis or trigeminal ganglia, drives headache initiation. Therefore, episodic DAGL inhibition, which reduces the time, cost, and invasiveness of currently accepted models of headache, may fill the need for episodic migraine/headache models

OPEN ACCESS

Edited by:
E. Alfonso Romero-Sandoval, Wake Forest School of Medicine, United States

Reviewed by:
Josee Guindon, Texas Tech University Health Sciences Center, United States
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Specialty section:
This article was submitted to Translational Pharmacology, a section of the journal Frontiers in Pharmacology

Received: *07 October 2020*
Accepted: *16 December 2020*
Published: *12 January 2021*

Citation:
Levine A, Liktor-Busa E, Karlage KL, Giancotti L, Salvemini D, Vanderah TW and Largent-Milnes TM (2021) DAGLα Inhibition as a Non-invasive and Translational Model of Episodic Headache. Front. Pharmacol. 11:615028. doi: 10.3389/fphar.2020.615028

mirroring clinical presentation. Moreover, use of this approach may provide an avenue to study the transition from episodic to chronic headache.

Keywords: headache, migraine, translational models, endocannabinoid system, DAGL, clinical endocannabinoid deficiency

INTRODUCTION

Headache disorders, including migraine and tension-type headache (TTH), affect nearly 46% of the global population (Stovner et al., 2007; IHS, 2018). While the exact pathophysiology of these disorders has yet to be elucidated, recent findings have suggested that the endocannabinoid system (eCB) may play an important role. Low levels of the two main eCB signaling molecules, 2-arachidonoylglycerol (2-AG) and anandamide (AEA), have been correlated with pain disorders such as migraine and fibromyalgia providing clinical evidence for the theory of Endocannabinoid Deficiency (ED) (Russo, 2016).

The eCB system comprises the small lipid signalers, 2-AG and AEA, which are primarily synthesized from the cellular lipid membrane via the enzymes diacylglycerol lipase (DAGL) and N-acyl-phosphatidylethanolamine hydrolyzing phospholipase D (NAPE-PLD), respectively (Ahn et al., 2008; Fezza et al., 2016). Two types of DAGLs, DAGLα and DAGLβ are described as sn-1 specific DAGLs that synthesize 2-AG in cells (Reisenberg et al., 2012). DAGLα was originally considered as primarily central in location, with DAGLβ as a peripheral DAGL. However, knock-out studies revealed that certain DAGLα knockout mice showed 80% reduction in 2-AG levels in the brain and spinal cord, and an approximately 60% reduction in the liver. A second line of DAGLα knockout mice revealed that cerebellum, hippocampus and striatum showed the lowest level of 2-AG; no appreciable changes in central 2-AG were observed in the DAGLβ knockout mice (Gao et al., 2010; Tanimura et al., 2010). These experiments concluded that DAGLα appears to be much more important in synapse-rich regions with limited contributions from DAGLβ. After synthesis, 2-AG and AEA act on the cannabinoid receptors CB1 and CB2, with CB1 representing the primary cannabinoid receptors in the central nervous system (CNS) (Ahn et al., 2008); CB2 receptors are reported in glial cells and in some neurons (Onaivi et al., 2006; Turcotte et al., 2016). Upon activation, the CB receptors canonically function as a presynaptic $G_{i/o}$ class of G protein coupled receptor to decrease neurotransmitter release (Mackie, 2006).

Migraine has been described as a state of CNS hyperexcitability (Aurora and Wilkinson, 2007); thus, it follows that decreased activation of presynaptic inhibitory mechanisms may lead to increased neuronal excitability, resulting in migraine induction. Several studies showed that the endocannabinoid system is centrally and peripherally engaged during pain signaling (Kaur et al., 2016; Woodhams et al., 2017). Some experimental evidence suggested eCB-dependent mechanisms underly the cause of migraine/headache; pharmacological manipulation of eCB tone by inhibiting its hydrolyzing enzymes, or directly targeting eCB receptors, seemed to represent a promising mechanistic tool and therapeutic approach to reduce migraine-like pain (Greco et al., 2018a; Tassorelli et al., 2019). Despite the mounting evidence, the real pathological connection between the eCB system and migraine with special emphasis on 2-AG signaling is not fully elucidated.

Based on the theory of CED and the therapeutic potential of elevated eCB tone in headache models (Lau et al., 2014; Nozaki et al., 2015; Zubrzycki et al., 2017; Greco et al., 2018b; Greco et al., 2020), we hypothesized that decreased levels of endocannabinoids, mainly 2-AG via exogenous inhibition of DAGL can induce migraine-like pain, and it can be utilized as a viable preclinical model for investigating headache-like pain. It can also be a useful tool to clarify the pathophysiological role of the eCB system in migraine and can support the validation of new therapeutic targets. The current study presents an original protocol for inducing headache-like pain by depletion of endocannabinoid tone, using non-selective DAGL and selective DAGLα inhibitors. Results indicate induction of cephalic hypersensitivity in the absence of extracephalic hypersensitivity following inhibition of DAGLα, but not DAGLβ, that are coupled to reductions in central, but not peripheral 2-AG levels. Allodynia induced by DAGL inhibition is responsive to two clinically relevant abortive anti-migraine agents, sumatriptan and olcegepant. DAGLα inhibition induced both head-tucking and photophobia, indicating both distress and visual sensitivity, characteristics of clinical headache. Thus, DAGL inhibition represents a new strategy to study headache that recapitulates the clinical features of headache and/or migraine as a reverse translational strategy to model CED.

MATERIALS AND METHODS

Drugs

Ketamine, xylazine, and isoflurane were purchased from VetOne (IL, United Stated). DH376 was a generous gift from Prof. Mario van der Stelt, Leiden University. LEI106 was purchased from Cayman Chemicals (Ann Arbor, MI). KT109, olcegepant, sumatriptan, and morphine were purchased from Sigma-Aldrich (St. Louis, MO). LEI106, KT109, and olcegepant were dissolved in DMSO-Tween80-saline 0.9% (1:1:8, v/v/v). LEI106 was injected intraperitoneally at doses of 10, 20, and 40 mg/kg. KT109 was interperitoneally administered at 5, 10, 20 mg/kg doses. DH376 was dissolved in ethanol-PEG-saline 0.9% (1:1:18, v/v/v). Sumatriptan succinate (0.6 mg/kg, dissolved in saline) was dosed subcutaneously 30 min before the injection of DAGL inhibitor. Olcegepant 0.8 mg/kg, dissolved in DMSO-Tween80-saline 0.9% (1:1:8, v/v/v) was injected intraperitoneally 30 min before the administration of DAGL inhibitor. AEA-d4 and 2-AG-d5 were purchased from Cayman Chemicals (Ann Arbor, MI).

Animals

Female and male Sprague Dawley rats (7–8 weeks old) were purchased from Envigo (Indianapolis, IN) and housed in a climate-controlled room on a regular 12/12 h light/dark cycle with lights on at 7:00 am with food and water available *ad libitum*. Animals were housed three per cage. All procedures were performed during the 12-h light cycle and according to the policies and recommendations of the International Association for the Study of Pain and the NIH guidelines for laboratory animals, and with IACUC approval from the University of Arizona. Justification for animal numbers was consistent with NIH policy (NOT-OD-15-102), and experiments were randomized to blinded treatment groups, giving 80% power to detect a treatment effect size of 20% compared to a baseline response of 5% at a significance level of 0.05 (Andrews et al., 2016). Numbers required to achieve statistical power were determined by G.Power3.1.

Medication Overuse Headache (MOH) Induced by Sustained Sumatriptan or Morphine Infusion

Alzet osmotic minipumps (Alzet, Cupertino, CA, United States; model 2001) with a flow rate of 1 μL/h for 7 days were used in female rats for drug infusion, as described in previous reports (De Felice et al., 2010; Bonnet et al., 2019). The minipumps were implanted subcutaneously under anesthesia with isoflurane (flow rate = 2 L/min in O_2). A 4–5 mm incision was made between the shoulder blades. The osmotic minipump was inserted under the skin. The incision site was closed with wound clips. The day of the pump implant was considered as day 0. Drugs administered by infusion were either sumatriptan (0.6 mg/kg/day) or morphine (5 mg/kg/day) or vehicle (NaCl, 0.9%). On day 7, spatially discrete brain regions (Ct, occipital cortex; PAG, periaqueductal gray; Vc, trigeminal nucleus caudalis; and TG, trigeminal ganglia) were harvested, as described below ("*Harvest of Tissue Samples*" Section).

Assessment of Periorbital and Hind-Paw Mechanical Allodynia

Mechanical allodynia was evaluated before and at 30, 60, 90, 120, 180, 360 min, and 24 h after drug treatment by an observer blinded to drug administration. Any rats exhibiting excessive allodynia at baseline (<75% of cut-off value; threshold <6 g for periorbital or <11.25 g for hind-paw) were removed from the study. Rats were acclimated to testing box 1 hour prior to evaluation of mechanical allodynia with calibrated von Frey filaments as previously described by Edelmayer and coworkers (Edelmayer et al., 2012). Behavioral responses of periorbital sensitivity were determined by applying calibrated von Frey filaments perpendicularly to the midline of the forehead at the level of the eyes with enough force to cause the filament to slightly bend while held for 5 s. A response was indicated by a sharp withdrawal of the head, vocalization, or severe batting at the filament with attempts to eat it. Hind-paw withdrawal thresholds were tested by perpendicular application of the filaments to the plantar surface of the left hind-paw. The withdrawal threshold was calculated using a modified version of the Dixon up-down method (Dixon, 1980).

Open Field Test

Open field test was performed 2 h post-injection of LEI106 to assess anxiety-associated behaviors. The open-field arena (90 cm × 90 cm × 40 cm) is a white box with an open top and a black floor. The test started by placing the animal in the center of the arena and allowing free movement for a 5 min duration. A consistent white noise (~55 dB) and a dim lighting (~24 lux) were applied during the test. The behavior of each rat was analyzed in real-time using a digital web camera mounted 1.5 m above the floor in conjunction with Any-Maze software (version 4.75, Stoelting, Wood Dale, IL), a video tracking system designed to automate testing in behavioral experiments. Using the software, the arena was divided into a scaled grid of equally sized squares spaced at 10 cm, resulting in a total of 28 squares. Eight of the inner squares were assigned as the center zone, while the remaining twenty were assigned as the perimeter zone. The main parameters analyzed were total distance traveled and the amount of time spent in each of the zones.

Light-Dark Box Test

Light-dark box test was conducted following the open-field test. For the light-dark box test (LDBT), a 3-compartment place preference box (San Diego Instruments, San Diego, CA) was utilized. This system consists of three adjoined chambers; one of the end chambers (27 cm × 21 cm × 34 cm) was covered with opaque black vinyl so that no light would pass through, while the opposing end chamber (27 cm × 21 cm × 34 cm) was open and illuminated (40 W); the center chamber (14 cm × 21 cm × 34) contained a 7 cm opening on either end to allow the animals to pass from one end to the other, it was also unlit and served as a transition zone to further remove the dark chamber from the light source. A 4 × 16 photobeam array runs along the bottom of each box; the beams are broken as the animal travels from one compartment to the other. The software (PAS Data Reporter, version 1.0.2.7, San Diego Instruments, San Diego, CA) which is run with these boxes records the amount of time the animal spends in each compartment by tracking the path and number of beam breaks. Each animal was placed into the center compartment and allowed to move freely for 15 min. The main parameters were duration of time spent in the light box; the latency to enter the dark box; and the number of transitions between the light and dark box.

Elevated Plus Maze (EPM) Anxiety Test

Immediately following the LDBT, elevated plus maze test was conducted, which can measure many relevant anxiety related behaviors such as, number of pokes and full entries into and duration spent in closed vs. open arm. The EPM consists of four elevated arms (50 cm long and 10 cm wide) with two opposing arms containing 30 cm high opaque walls. The arms and central platform of the apparatus are elevated to a height of 62 cm. Each rat was allowed 5 min to explore the EPM and then returned to its home cage. Between animals the EPM was cleaned thoroughly

with Versa-Clean (Fisher Scientific). EPM performance was video recorded for later analyses using ANYmaze software (version 4.75, Stoelting, Wood Dale, IL). The main parameters were duration of time spent in the open and closed arm and number of entries into the arms.

Harvest of Tissue Samples

Spatially discrete brain regions (occipital cortex-Ct, periaqueductal gray-PAG, trigeminal nucleus caudalis-Vc, and trigeminal ganglia-TG), involved in pain signaling were harvested 2 h after the injection of LEI106 (20 mg/kg, ip.) or KT109 (20 mg/kg, ip.), along with vehicle-treated controls. The animals were anesthetized with ketamine:xylazine mix (80: 10 mg/kg, ip.), then transcardially perfused with ice cold 0.1 M phosphate buffer at rates to not burst brain microvasculature (i.e., 3.1 ml/min). After decapitation, tissue samples were harvested, flash frozen in liquid nitrogen and stored at −80°C until further application.

Quantification of 2-AG and AEA by LC-MS

The brain samples for LC-MS were purified by organic solvent extraction, as described by Wilkerson et al. (Wilkerson et al., 2016). Briefly, tissues were harvested, snap-frozen in pre-weighted tubes, and stored at −80°C. On the day of processing, tissues were weighed and homogenized in 1 ml of chloroform/methanol (2:1 v/v) supplemented with phenylmethylsulfonyl fluoride (PMSF) at 1 mM final concentration to inhibit the degradation by endogenous enzymes. Dounce homogenizer was used for homogenization. Homogenates were then mixed with 0.3 ml of 0.7% w/v NaCl, vortexed, and then centrifuged for 10 min at 3,200 × g at 4°C. The aqueous phase plus debris were collected and extracted two more times with 0.8 ml of chloroform. The organic phases from the three extractions were pooled and internal standard was added to each sample. Mixed internal standard solutions were prepared by serial dilution of AEA-d4 and 2-AG-d5 in 80% acetonitrile. The organic solvents were evaporated under nitrogen gas. 6 uL of 30% glycerol in methanol per sample was added before evaporation. Dried samples were reconstituted with 0.2 ml of chloroform and mixed with 1 ml of ice-cold acetone to precipitate proteins. The mixtures were then centrifuged for 5 min at 1,800 × g at 4°C. The organic layer of each sample was collected and evaporated under nitrogen.

Analysis of 2-AG and AEA was performed on an Ultivo triple quadrupole mass spectrometer combined with a 1290 Infinity II UPLC system (Agilent, Palo Alto, CA). The instrument was operated in electrospray positive mode with a gas temperature of 150°C at a flow of 5 L/min, nebulizer at 15 psi, capillary voltage of 4,500 V, sheath gas at 400°C with a flow of 12 L/min and nozzle voltage of 300 V. Transitions monitored were 348.3 → 287.3 and 62, 352.3 → 287.4 and 65.9, 379.3 → 287.2 and 269.2, and 384.3 → 287.2 and 296.1 for AEA, AEA-d4, 2-AG, and 2-AG-d5. The first fragment listed was used for quantification and the second fragment was used for confirmation. The first 3 min of analysis time was diverted to waste. Chromatographic separation was achieved using an isocratic system of 21% 1 mM ammonium fluoride and 79% methanol on an Acquity UPLC BEH C-18 1.7u 2.1 × 100 mm column (Waters, Milford, MA) maintained at 60°C. After each injection the column was washed with 90% methanol for 1 min then re-equilibrated for 5 min prior to the next injection. Samples were maintained at 4°C. Mixed calibration solutions were prepared by serial dilution of AEA and 2-AG stock solutions in 80% acetonitrile. Calibration curves were prepared for each analysis by adding 10 µL internal standard solution to 20 µL standard solution. Prior to sample analysis, 200 µL of 80:20 acetonitrile:water was added to dried samples which were then vortexed and sonicated. The samples were centrifuged at 15,800 × g at 4°C for 5 min. Supernatant was transferred to autosampler vials and 5 µL was injected for analysis.

Quantification of Diacylglycerol (DAG) by ELISA

Quantification of diacylglycerol (DAG) ELISA kit (Aviva System Biology, OKEH02607) was used according to manufacturer's instruction. Briefly, tissue samples were weighed, homogenized in 1× PBS buffer and stored overnight at ≤−20°C. Two freeze-thaw cycles were performed to break the cell membranes then homogenates were centrifuged at 5,000 × g at 4°C for 10 min. 5 µL of the supernatant was applied in the immunoassay.

Data Analysis and Statistics

GraphPad Prism 7.0 software (GraphPad Software) was used for statistical analysis. Unless otherwise stated, the data were expressed as mean ± SEM. Mechanical allodynia measurements were assessed using a repeated measure two-way ANOVA to analyze differences between treatment groups over time with either a Bonferroni or Tukey test applied post hoc. Molecular studies were compared by Student's *t*-test or one-way ANOVA, as indicated. Differences were considered significant if $p \leq 0.05$ to give 80% power to detect at 20% difference and prevent a type II error (GPower3.1).

RESULTS

2-AG, AEA Levels in Medication Overuse Headache Models

Endocannabinoid deficiency is primarily reported in chronic migraine patients (Burston and Woodhams 2014; McPartland et al., 2014; Gouveia-Figueira et al., 2017). To determine if this observation was recapitulated in preclinical medication overuse headache with regional selectivity in the CNS, female rats were continuously administered with either sumatriptan (0.6 mg/kg/day) or morphine sulfate (5 mg/kg/day) via osmotic minipump for 7 days. Following tissue harvest on D7, levels of 2-AG and AEA were determined in the V1M cortex (cortex), PAG, Vc, and TG using LC-MS (**Figure 1**). Mean levels of AEA in tissues for control (vehicle-treated) animals were as follows in pmol/g tissue: cortex: 17.24 ± 3.20, PAG: 21.43 ± 4.06, Vc: 13.12 ± 0.74, and TG: 23.64 ± 2.61. Chronic infusion of both sumatriptan and morphine for 7 days significantly increased levels of AEA in the cortex without changing levels in the PAG, Vc, or TG (two-way ANOVA $F(2,51) = 4.37$; $p = 0.017$; Tukey post-hoc $p = 0.031$

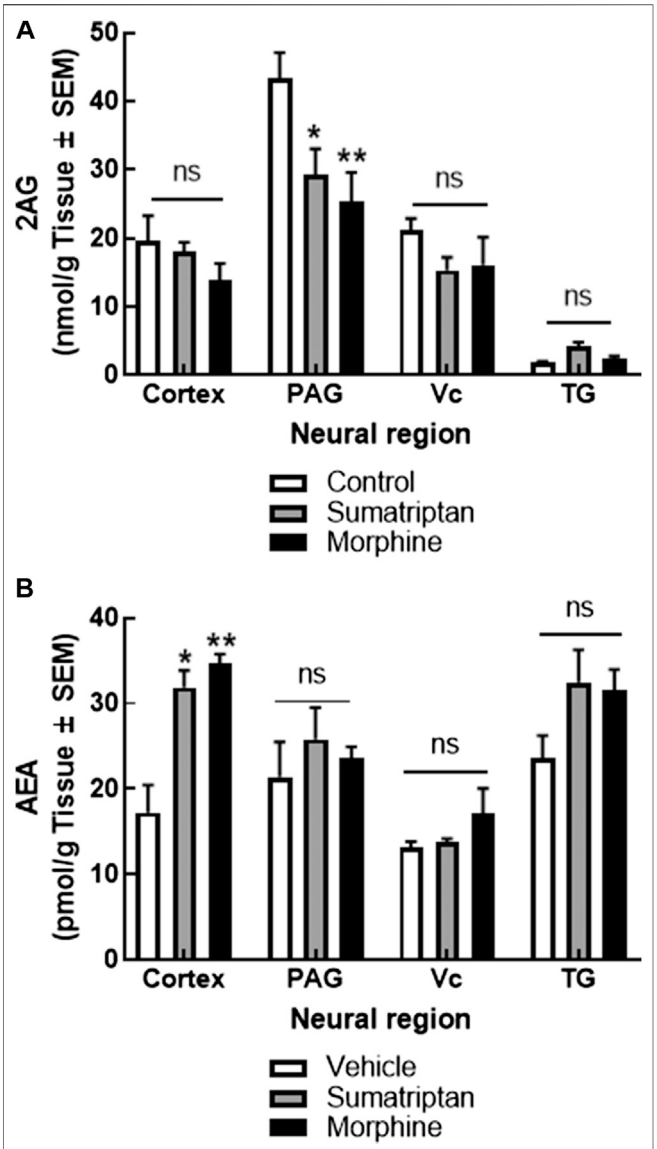

FIGURE 1 | Sustained administration of sumatriptan or morphine, as models of medication overuse headache, changed eCB levels in the cortex and PAG. 2-AG levels in the PAG, but not cortex, Vc, or TG were reduced after 7 days of sumatriptan (0.6 mg/kg/day, SC) or morphine (5 mg/kg/day, SC) infusion by osmotic minipump in female rats. **(A)** AEA levels were increased in the cortex, but not PAG, Vc, or the TG, in the same animals. **(B)** Data are expressed mean ± SEM (n = 3–9/group), analyzed by two-way ANOVA, $^{*}p < 0.05$, $^{**}p < 0.01$, ns, non-significant.

and $p = 0.004$, respectively) when compared to chronic vehicle treated animals.

Quantified mean control levels of 2-AG in nmol/g tissue were: cortex: 19.16 ± 3.64, PAG: 43.31 ± 3.85, Vc: 21.17 ± 1.73, and TG: 1.80 ± 0.17. Chronic infusion of both sumatriptan and morphine significantly reduced 2-AG levels in the PAG without changing levels in the cortex, Vc, or TG (Two-way ANOVA $F(2,48) = 6.80$; $p < 0.003$; Tukey post-hoc $p = 0.018$ and $p = 0.004$, respectively) when compared to chronic vehicle infusion. Thus, sustained administration of two analgesics associated with medication overuse headache altered eCB levels. Since clinically, endocannabinoid deficiency is associated with headache, the next experiments focused on recapitulating 2-AG depletion.

Selective Blockade of DAGLα, but Not DAGLβ, Induced Facial Allodynia Without Causing Peripheral Sensitivity

To investigate the possible connection between endocannabinoid depletion and headache-like pain, we tested the effect of a non-selective DAGL inhibitor, DH376, in female rats. DH376 was intraperitoneally injected at 10 mg/kg dose. The ED50 value of DH376 after intraperitoneal injection was determined between 5–10 mg/kg, showing blockade of DAGLα and DAGLβ in mice (Ogasawara et al., 2016). The periorbital and peripheral mechanical allodynia was assessed before and at 30, 60, 90, 120, 180, 360 min, and 24 h after drug treatment by calibrated von Frey filaments. DH376 (10 mg/kg, ip) significantly decreased periorbital withdrawal threshold at 60, 90, 120, and 180 min post-injection compared to vehicle-treated controls (**Figures 2A,B**; DH376 vs. vehicle, $p = 0.01$ at 60 min, $p = 0.013$ at 90 min, $p < 0.0001$ at 120 min, and $p = 0.02$ at 180, two-way ANOVA with Tukey's post-test, n = 11–17/group, $F(7,160) = 2.648$); (AUC-DH376: 39.61 ± 3.82 vs. vehicle: 52.67 ± 2.39, $p < 0.0001$, Student's *t*-test). All DH376-treated animals showed facial sensitivity, which was defined as FWT < 6 g at two consecutive time-points (**Figure 2C**). The treatment of non-selective DAGL inhibitor did not cause significant changes in hind-paw withdrawal threshold (**Figures 2D–F**; AUC- DH376: 78.93 ± 5.51 vs. vehicle: 77.16 ± 6.33, $p = 0.53$, Student's *t*-test), suggesting that loss of DAGL activity plays a role in facial, but not hind-paw, mechanical allodynia.

Next, the contributions of the individual DAGL isoforms to cephalic vs. hind-paw allodynia was tested. Female rats were treated with selective DAGL inhibitors, and the mechanical allodynia was evaluated before and at 30, 60, 90, 120, 180, 360 min, and 24 h after drug treatment in facial and left hind-paw, as described above. Selective inhibition of DAGLα, by LEI106 induced facial allodynia (**Figure 3**). LEI106 at 20 and 40 mg/kg significantly reduced periorbital withdrawal thresholds 120 and 180 min post-injection as compared to vehicle control (**Figure 3A**) (two-way ANOVA with Bonferroni post-test, n = 9–13/group, Ftime x treatment(21, 284) = 3.472; $p = 0.017$–0.039). The 10 mg/kg LEI dose reduced periorbital withdrawal thresholds by ~35% 120- and 180-min post administration; this did not reach statistical significance ($p = 0.07$ and $p = 0.06$, respectively; **Figure 3A**). Moreover, the highest dose of LEI106 showed the longest duration of effect, the facial threshold was significantly reduced at 24 h time-point after the injection of LEI106 (**Figure 3A**). While magnitude was similar between 10 and 20 mg/kg, escalating doses of LEI106 increased the percentage of animals exhibiting facial sensitivity, defined as FWT < 6 g at two consecutive time-points (**Figure 3E**). Calculation of the area under the curves revealed that LEI106 induced significant dose-dependent periorbital allodynia (**Figure 3B**, one-way ANOVA F = 10.85, $p < 0.001$; Tukey post-hoc $p = 0.036$ and $p < 0.0001$).

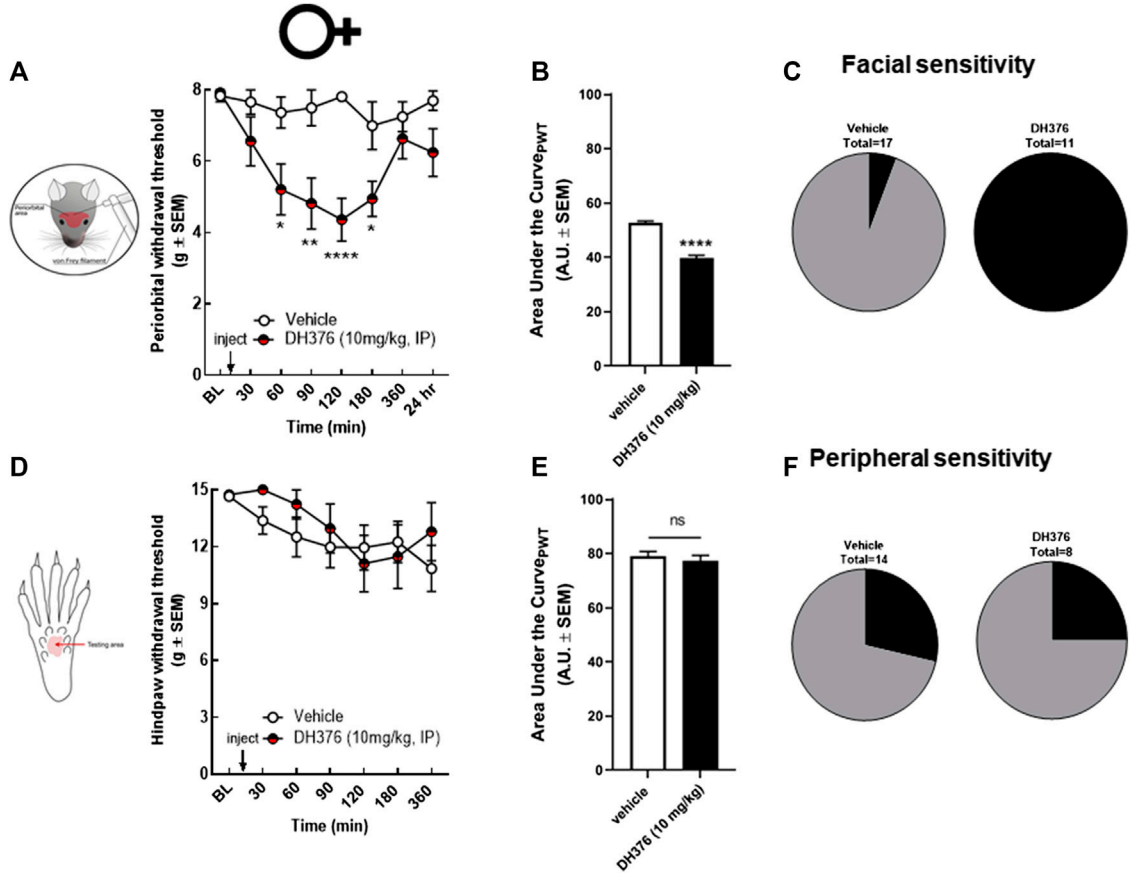

FIGURE 2 | Non-selective inhibition of DAGL induced periorbital but not peripheral mechanical allodynia in female rats. Intraperitoneal injection of DH376 (10 mg/kg, IP) significantly decreased periorbital withdrawal threshold **(A)** at 60, 90, 120, and 180 min post-injection compared to vehicle-treated controls. Data are expressed mean ± SEM (n = 11–17/group), analyzed by two-way ANOVA, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$. Hind-paw withdrawal thresholds after injection of DH376 were not significantly different compared to vehicle-treated controls **(D)**. Data are expressed mean ± SEM (n = 8–14), analyzed by two-way ANOVA, ns, non-significant. The area under the curve for the corresponding time-course experiments **(B,E)**, assessed by Student's *t*-test, $^{****}p < 0.0001$, ns, non-significant. The percentage of animals in vehicle-, and in DAGL-inhibitor treated groups, showing facial **(C)** and peripheral **(F)** sensitivity, as defined FWT < 6 g at two consecutive time-points in facial area.

To assess sex as a behavioral variable in this model, male rats were injected with calculated A50 dose from females of LEI106 (20 mg/kg, IP) and evaluated for periorbital and hind-paw allodynia over time (**Figures 3C,D,F,H**). LEI106 (20 mg/kg) induced significant periorbital allodynia in 3/8 rats (FWT < 6 g at two consecutive timepoints (**Figure 3F**). Collectively, facial withdrawal thresholds were reduced by 24 and 25% of baseline at 120 and 180 min, respectively; this was not statistically significant (n = 8, two-way ANOVA, Ftime x treatment(7,89) = 1.913, $p = 0.077$, Bonferroni post-hoc). Area under the curve analysis showed a significant reduction in facial threshold over time (**Figure 3D**, Student's *t*-test $p = 0.0006$).

To determine if DAGLα inhibition induced peripheral sensitivity, hind paw allodynia was assessed. Similar to the non-selective blockade of DAGL, selective inhibition of DAGLα did not induce hind-paw sensitivity in female rats at any dose tested as compared to vehicle (**Figure 3G**) (two-way ANOVA Ftime x treatment(18,209) = 0.845, $p = 0.65$; Tukey post-hoc; n = 8–13). Hind-paw allodynia was observed in 2/8 male rats (two consecutive timepoints <12 g), but overall, no significant hind-paw allodynia was observed in the cohort (n = 7, two-way ANOVA, Ftime x treatment(7,91) = 1.427, $p = 0.20$; **Figure 3H**). Periorbital allodynia was not observed in vehicle control animals of either sex (**Figures 3E–H**).

KT109, a selective inhibitor, was used to assess DAGLβ contributions to the development of periorbital allodynia (**Figure 4**). Increasing doses of KT109 (5, 10, 20 mg/kg, IP) did not induce significant periorbital (**Figure 4A**) or hind paw allodynia (**Figure 4C**) across the experimental duration in female rats. Dosing of male rats with KT109 (20 mg/kg) showed similar results (**Figures 4B,D**). Together, these data indicate that inhibition of DAGLα, but not DAGLβ, is enough to induce cephalic, but not extracephalic, mechanical sensitivity with a pronounced effect in females.

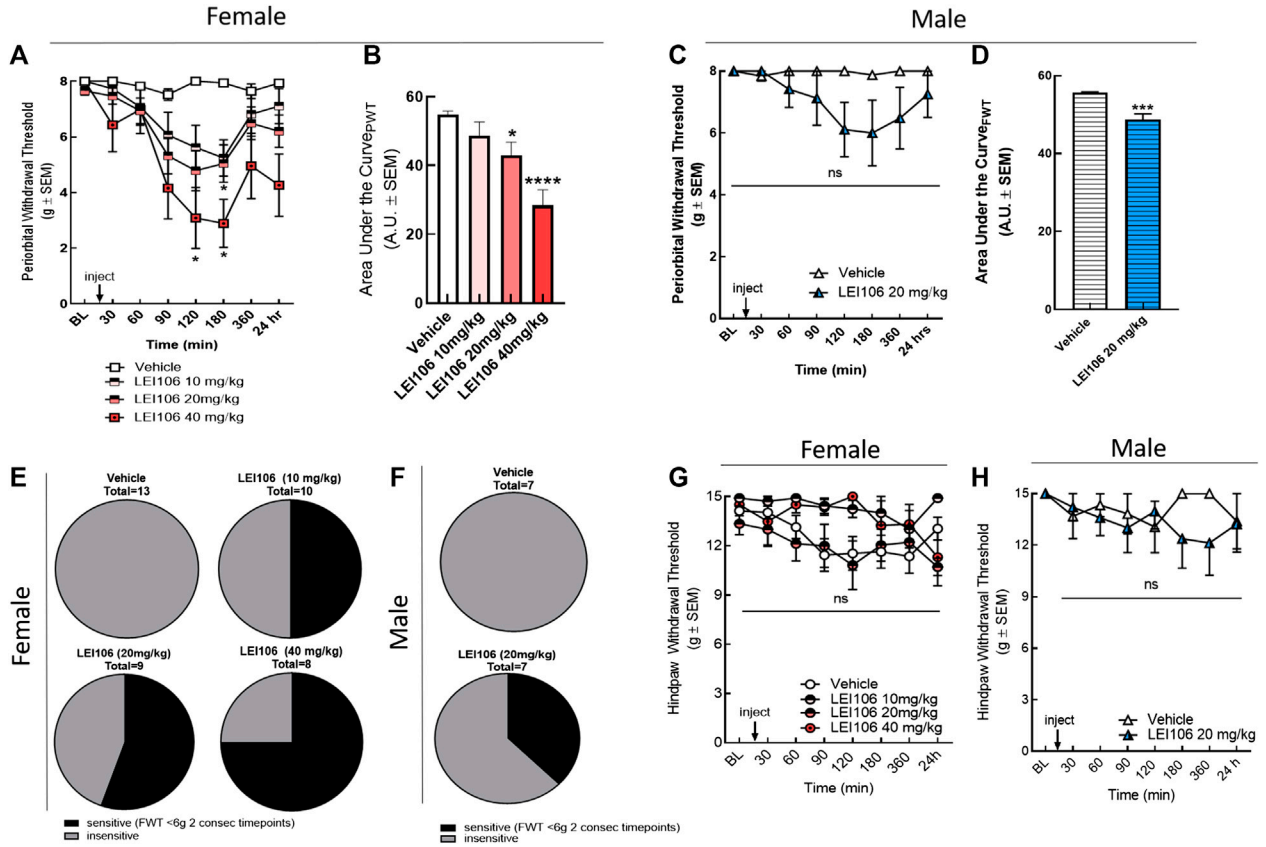

FIGURE 3 | DAGLα inhibition with LEI106 induced significant periorbital but not hind-paw allodynia. Intraperitoneal injection of LEI106 at 10, 20, and 40 mg/kg induced facial allodynia in female animals **(A)**. LEI106 at 20 and 40 mg/kg doses caused significant reduction in facial withdrawal thresholds at 120 and 180 min post-injection compared to vehicle control. Data are expressed mean ± SEM (n = 8–13), two-way ANOVA, $^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$. In male animals, intraperitoneal injection of LEI106 at 10 mg/kg did not cause significant changes in facial withdrawal thresholds in any time-point **(C)**. Data are expressed mean ± SEM (n = 7), two-way ANOVA, ns, non-significant. The area under the curve for the corresponding time-course experiments **(B,D)**, assessed by one-way ANOVA (female) or Student's *t*-test (male), $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. The percentage of animals in vehicle-, and in LEI106-treated groups, showing facial sensitivity **(E,F)**, as defined FWT < 6 g at two consecutive time-points in facial area. LEI106 did not significantly influence the hind-paw withdrawal thresholds in either doses in either sexes **(G,H)**, suggesting the absence of peripheral action.

Periorbital Allodynia Induced by DAGLα Inhibition Is Sensitive to Anti-Migraine Agents

Responsivity to the acute dosing of abortive antimigraine agents, sumatriptan and olcegepant were used to validate DAGLα inhibition as a non-invasive, preclinical model of headache. Female rats were treated with LEI (20 mg/kg, IP) and the mechanical allodynia allowed to develop. Thirty minutes after LEI106 dosing, female rats were injected with either sumatriptan (0.6 mg/kg, SC) or olcegepant (0.8 mg/kg, IP) and facial mechanical sensitivity reassessed (**Figure 5**). As in **Figure 3**, LEI + saline induced significant periorbital allodynia in 5/7 rats. Sumatriptan significantly mitigated this allodynia 90, 120, and 180 min after dosing in 6/7 rats (**Figures 5C,D**) (LEI106 + sumatriptan vs. LEI106 + saline, two-way ANOVA with Tukey's post-test, n = 8/group, F(6,61) = 4.439, $p = 0.0009$). Responsivity to the CGRP receptor antagonist was assessed in a separate set of rats (**Figures 5E,F**). LEI106 + vehicle (10%DMSO: 10% Tween80: 80% saline) induced significant periorbital allodynia in 5/8 rats. Olcegepant dosed after DAGLα significantly attenuated the periorbital allodynia associated with LEI106 at 90, 120, 180 and 360 min after LEI dosing; allodynia was observed in 1/8 rats (LEI106 + olcegepant vs. LEI106 + saline, two-way ANOVA with Tukey's post-test, n = 8/group, F(6,74) = 3.021, $p = 0.011$). These results suggest that periorbital allodynia induced by DAGLα inhibition is reversed by two clinically relevant, antimigraine agents.

DAGLα Inhibition Induces Non-Allodynic Measures of Pain Linked to Headache but Not Anxiety

Head pressing against a hard surface is a sign of neurological dysfunction in animals and is linked to severe pain in rodents (Kohn et al., 2007). Administration of LEI106 dose-dependently increased the number of animals exhibiting head-pressing

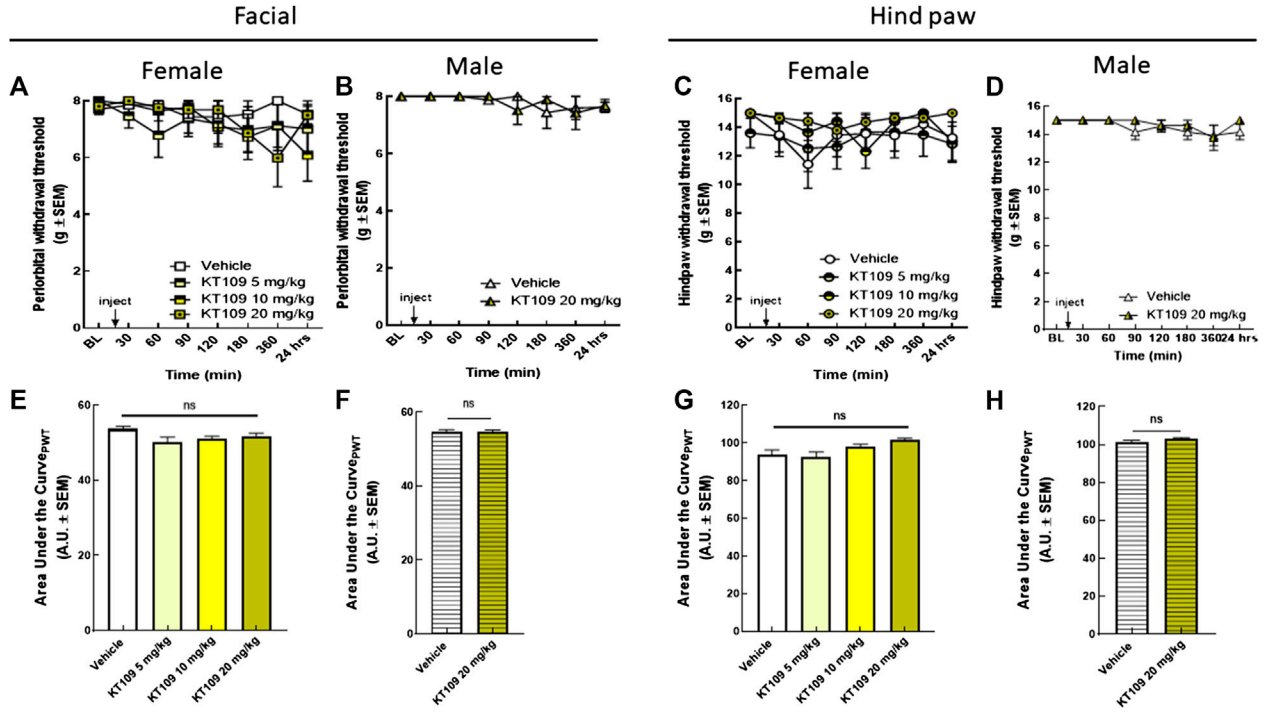

FIGURE 4 | Inhibition of DAGLβ did not induce periorbital or hind-paw allodynia in either sex. Injection of KT109 (5, 10, 20 mg/kg, IP) did not significantly influence periorbital **(A)** or hind-paw withdrawal thresholds **(C)** in female rats. Data are expressed mean ± SEM (n = 6–9), two-way ANOVA, ns, non-significant. Intraperitoneal injection of KT109 (20 mg/kg) did not cause significant changes of facial **(B)** or hind-paw **(D)** thresholds in male animals. Data are expressed mean ± SEM (n = 8), two-way ANOVA, ns, non-significant. The area under the curve for the corresponding time-course experiments **(E–H)**, assessed by one-way ANOVA or Student's *t*-test, ns, non-significant.

behaviors (**Figures 6A,B**). Neither vehicle, nor the DAGLβ induced head pressing behaviors after systemic dosing.

In addition to facial sensitivity, migraine headache is associated with photophobia. To determine if DAGLα inhibition induced sensitivity to light, rats could choose between light and dark environments in the light/dark preference assay at the time of peak periorbital allodynia, 120 min post-injection. Female rats administered LEI106 (20 mg/kg, IP) spent significantly more time in the dark chamber as compared to the light despite the number of entries being the same (**Figure 6C**; LEI106 vs. vehicle, Student's *t*-test $p = 0.03$, n = 8/group).

Given that the light-dark box is used for both photophobia and anxiety measures (Albani et al., 2015), the same rats were assessed for anxiety like behaviors using the elevated plus maze. LEI106 (20 mg/kg, IP) did not statistically change the number of entries between the open, closed and transition square when compared to vehicle treated animals (**Figure 7A**); the total time in each arm was also similar between vehicle and LEI106 treatments (**Figure 7B**). LEI106 administration (20 mg/kg, IP) significantly reduced the total distance traveled in the open arms, but not closed (**Figures 7C,D**; Student's *t*-test, n = 8/group, $p = 0.04$).

Several headache models have reported changes in open field parameters as a measure of motor skill, moreover, the ability to choose a preferred environment relies on the ability to move voluntarily (Vuralli et al., 2019). To assess whether reduced movements in the light-dark assay and elevated plus maze were confounds by reduced movement, rats were evaluated using the open field test. LEI106 dosing did not statistically change the total distance traveled, sped of travel, or time spent in the center vs. the perimeter zone (**Figure 8**; LEI106 vs. vehicle, total distance traveled: $p = 0.08$, speed of travel: $p = 0.07$; time spent in the center vs. the perimeter zone: $p = 0.07$, Student's *t*-test, n = 8/group). Together, these data suggest DAGLα inhibition produces migraine-like behaviors of allodynia and photophobia without reducing voluntary movement or acting as an anxiogenic.

Regional Differences in 2-AG, AEA, but Not DAG Levels After DAGL Inhibition

To determine if behavioral outcomes aligned with differences in 2-AG and AEA levels, cortex, PAG, Vc, and TG tissue samples from female rats were collected 120 min after drug administration, and analyzed by LC-MS. Systemic administration of the DAGLα selective inhibitor, LEI106 (20 mg/kg, IP) significantly reduced 2-AG levels in the cortex (V1M) and PAG, but not in Vc or TG, as compared to vehicle treated controls (**Figure 9A**; LEI106 vs. vehicle Student's *t*-test by region: Ct: $p = 0.02$, PAG: $p = 0.03$, TG: $p = 0.68$, Vc: $p = 0.06$, n = 3/group). AEA levels were not significantly different between

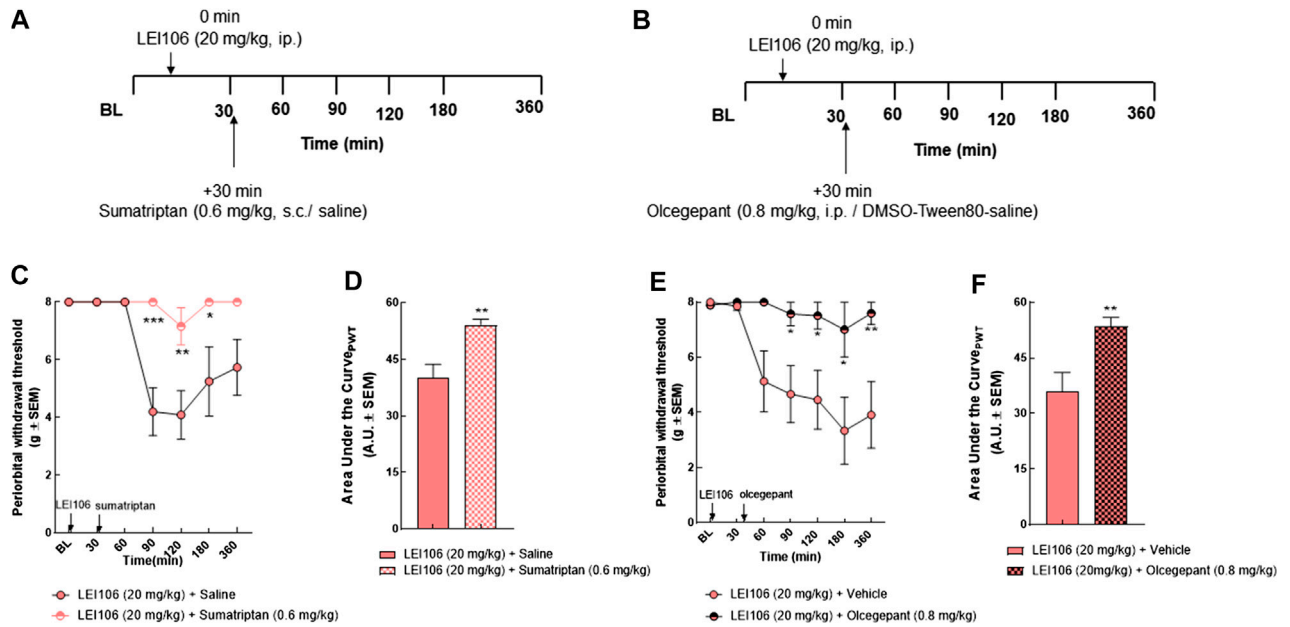

FIGURE 5 | Periorbital allodynia induced by DAGLα inhibition is sensitive to anti-migraine agents, sumatriptan and olcegepant. Timeline of experiments **(A,B)**. Injection of sumatriptan (0.6 mg/kg, SC) significantly mitigated the periorbital allodynia induced by LEI106 (20 mg/kg, IP) at 90, 120, and 180 min after dosing **(C)**. Data are expressed mean ± SEM (n = 8), two-way ANOVA, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Administration of olcegepant (0.8 mg/kg, IP) significantly attenuated the periorbital allodynia induced by LEI106 (20 mg/kg, IP) at 90, 120, 180, and 360 min after dosing **(E)**. Data are expressed mean ± SEM (n = 8), two-way ANOVA, $*p < 0.05$, $**p < 0.01$. The area under the curve for the corresponding time-course experiments **(D,F)**, assessed by Student's *t*-test, $**p < 0.01$.

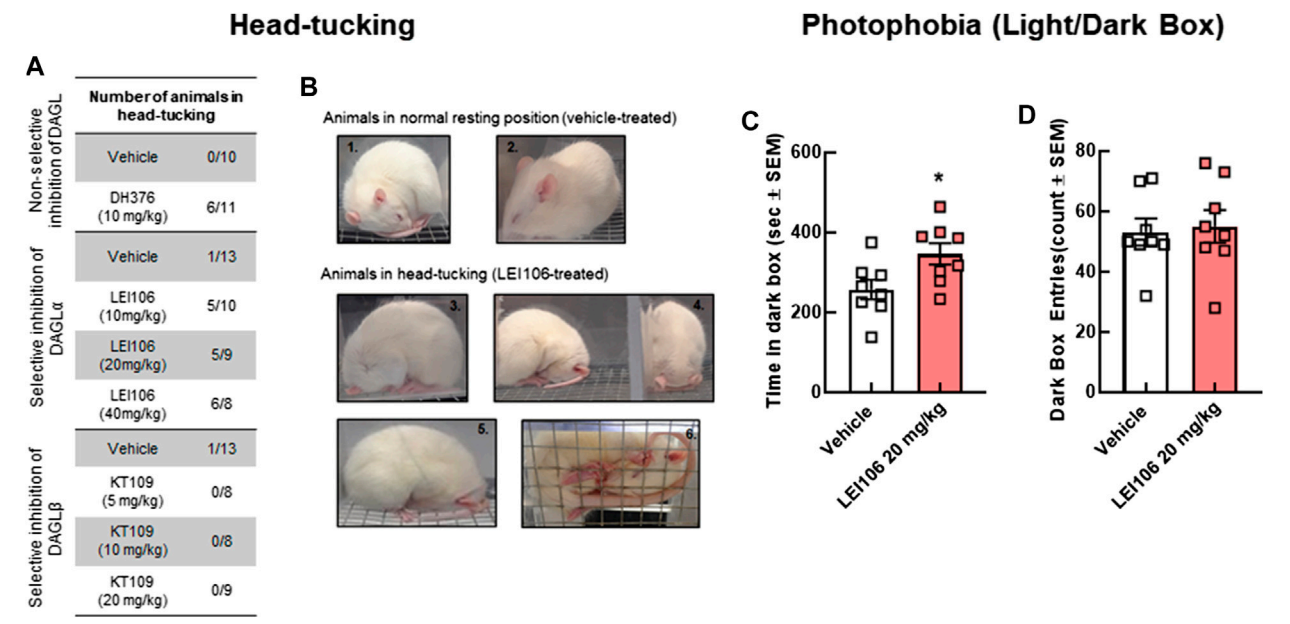

FIGURE 6 | DAGLα inhibition induces non-allodynic measures of pain linked to headache in female rats. The animals treated with non-selective inhibitor, DH376 (10 mg/kg, IP) or DAGLα-selective, LEI106 (10, 20, and 40 mg/kg, IP) showed spontaneous pain-related behavior, known as head-tucking. After injection of DAGLβ inhibitor, KT109, head-tucking behavior was not observed. The number of animals, showing head-tucking/total number of animals in each treatment group **(A)**, representative photographs about animals in normal resting position **(B1,B2)** after vehicle-injection, and in head-tucking-position **(B3–B6)** after treatment with LEI106. In light-dark box experiment, female rats administered LEI106 (20 mg/kg, IP) spent significantly more time in the dark chamber as compared to the light side **(C)**. However, there was no significant differences in the number of entries **(D)**. Data are expressed mean ± SEM (n = 8), Student's *t*-test, $*p < 0.05$, ns, non-significant.

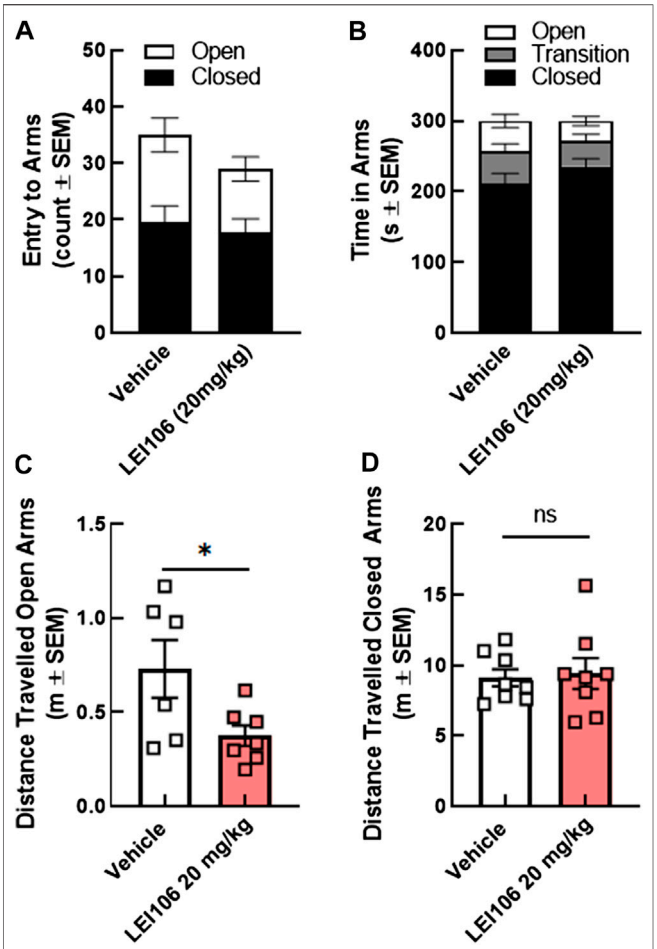

FIGURE 7 | DAGLα inhibition did not induce anxiety in the elevated plus maze tests in female rats. LEI106 (20 mg/kg, IP) did not statistically influence the number of entries between the open, closed and transition square when compared to vehicle treated animals **(A)**. No statistically significant differences between vehicle and LEI106 treatments were observed in the total time in each arm **(B)**. However, administration of LEI106 (20 mg/kg, IP) significantly reduced the total distance traveled in the open arms, but not closed **(C,D)**. Data are expressed mean ± SEM (n = 8), Student's *t*-test, $^{*}p < 0.05$, ns, non-significant.

vehicle and LEI106-treated rats in any sample evaluated (**Figure 9B**; LEI106 vs. vehicle Student's *t*-test by region: Ct: $p = 0.87$, PAG: $p = 0.35$, TG: $p = 0.49$, Vc: $p = 0.94$, n = 3/group). Dosing with the DAGLβ selective inhibitor, KT109 (20 mg/kg, IP), significantly increased 2-AG levels in the cortex relative to vehicle dosed rats; levels in PAG, Vc, and TG were not changed (**Figure 9C**; KT109 vs. vehicle Student's *t*-test by region: Ct: $p = 0.01$, PAG: $p = 0.91$, TG: $p = 0.01$, Vc: $p = 0.28$, n = 3/group). In contrast, KT109 significantly reduced AEA levels in both cortex and PAG, but not Vc or TG (**Figure 9D**; KT109 vs. vehicle Student's *t*-test by region: Ct: $p = 0.008$, PAG: $p = 0.03$, TG: $p = 0.19$, Vc: $p = 0.40$, n = 3/group).

To determine if LEI106 induced reductions in 2-AG were coupled to changes in DAG levels, quantitative ELISA was employed. Analysis showed DAG levels (ng/g tissue) in the

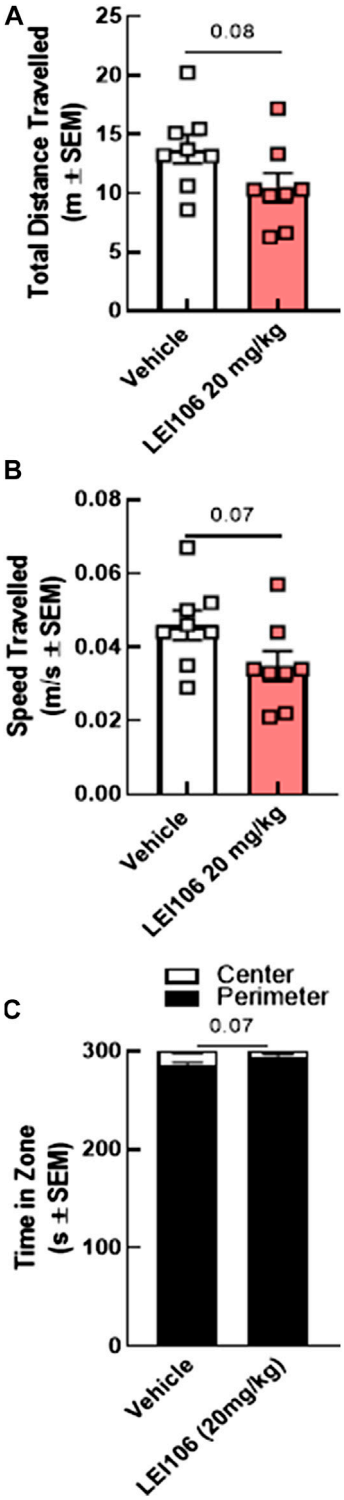

FIGURE 8 | DAGLα inhibition did not influence voluntarily movements and motor skills in the open field tests in female rats. The injection of LEI106 (20 mg/kg IP) did not statistically change the total distance traveled **(A)**, the sped of travel **(B)**, or the time spent in the center vs. the perimeter zone **(C)**. Data are expressed mean ± SEM (n = 8), Student's *t*-test, ns, non-significant.

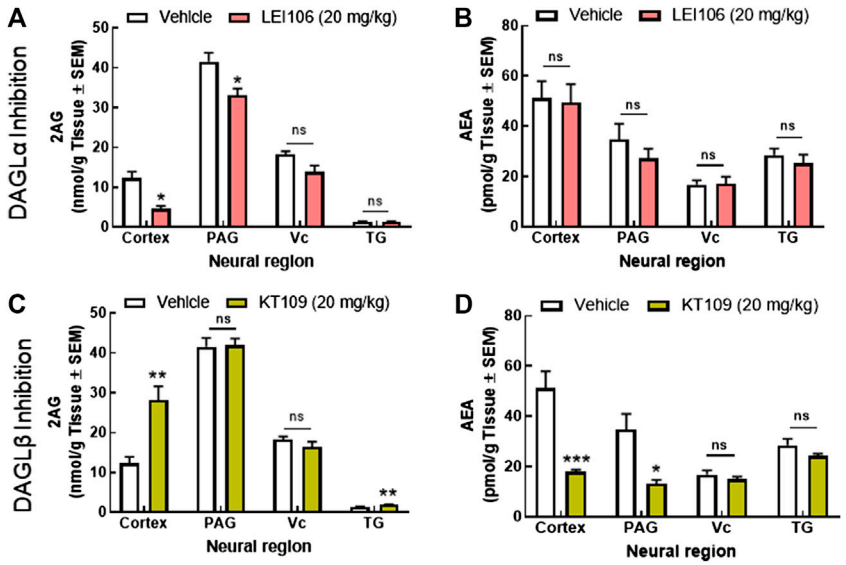

FIGURE 9 | Reduction in cortical and PAG 2-AG, but not AEA is associated with the induction of headache-like pain in female rats. Systemic administration of the DAGLα selective inhibitor, LEI106 (20 mg/kg, IP) significantly reduced 2-AG levels in the cortex and PAG, but not in Vc or TG, as compared to vehicle treated controls **(A)**. There were no significant differences in the level of AEA between vehicle and LEI106-treated rats in any sample evaluated **(B)**. Injection of the DAGLβ selective inhibitor, KT109 (20 mg/kg, IP), significantly increased 2-AG levels in the cortex relative to vehicle treated rats; levels in PAG, Vc, and TG were not changed **(C)**. KT109 significantly reduced AEA levels in both cortex and PAG, but not Vc or TG **(D)**. Data are expressed mean ± SEM (n = 3), one-way ANOVA, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, ns, non-significant.

cortex (Vehicle = 192.17 ± 38.44; LEI106: 145.66 ± 6.29) and PAG (Vehicle = 168.21 ± 7.18; LEI106: 193.21 ± 28.30) that were not significantly different (Student's *t*-test, cortex $p = 0.30$; PAG $p = 0.44$) (data not shown). Together, these data support loss of 2-AG via inhibition of DAGL in the PAG as a driver of headache pain in this model.

DISCUSSION

Clinical migraine is associated with sensory sensitivities such as allodynia, photophobia, phonophobia, and hyperosmia (Goadsby et al., 2017). Several animal models have been developed with the aim to understand migraine disorder and to develop new therapeutic strategies (Munro et al., 2017; Harriott et al., 2019; Vuralli, et al., 2019). Although most of these models display similar phenotype to human migraine, there is currently no animal model able to replicate all its features, since migraine is considered a complex disorder with variable phenotypes. Recent clinical studies of migraine sufferers have demonstrated that while episodic migraineurs primarily experience hypersensitivity of solely cephalic sites, chronic migraineurs are more likely to experience both cephalic and extracephalic hypersensitivity (Mathew et al., 2004; Kitaj and Klink, 2005; Bigal and Lipton, 2008; Guy et al., 2010). Similarly, patients with TTH (tension-type headache) solely experience cephalic pain (IHS, 2018). Thus, a reverse translational approach to verifying models for these pain states should focus on the ability of these models to solely induce cephalic hypersensitivity.

Recent reports suggest that functional pain disorders, including migraine/chronic headache, result from deficiencies in the eCB system (Greco et al., 2018a; Greco et al., 2018b; Russo 2016; Tassorelli et al., 2019). Though numerous animal models of migraine-like pain exist (Munro et al., 2017; Harriott et al., 2019; Vuralli, et al., 2019), these models often are invasive or minimally invasive and have not investigated contributions of the ECS to migraine pain. Thus, the studies above investigated the hypothesis that pharmacologic inhibition of DAGL, the enzyme implicated in the synthesis of 2-AG, would induce symptomology aligning with clinical episodic headache. In two established models of medication overuse headache2-AG was reduced in the PAG, while AEA was increased in the cortex. Selective inhibition of DAGLα, but not DAGLβ, induced significant periorbital allodynia in a dose-dependent manner, accompanied with increased secondary behavioral measures of distress (i.e., head pressing) and photophobia, but not reduced movement or anxiety. Behavioral outcomes were associated with significant reductions in 2-AG levels in the cortex and PAG, but not within the trigeminal ganglia or Vc, indicating a CNS origin of headache-like pain; AEA levels were unchanged. Together, these data support 1) DAGLα inhibition as a non-invasive model of episodic headache and 2) identify the 2-AG-ECS system within the PAG as necessary for headache pathology.

The PAG is a point of integration in the descending pain modulatory systems that receives information from cortical and subcortical brain regions and projects to the rostroventromedial medulla (RVM) (Yam et al., 2018). Changes in PAG connectivity have previously been implicated in migraine as well as cannabinoid signaling in this brainstem region (Goadsby et al.,

2017). CB_1R agonists applied in the ventrolateral PAG attenuated dural Aδ trigeminal afferent activation of second order neurons without altering cutaneous activity in the V1 region of the trigeminal nerve; similar effects were observed with the 5HT1B/1D agonist, naratriptan (Akerman et al., 2013). Application of a CB1R antagonist reversed these observations (Akerman et al., 2013). Moreover, AEA did not mediate the same effects in these studies, suggesting that that a second eCB activates these CBRs endogenously. It is also known that 2-AG induces retrograde inhibition (disinhibition) of GABA release via presynaptic CB1 receptors in PAG which leads to antinociception by activating the descending pain inhibitory pathways (Ho et al., 2011; Liao et al., 2011). Interestingly, immunofluorescent staining in these studies demonstrated the presence of DAGLα, but not DAGLβ in PAG. The data collected herein suggest that systemic application of a DAGLα inhibitor selectively depleted 2-AG in the cortex and PAG, whereas DAGLβ increased 2-AG levels in the cortex and reduced AEA levels in the PAG and cortex; in both conditions, eCB levels in the Vc and TG were unchanged. These results implicate 2-AG within the PAG as the endogenous eCB exerting regulation over descending pain modulatory circuits to the trigeminal brainstem complex during headache.

Under normal physiology, 2-AG release is considered on-demand in response to stress, inflammation, and to restore homeostasis (Horne and Stella, 2008). 2-AG acts at CB1R and CB2R to reduce transmitter release with Gαi signaling (Reggio, 2010). Hydrolysis of 2-AG generates arachidonic acid, the precursor substrate for both AEA, and eicosanoid inflammatory signaling (Murataeva et al., 2014). Depletion of 2-AG via DAGLα inhibition with LEI106 is reported to reduce arachidonic acid and eicosanoid levels, elevate DAGs, and impair synaptic plasticity without altering AEA levels *in vitro* and in mice (Janssen et al., 2014; Kohnz and Nomura, 2014); findings above confirm these observations in rats in the cortex and PAG. The pharmacological blockade of DAGLβ by KT109 showed lower levels of 2-AG, arachidonic acid, and prostaglandins in Neuro2A cells, mouse peritoneal macrophages, mouse liver, and human prostate cancer cells (Hsu et al., 2012; Hsu et al., 2013). Activity-based protein profiling confirmed that KT109 is a systemically active DAGLβ inhibitor. DAGLβ in the lumbar spinal cord and brain was blocked after intraperitoneal injection of KT109 at 30 mg/kg dose in rats. However, the treatment with KT109 did not significantly reduce 2-AG, AA, and PGE2 levels in brain, indicating different role of DAGLβ in the central endocannabinoid system (Luk et al., 2018). Our LC-MS results correspond well with those findings. The genetic studies also provided evidence of the different roles of DAGLs in the central nervous system. Dramatic reduction of 2-AG in cortex, cerebellum, hypothalamus, and hippocampus were observed in DAGLα knockout mice, while DAGLβ knockout mice showed lower 2-AG levels only in the hypothalamus, proofing the different contributions of the two isoforms within different brain regions (Gao et al., 2010; Tanimura et al., 2010).

Alternatively, DAGLα inhibition may lead to accumulation of DAG. DAG can activate nonselective cation channels, including TRPV1 and TRPC3, as well as receptor- and store- operated calcium channels to activate nociceptive fibers (Brown and Passmore, 2010; Lafreniere and Kelly, 2018; Yasko et al., 2019). Inhibition of DAGLα did not significantly change DAG levels in the cortex or PAG suggesting that this mechanism may not play a role in the periorbital allodynia resulting from DAGLα inhibition. Rather, periorbital allodynia after blockade of DAGLα may reflect a loss of CB receptor activation on descending pain inhibitory pathways or reduced vascular tone by 2-AG; this is supported by results with sumatriptan and olcegepant. Together, these data point to a central mechanism driving headache pain that is regulated by the 2-AG-eCB system.

Preclinically, medication overuse induced allodynia in animals is observed in both periorbital (central) and hind-paw (peripheral) sites (De Felice et al., 2011). In other acute models, including NO-donor and CSD induction, whole body allodynia is also observed (Fioravanti et al., 2011; Kim et al., 2018). Here, and in contrast to other preclinical models, acute DAGL inhibition induced cephalic hypersensitivity in the absence of hind-paw allodynia, suggesting differences in the role of DAG/DAGL/2-AG signaling in facial vs. somatic regions. Importantly, female rats showed greater sensitivity to DAGLα inhibition as compared to males mirroring sex differences in the clinical presentation of migraine. Relevant to clinical translation, animals with sensitivity after DAGLα inhibitor administration showed positive responses to the clinically used antimigraine agent sumatriptan and the CGRP antagonist, olcegepant.

Non-sensory features of migraine/chronic headache include anxiety, depression, and obesity that increase distress and reduce quality of life (Katsarava et al., 2012). Anxiety disorders are more prevalent in chronic migraineurs (30–50%) vs. episodic (18%) (Katsarava et al., 2012), and depression is reported to precede the transition of episodic migraine to chronic (Ashina et al., 2012). DAGLα inhibition did not reduce voluntary movement or induce anxiety in the open field and elevated plus maze tests, two assays shown to assess behavioral distress (Belovicova et al., 2017), suggesting that acute DAGLα is not necessary for these behaviors. This is notable since these clinical features are indicative of the transition of episodic to chronic migraine (Katsarava et al., 2012). In conclusion, this model recapitulates the pain presentation and behavioral symptomology of episodic, but not chronic, headache.

Limitations: The authors acknowledge that the studies herein have limitations. The drugs applied in these studies might have off-target(s) effects. It is known that LEI106 inhibits the hydrolysis of 2-AG by ABHD6 in mouse brain membrane homogenates and in HEK293T cell membrane preparation (Ki = 0.8 μM) (Janssen et al., 2014). However, the IC50 value of LEI106 (18 nM), targeting sn-1 DAGLα is 40-fold lower, than its off-target ABHD6, proving the higher affinity of LEI106 toward DAGLα. Our LC-MS data showed reduced 2-AG levels in cortex and PAG after the administration of LEI106, aligning with the more potent DAGLα inhibitory effect of LEI106. Notably, KT109 was also reported to inhibit ABHD6 in rodent brain (Hsu et al., 2012; Luk et al., 2018), but our LC-MS results as well as previous report (Luk et al., 2018) did not detect changes in brain 2-AG levels after injection of KT109. KT109 also showed inhibitory activity against PLA2G7 and MAGL at higher concentrations (Kohnz and Nomura, 2014).

Secondly, a genetic approach, targeting Dagla gene would provide additional mechanistic insight into DAG/DAGL/2-AG signaling. Jenniches and co-workers investigated the consequences of the deletion of Dagla gene (Jenniches et al., 2016). 2-AG levels were significantly decreased (by 80–90%) in cortex, hippocampus, striatum, and amygdala in Dagla−/− mice, but reduced AEA levels in cortex and amygdala were also observed. The deletion of Dagla enhanced anxiety, stress, and fear responses, including reduced exploration of the central area of the open field, and increased anxiety-related behaviors in the light/dark box, which were not observed after the acute administration of DAGLα inhibitor, LEI106 in our study. Dagla−/− mice did not show increased pain sensitivity but altered pain response in the hot plate test, drawing the attention to the possible involvement of pain signaling. It is notable that reduced hippocampal neurogenesis was also detected in Dagla−/− mice. The behavioral phenotype of Dagla knock-out animals suggest that the systemic deletion of Dagla affect the emotional state in addition to pain signaling. However, it is not known that this phenotype is due to the acute disruption of 2-AG biosynthesis or rather a consequence of developmental effects. The genetic manipulation of Dagla gene in certain brain area(s) by microinjections would clarify the different roles of DAGLα in pain signaling, anxiety, and stress-responses, but one of the strengths of the present study is the non-invasive feature of pharmacological manipulation of DAGLα would be lost in those experiments.

Our model captured the main features of migraine-like headache, including cutaneous allodynia at the cephalic site, spontaneous pain behavior, like head-pressing and photophobia, however, non-sensory features of migraine such as anxiety, depression were not observed with acute DAGLα inhibition. It is notable that not all the sensory sensitivities associated with clinical migraine, such as phonophobia or hyperosmia were studied in our model system. Several well-validated animal models of headache-like pain exist, each of them has its own advantage and limitation, but nonecan cover all migraine-associated symptomology (Munro et al., 2017; Harriott et al., 2019; Vuralli, et al., 2019; Levine et al., 2020). It is known that 70% of migraineurs experience cephalic allodynia, although extracephalic allodynia in the arms and legs was reported in more severe and chronic cases (Burstein et al., 2000). Anxiety and depression are also more prevalent symptoms in patients with chronic migraine (Ashina et al., 2012; Katsarava et al., 2012). Therefore, symptoms observed after the acute pharmacological blockade of DAGLα more likely represent the episodic stage of migraine-like pain. Whether chronic administration of DAGLα inhibitor could capture signs of chronic form of migraine is an exciting question that needs to be addressed in future experiments.

CONCLUSION

Clinical endocannabinoid deficiency is one theory underlying migraine/chronic headache pathology. Drawing on this idea, studies here selectively blocked DAGL to reduce 2-AG which induced symptomology reflective of clinical episodic migraine. DAGLα, rather than DAGLβ, was identified as the critical isoform needed for cephalic pain. Importantly, periorbital sensitivity was reversed by clinical abortive antimigraine agents further supporting the utility of this model to study migraine/chronic headache pathology. Finally, this model of headache via DAGLα inhibition revealed that loss of 2-AG within the PAG is necessary for induction of headache pain. Incorporation of this non-invasive headache model in the field may improve the translation of novel therapeutics and understanding of headache pathology.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by IACUC approval from the University of Arizona (17-223).

AUTHOR CONTRIBUTIONS

AL: Experimental design, data collection/analysis, manuscript preparation. EL-B: Experimental design, data collection/ analysis/ and interpretation, manuscript preparation. KK: Data collection and analysis. LG: Data collection and analysis. DS: Experimental design, data interpretation, manuscript preparation. TV: Experimental design, data interpretation, manuscript preparation. TL-M: Experimental design, data analysis/interpretation, manuscript preparation.

FUNDING

This work was supported by grants from the National Institute of Neurological Disorders and Stroke (R01NS099292, TL-M) of the National Institutes of Health, Arizona Biomedical Research Commission (ABRC45952, TL-M), with monies from the Department of Pharmacology at the University of Arizona, the M.D.-Ph.D. Program at the University of Arizona, and the University of Arizona Comprehensive Pain and Addiction Center. Research reported in this publication was also supported by the National Cancer Institute of the National Institutes of Health under award number P30 CA023074.

ACKNOWLEDGMENTS

The authors would like to acknowledge Mario van der Stelt from Leiden University for providing DH376. University of Arizona Cancer Center's Analytical Chemistry Shared Resources is acknowledged for the assistance of LC-MS service.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 29 January 2021
doi: 10.3389/fphar.2020.615782

Resistance of Food-Maintained Operant Responding to Mechanical Punishment in Rats: Further Evidence for Weak "Affective/Motivational Pain" in Rat Models of Inflammatory and Neuropathic Pain

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Clinically relevant chronic pain is often associated with functional impairment and behavioral depression as an "affective/motivational" sign of pain; however preclinical animal models of inflammatory and neuropathic pain often produce weak evidence of impaired function. We hypothesized that hindpaw mechanical stimulation produced by a requirement to rear on a textured "NOX" plate would punish operant responding in rats treated with intraplantar complete Freund's adjuvant (CFA, a model of inflammatory pain) or the chemotherapeutic paclitaxel (PTX, a model of neuropathic pain) and produce sustained pain-related depression of operant behavior. Male Sprague–Dawley rats were trained under a progressive-ratio (PR) schedule of food-maintained operant responding, then treated with CFA (100 µL in left hindpaw), PTX (2.0 mg/kg IP on alternate days for four total injections; 6.6 mg/kg IV on alternate days for three total injections), or saline vehicle. PR break points and mechanical thresholds for paw withdrawal from von Frey filaments were then tracked for 28 days. Subsequently, rats were tested with the opioid receptor antagonist naltrexone to assess latent sensitization and with the kappa opioid receptor (KOR) agonist U69593 to assess KOR function. CFA produced significant mechanical hypersensitivity for 3 weeks but decreased PR breakpoints for only 1 day. Both IP and IV PTX produced mechanical hypersensitivity for at least three weeks; however, only IV PTX decreased PR breakpoints, and this decrease was not alleviated by morphine. After recovery, naltrexone reinstated mechanical hypersensitivity in CFA- but not PTX-treated rats, and it did not reinstate depression of breakpoints in any group. U69593 dose-dependently decreased PR breakpoints in all groups with no difference between control vs. CFA/PTX groups. These results suggest that rearing on a textured NOX plate was not sufficient to punish operant responding in CFA- and PTX-treated rats despite the presence of sustained mechanical hypersensitivity. The rapid recovery of operant responding could not be attributed to latent sensitization, KOR downregulation, or behavioral tolerance. These results extend the range of conditions under which putative

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Edited by:
E. Alfonso Romero-Sandoval, Wake Forest School of Medicine, United States

Reviewed by:
Abdallah El-Sayed Allam, Tanta University, Egypt
Ainhoa Bilbao, University of Heidelberg, Germany

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Specialty section:
This article was submitted to Translational Pharmacology, a section of the journal Frontiers in Pharmacology

Received: *09 October 2020*
Accepted: *29 December 2020*
Published: *29 January 2021*

Citation:
Negus SS, Marsh SA and Townsend EA (2021) Resistance of Food-Maintained Operant Responding to Mechanical Punishment in Rats: Further Evidence for Weak "Affective/Motivational Pain" in Rat Models of Inflammatory and Neuropathic Pain. Front. Pharmacol. 11:615782. doi: 10.3389/fphar.2020.615782

chronic pain manipulations produce weak evidence for depression of operant responding as a sign of the "affective/motivational" component of pain in rats.

Keywords: complete freund adjuvant, paclitaxel, Operant, punishment, naltrexone, U69,593, morphine

INTRODUCTION

Clinically relevant chronic pain states in human and veterinary medicine are often associated with functional impairment, and restoration of function is a common goal of pain treatment (Dworkin et al., 2005; Jensen et al., 2007; Brown et al., 2008). Experimental models of inflammation and neuropathy have been developed in laboratory animals with the intent of inducing clinically relevant chronic pain states that include the so-called "affective/motivational" components of chronic pain; however, these models often produce surprisingly weak and transient evidence of impaired function (Negus, 2019; Tappe-Theodor et al., 2019; Gonzalez-Cano et al., 2020). For example, paclitaxel treatment to model chemotherapy-induced neuropathic pain in rats produced mechanical hypersensitivity of paw-withdrawal responses from von Frey filaments for at least four weeks, but it produced no significant changes in operant responding for electrical brain stimulation in an assay of intracranial self-stimulation (ICSS) and only weak and morphine-insensitive decreases in food-maintained operant responding (Legakis et al., 2018; Legakis et al., 2019). Similarly, spinal nerve ligation models of mononeuropathy that produced sustained mechanical hypersensitivity in rats for weeks failed to decrease either ICSS or food-maintained operant responding (Ewan and Martin, 2014; Okun et al., 2016).

It may be possible to enhance expression of functional impairment in operant procedures by supplementing the primary inflammatory or neuropathic insult with acute stimuli that are delivered as a consequence of behavior and that can function as punishers of behavior. As one example, Fuchs and colleagues developed a "place escape-avoidance procedure" (PEAP) in which inflammatory and neuropathic pain models were supplemented by acute, response-contingent mechanical stimulation to the affected paw (LaBuda and Fuchs, 2000a; LaBuda and Fuchs, 2000b). In this procedure, rats received intraplantar injection of complete Freund's adjuvant (Ipl CFA, an inflammatory pain model) or unilateral L5 nerve ligation (a surgical mononeuropathy model) one or two days, respectively, before a sequence of two test days. On the first test day, mechanical paw withdrawal thresholds were assessed, and both Ipl CFA and L5 nerve ligation produced mechanical hypersensitivity in the injured paw. On the second day of testing, rats were placed into a chamber with equally sized light and dark compartments and a mesh floor that provided access to the subject's feet from below. Locomotion in the normally preferred dark compartment resulted in delivery of an acute mechanical stimulus (476 mN von Frey filament) to the injured paw every 15 s, whereas locomotion in the light compartment resulted in delivery of the same stimulus to the uninjured paw every 15 s. Thus, delivery of the acute mechanical stimulus to the injured paw was contingent on locomotion in the dark compartment. This behaviorally contingent mechanical stimulation significantly reduced time in the dark compartment, indicating that mechanical stimulation of the injured paw functioned as a punisher of locomotion in the dark compartment, and a parallel study found that morphine blocked this punishment. This type of acute stimulus delivery is labor intensive, but Boada and colleagues recently developed a strategy to simplify mechanical stimulus delivery to the feet of rats with a nerve injury model of neuropathic pain (Boada et al., 2016). Specifically, they created a locomotor activity field divided into quadrants, and the floor of each quadrant consisted of a metal plate (called a "NOX plate") covered with a grid of pyramids with increasing degrees of sharpness at their apex (apex areas of 1.5, 1.0, 0.6, 0.2 mm^2). Mechanical stimulation (delivered via the rat's body weight pressing its paw onto the pyramids) was contingent on locomotion in the different quadrants, and nerve-injured rats avoided the quadrant with the sharpest (0.2 mm^2) pyramids, indicating that mechanical stimulation associated with these sharper pyramids punished locomotion in those quadrants.

The goal of the present study was to evaluate the degree to which acute mechanical stimulation delivered via NOX plates might function as a punisher of food-maintained operant responding and increase expression of behavioral depression in rats that had received Ipl CFA or repeated paclitaxel treatment. In addition to the NOX plates, several additional steps were taken in an effort to increase sensitivity of the procedure to pain-related behavioral depression relative to our previous study of chemotherapy effects on food-maintained operant responding (Legakis et al., 2019). First, only male rats were used, because only males showed evidence for paclitaxel-induced decreases in food-maintained operant responding in our previous study. Second, we used unsweetened grain pellets rather than sweetened pellets to reduce reinforcing effectiveness of the food reinforcer (Warner et al., 2015). Third, responding was maintained under a progressive-ratio (PR) schedule rather than a fixed-ratio schedule to permit determination of PR break points as a sensitive measure of CFA and paclitaxel effects on food reinforcing strength (Hodos, 1961; Richardson and Roberts, 1996). Fourth, the response lever was elevated to require rats to rear and place their full body weight on their hindpaws during operant responding, thus maximizing mechanical stimulation delivered via contact with the NOX plate. Lastly, we compared effects of our standard paclitaxel dosing regimen (4 alternate-day IP injections of 2.0 mg/kg/day paclitaxel; total dose = 8.0 mg/kg) with a more aggressive treatment regimen (3 alternate-day IV injections of 6.6 mg/kg/day; total dose = 19.8 mg/kg) shown to produce clinically relevant leukopenia as well as robust pain behaviors, including sensitivity to mechanical punishment in a PEAP apparatus (Hamity et al., 2017).

METHODS

Subjects

Adult male Sprague-Dawley rats (Envigo, Somerset, NJ) that weighed 275–300 g upon arrival at the laboratory and were housed individually in an AAALAC International-accredited housing facility maintained on a 12-h light/dark cycle with lights on from 6:00 AM to 6:00 PM. All testing occurred during the light phase. Daily food rations (Teklad standard diet—19% protein; Envigo) were provided immediately after behavioral sessions and were titrated to maintain daily body weights within 5% of the running mean for each subject. In addition, rats had access to 45 mg food pellets (BioServ Dustless Precision Grain Pellets, #F0165, Flemington, NJ) during operant behavior sessions. Rats receiving IV paclitaxel received supplemental food as described below during paclitaxel treatment. Water was available ad libitum in the home cage. Animal-use protocols were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee and were in accordance with the National Academy of Science's Guide for the Care and Use of Laboratory Animals (National Research Council, 2011).

Drugs and Noxious Stimuli

Naltrexone HCl and U69593 (National Institute on Drug Abuse Drug Supply Program, Bethesda, MD) were dissolved in saline and administered subcutaneously (SC) in a volume of 1 ml/kg. Complete Freund's adjuvant (CFA; Sigma Aldrich, St. Louis, MO) was administered to the intraplantar (Ipl) region of the left hindpaw in a volume of 100 µL. Controls received the same volume of Ipl saline.

Paclitaxel was obtained as a clinically available 6.0 mg/ml solution (Cardinal Health, Richmond, VA), and it was administered in one of two ways. One set of rats received a series of four intraperitoneal (IP) injections administered every other day. Each injection delivered 2.0 mg/kg paclitaxel (6.0 mg/ml stock solution × 0.33 ml/kg/day injection volume), and the total dose of 8.0 mg/kg was delivered over seven days as we have described previously (Legakis et al., 2018; Legakis et al., 2019). For a second set of rats, each rat was implanted with an intravenous (IV) catheter in the right jugular vein as described previously (Townsend et al., 2015; Townsend et al., 2019). Briefly, rats were anesthetized with isoflurane (2–3%), and the right jugular vein was isolated, punctured, and implanted with a custom-made polyethylene catheter. The unsecured end of the catheter was subcutaneously routed to the midscapular region of the back and connected to a subcutaneous vascular access port (Model VABR1B/22, Instech Laboratories, Plymouth Meeting, PA, United States). Subjects received one dose of ketoprofen (5 mg/kg) immediately following surgery and a second dose 24-h post-operatively. Catheters were flushed each weekday before and during paclitaxel injections with an antibiotic (gentamicin, 0.08 mg) followed by 0.1 ml of heparinized saline (30 U/ml). Beginning one week after surgery, rats received a series of three IV injections administered every other day. Each injection delivered 6.6 mg/kg paclitaxel, and the total dose of 19.8 mg/kg was delivered over 5 days as described previously (Hamity et al., 2017). For each day of IV treatment, the 6.0 mg/ml stock solution of paclitaxel was diluted in saline to a concentration of 0.66 mg/ml, and a 1.0 ml/kg injection was administered once every 10 min for a total of 10 injections (total daily injection volume of 10 ml/kg). Pilot studies with this IV paclitaxel dosing regimen indicated a risk of substantial weight loss; accordingly, during the week of IV paclitaxel treatment, rats received a supplemental diet of powdered grain and sucrose pellets mixed with peanut butter and DietGel®31 M (ClearH2O, Portland, ME) and placed on the cage floor for easy access. Paclitaxel controls received four IP injections of saline.

Overview of Experimental Design

The main goal of the study was to compare the time courses of mechanical hypersensitivity and mechanical punishment of operant responding in rats after treatment with CFA, paclitaxel, or their respective controls. **Figure 1** shows the timeline of experimental events. After collection of baseline data, rats received their respective treatments, and both mechanical sensitivity and operant responding were assessed over a period of 28 days. Sessions of food-maintained operant responding were generally conducted during all weekdays, whereas mechanical sensitivity was assessed approximately 1 h after operant behavioral sessions and after delivery of supplemental food rations on the days indicated in **Figure 1**. After the conclusion of this 28-days test period, rats were treated with 3.2 mg/kg naltrexone 15 min before an operant behavioral session or before mechanical sensitivity testing on Days 29 and 31. The order of naltrexone testing with operant vs. mechanical sensitivity assessments was counterbalanced across rats. When naltrexone was tested before an operant session, mechanical sensitivity testing was omitted afterward. When naltrexone was tested before mechanical sensitivity assessments, rats had their usual operant session followed by naltrexone administration and then by mechanical sensitivity assessments. Effects of the opioid receptor antagonist naltrexone were evaluated to assess potential presence of latent sensitization (Corder et al., 2013). Next, on Days 35–38, each rat was tested with a range of U69593 doses (vehicle, 0.1, 0.32, and 1.0 mg/kg SC) administered 15 min before operant behavioral sessions. All rats received all doses on four consecutive days, and dose order was randomized across rats using a Latin Square design. Effects of the kappa opioid receptor (KOR) agonist U69593 were examined to assess potential changes in KOR agonist effects that might be indicative of pain-related changes in KOR signaling (Liu et al., 2019). A final study with the mu opioid receptor (MOR) agonist morphine was conducted in rats treated with high-dose IV paclitaxel. On Days 42–45 after initiation of IV paclitaxel, each rat was tested with a range of morphine doses (vehicle, 0.32, 1.0, 3.2 mg/kg SC) administered 30 min before operant behavioral sessions. As with U69593, all rats received all morphine doses on four consecutive days, and dose order was randomized across rats using a Latin Square design. Morphine was tested to evaluate effectiveness of a clinically effective analgesic to increase operant responding in IV paclitaxel-treated rats (the only group to show a sustained decrease in operant responding) and hence to evaluate the role of

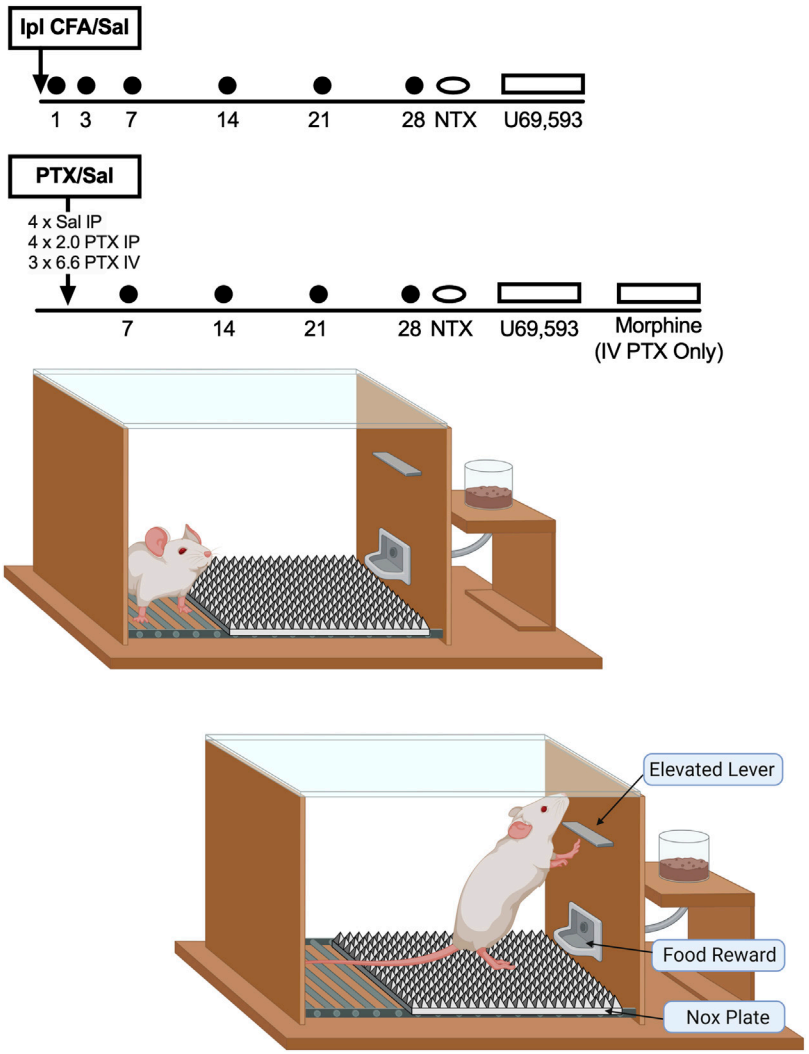

FIGURE 1 | Experimental design and apparatus. Timelines at the top show the sequence of treatments and tests in each group. Operant responding was assessed each weekday, and mechanical sensitivity to von Frey filaments was assessed on days indicated by the filled circles. Schematics of the operant chamber and position of the NOX plate are shown at the bottom. Rats had to rear on the NOX plate to reach the lever and complete the response requirement.

pain in mediating that decrease. Morphine was tested at doses shown previously to reverse paclitaxel-induced mechanical hypersensitivity (Legakis and Negus, 2018).

Mechanical Sensitivity Testing With von Frey Filaments

On test days, rats were placed on an elevated mesh galvanized steel platform in individual chambers with a hinged lid and allowed to acclimate for at least 20 min before exposure to mechanical stimuli. Von Frey filaments (ranging from 0.4 to 15.0 g and increasing in ~0.25 log increments; North Coast Medical, Morgan Hill, CA) were applied to the plantar surface of each hindpaw, and the threshold stimulus to elicit paw withdrawal was determined in log grams using the "up-down" method as previously described (Chaplan et al., 1994; Leitl et al., 2014b; Legakis et al., 2019). Filament forces greater than 15.0 g were not used because they physically lifted the paw, and as a result, paw movement could not be reliably attributed to a withdrawal response by the subject. For rats receiving Ipl CFA or saline, only data from the injected paw were subjected to data analysis; thresholds in the uninjected paw were nearly always at the cutoff value of 15.0 g (data not shown). For rats receiving paclitaxel or saline, data from both paws were averaged within a rat and then across rats for subsequent analysis.

Food-Maintained Operant Responding in Food-Restricted Rats

Apparatus

Studies were conducted in sound-attenuating boxes containing modular acrylic and metal test chambers (Med Associates, St

Albans, VT), and major features of the operant chambers are illustrated in **Figure 1**. Each chamber contained one active response lever, three stimulus lights (red, yellow, and green) centered above the lever, a 2-W house light, a floor made of parallel bars, and a pellet dispenser that delivered 45 mg grain pellets to an aperture beside the lever. Control of stimulus delivery in the operant chamber and collection of data on lever presses emitted and reinforcements earned were accomplished with a computer, interface, and custom software (Med PC-IV, Med Associates).

Training

Training progressed in three phases in two different sets of operant chambers that were identical except for the height of the response lever (6 vs. 15 cm above the floor in the low-lever and high-lever chambers, respectively). Phase 1 of training was conducted in the low-lever chambers. Operant responding was established for a series of increasing fixed-ratio (FR) values from FR 1 to FR 10, and the schedule of reinforcement was then changed to a progressive-ratio (PR) schedule such that the response increment for each reinforcer increased exponentially (1, 2, 4, 8, 16, etc.) with each set of eight reinforcers. Thus, the response requirement increased by an increment of 1 for each of the first eight reinforcers (1, 2, 3, 4, 5, 6, 7, 8), an increment of two for the second set of eight reinforcers (10, 12, 14, 16, 18, 20, 22, 24), an increment of four for the third set of eight reinforcers (28, 32, 36, 40, 44, 48, 52, 56), and so on. The break point was defined as the number of reinforcers earned during a session, and training on the PR schedule in the low-lever boxes was considered complete when the breakpoints over three consecutive days varied by ≤ 10% of the mean with no upward or downward trends. In Phase 2 of training, rats were moved to the high-lever boxes, which required rats to rear to reach the lever (see **Figure 1**), and training progressed again through the same sequence of schedules until responding again stabilized under the PR schedule. For the third and final phase of training, a textured aluminum "NOX" plate (Control Technologies, Hickory, NC) was inserted onto the floor of the high-lever chambers (see **Figure 1**). The 23.5 × 18.5 cm NOX plate spanned the width of the floor beneath the response lever and extended across approximately 2/3 of the cage floor toward the opposite wall. The remaining 10.5 cm adjacent to the opposite wall was not covered and provided a location where the rat could avoid the NOX plate. The NOX plate surface consisted of a grid of pyramids (5 mm^2 at base and 5 mm tall), each with an apex of 0.2 mm designed to activate sensitized nociceptors in a partial spinal-nerve-ligation model of neuropathy (Boada et al., 2016). To reach the lever, each rat had to rear on the NOX plate and experience the mechanical stimulation associated with the force of its body weight pressing its hindpaws against the apices on the NOX plate. Training continued until responding again stabilized under the terminal PR schedule. With one exception (see below), all remaining sessions of food-maintained operant responding took place in the high-lever chambers with the NOX plate in place beneath the lever.

Testing

Once stable baseline break points had been established under the high-lever/NOX plate conditions, groups of $N = 6$ rats each were treated with 1) Ipl CFA (single injection), 2) Ipl saline (single injection), 3) IP paclitaxel (four doses of 2.0 mg/kg/day administered on alternate days), 4) IV paclitaxel (three doses of 6.6 mg/kg/day administered on alternate days), and 5) IP saline (four injections on alternate days). Operant behavioral testing then proceeded as shown in **Figure 1**. As described below, results suggested that depression of operant responding was more transient than mechanical hypersensitivity after both CFA and paclitaxel. To assess possible behavioral factors in this resilience of operant responding, two additional groups ($N = 5$ per group) were tested with CFA using a modification of the design shown in **Figure 1**. For the first group, operant behavioral sessions were suspended for seven days after CFA injection before resuming from Day 7–14. This manipulation was implemented to delay any learning of postural adjustments that might relieve pressure on the inflamed paw and thereby permit recovery of depressed operant responding. If learned postural adjustments contributed to rapid recovery of operant responding, then responding was expected to be lower on Day 7 in the group that did not have that opportunity for learning between Days 1 and 7. For the second group, rats were trained as described above, but then returned to the low-lever chambers and rebaselined with the low lever and bar floor (i.e. no NOX plate). CFA was then administered, and operant responding was assessed for 14 days. This manipulation was implemented to evaluate the degree to which mechanical stimulation produced by rearing on the NOX plate functioned as a punisher of operant responding. If rearing on the NOX plate functioned as a punisher, then operant responding would be lower in the high-lever/NOX plate condition where this stimulus was present than in the low-lever/bar-floor context where this stimulus was absent.

Data Analysis

Baseline mechanical thresholds and operant break points were compared by one-way ANOVA. For analysis of CFA, paclitaxel, and control treatment effects on operant responding, break points were expressed as a percent of each rat's baseline break point determined before CFA, paclitaxel, or control treatment. Mechanical thresholds and % baseline operant break points were evaluated by two-way ANOVA, with time as a within-subject variable and treatment as a between-subject variable. Similarly, naltrexone and U69593 effects were compared across groups with dose as a within-subject variable and treatment as a between-subject variable. Morphine was tested in only one group, and effects were analyzed by one-way ANOVA. A significant one-way ANOVA or a significant treatment × day interaction in a two-way ANOVA was followed by a Holm–Sidak post hoc test, and the criterion of significance for all tests was $p < 0.05$.

RESULTS

When data were collapsed across groups for each of the three different phases of operant training, there were no significant differences in break points under Phase 1 low-lever/bar-floor, Phase 2 high-lever/bar-floor, or Phase 3 high-lever/NOX plate contexts (mean ± SEM break points = 25.8 ± 0.8, 24.3 ± 0.5, and 24.9 ± 0.7 pellets per session, respectively). Additionally,

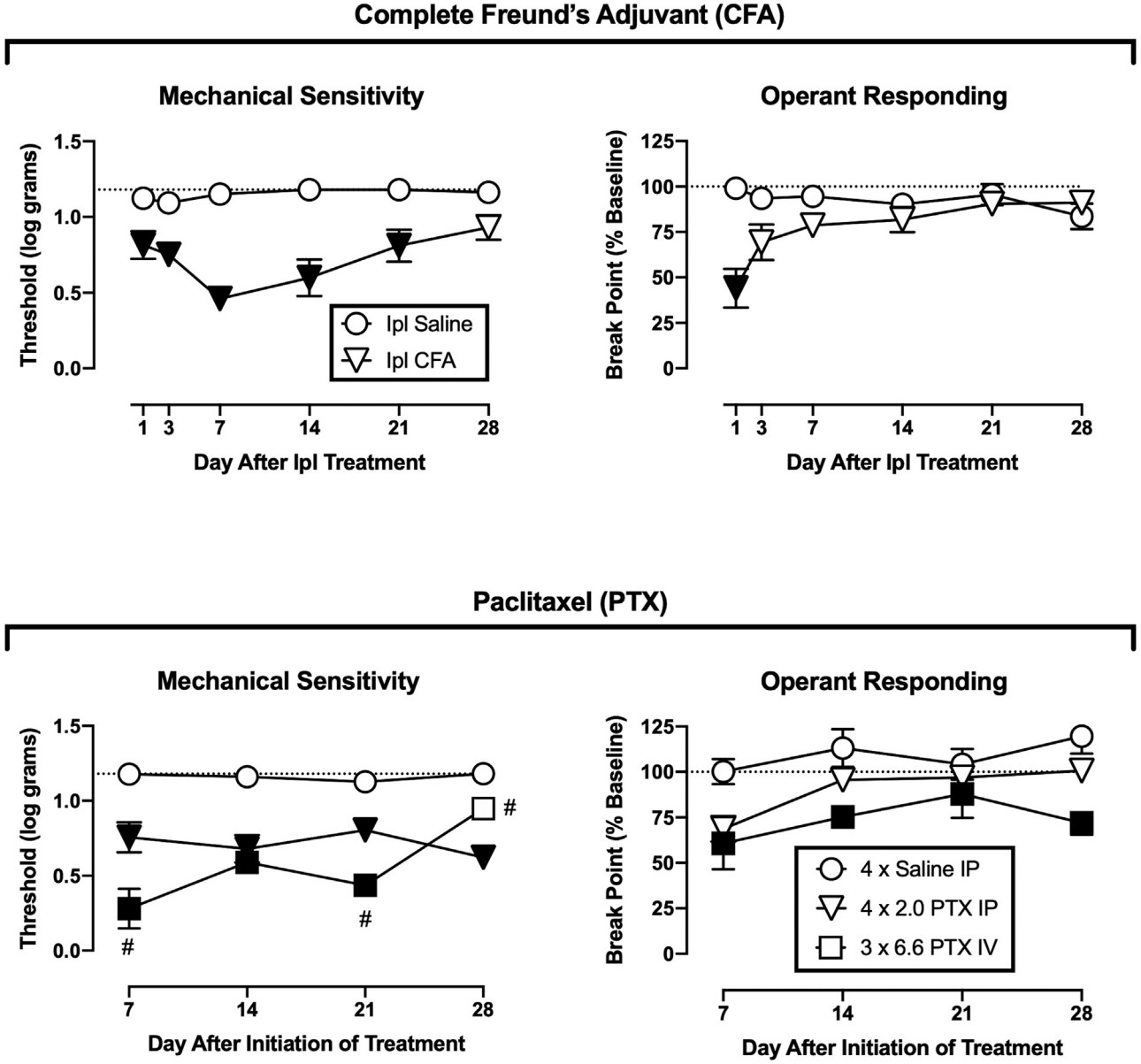

FIGURE 2 | Time course of changes in mechanical sensitivity and operant responding after treatment with CFA **(top panels)** or paclitaxel **(bottom panels)**. Abscissae: days after CFA treatment or initiation of paclitaxel treatment. Ordinates: Threshold von Frey stimulus to elicit paw withdrawal (expressed as log g, **left panels**) or PR break point (expressed as a % of the pre-CFA/PTX baseline, **right panels**). All points show mean ± SEM from $N = 6$ rats except IV PTX ($N = 5$). Filled points indicate a significant difference from saline controls, and the hashtag (#) indicates a significant difference from IP PTX, $p < 0.05$.

there were no significant differences in break points across groups at the conclusion of operant training, and similarly, there were no differences across groups in baseline mechanical thresholds (overall mean ± SEM = 1.13 ± 0.02 log g). Rats in the IV paclitaxel group were surgically implanted with IV catheters and allowed to recover for one week prior to initiation of paclitaxel treatment, and mean ± SEM break points in this group did not differ at the conclusion of operant training (22.9 ± 1.5), on the day after surgery (22.0 ± 2.9), or after the one-week recovery period (24.9 ± 2.6).

Figure 2 shows the time course of changes in mechanical paw-withdrawal thresholds and operant break points after treatment with CFA or its vehicle (top panels) or paclitaxel or its vehicle (bottom panels). For Ipl CFA, decreases in operant break points were significant but more transient than decreases in mechanical thresholds. CFA significantly reduced mechanical thresholds from Days 1–21 [treatment × day interaction, $F(5,50) = 4.59$, $p = 0.002$], whereas operant breakpoints were reduced only on Day 1 [treatment × day interaction, $F(5,50) = 10.110$, $p < 0.001$].

For paclitaxel, one rat in the IV treatment group lost sufficient body weight to meet IACUC-established moribundity criteria.

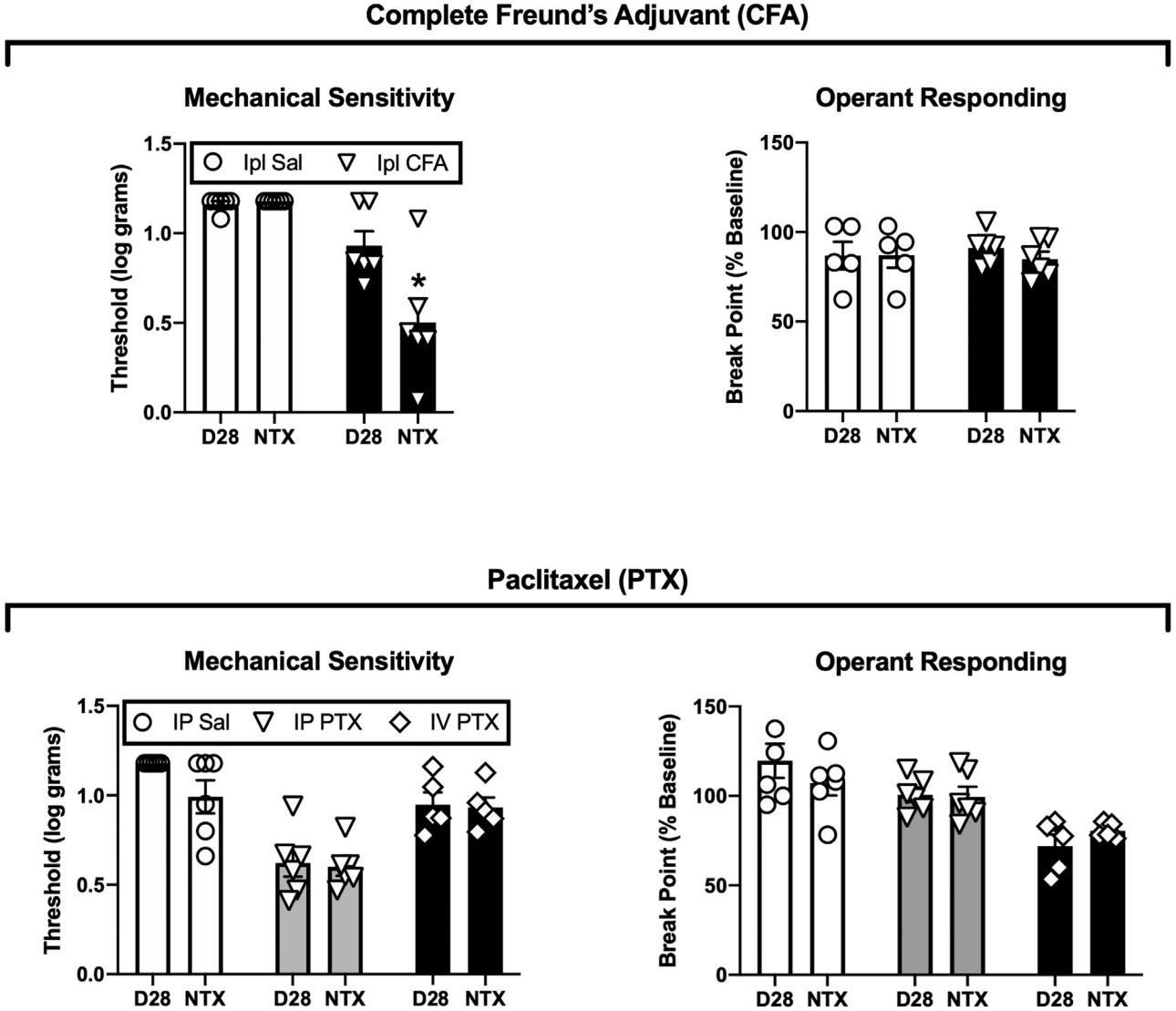

FIGURE 3 | Effects of naltrexone on mechanical sensitivity and operant responding after treatment with CFA **(top panels)** or paclitaxel **(bottom panels)**. Abscissae: Data from Day 28 (D28) or the naltrexone-treatment day (NTX, either Day 29 or 31). Ordinates: Threshold von Frey stimulus to elicit paw withdrawal (expressed as log g, **left panels**) or PR break point (expressed as a % of the pre-CFA/PTX baseline, **right panels**). All bars show mean ± SEM from $N = 6$ rats except IV PTX ($N = 5$). The asterisk indicates a significant difference from D28, $p < 0.05$.

As a result, this rat was euthanized on Day 11 after initiation of paclitaxel treatment, its data are omitted from all data analysis, and the IV paclitaxel results show data only from the remaining five rats. Both the lower dose IP regimen and the higher dose IV regimen of paclitaxel treatment significantly reduced mechanical thresholds [treatment × day interaction, $F(6,42) = , p < 0.001$]. Relative to IP saline treatment, mechanical thresholds were reduced across all test days by IP paclitaxel and across Days 7–21 by IV paclitaxel. Additionally, IV paclitaxel produced greater mechanical hypersensitivity than IP paclitaxel on Days 7 and 21, but significantly less on Day 28. Paclitaxel also significantly reduced operant responding, and because there was a main effect of treatment [$F(2,14) = 7.821$, $p = 0.005$] but not a significant treatment × day interaction, analysis defaulted to a one-way ANOVA by treatment. Relative to IP saline treatment, operant responding was significantly reduced by IV paclitaxel. Differences between IP saline and IP paclitaxel ($p = 0.089$), and between IP and IV paclitaxel (also $p = 0.089$), approached but did not meet the criterion for significance. Thus, IP paclitaxel produced mechanical hypersensitivity without significantly altering operant responding, whereas IV paclitaxel produced lethality in one rat and both mechanical hypersensitivity and reduced operant responding in the remaining rats.

Figure 3 shows the effects 3.2 mg/kg naltrexone on mechanical thresholds and operant break points on Days 29 and 31 after Ipl CFA or initiation of paclitaxel treatment. In CFA-treated rats, naltrexone reinstated mechanical hypersensitivity

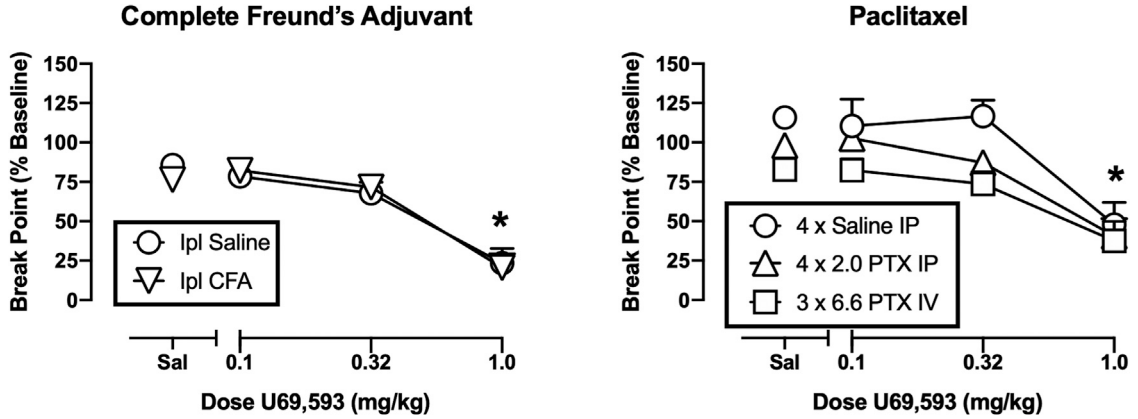

FIGURE 4 | Effects of U69593 on operant responding after treatment with CFA (**left panel**) or paclitaxel **(right panel)**. Abscissae: Dose U69593 (mg/kg, log scale). Ordinates: PR break point expressed as a % of the pre-CFA/PTX baseline. All points show mean ± SEM from *N* = 6 rats except IV PTX (*N* = 5). The asterisk indicates a significant difference from saline (Sal) for all groups, $p < 0.05$.

TABLE 1 | Morphine did not increase operant responding in rats treated with high-dose IV paclitaxel, and instead tended to decrease break points further.

Morphine dose (mg/kg)	% Baseline breakpoint (Mean ± SEM)
Saline	92.33 ± 2.53
0.32	91.74 ± 3.5
1.0	81.95 ± 4.78
3.2	64.36 ± 11.50

[treatment × naltrexone interaction, $F(1,10) = 15.52$, $p = 0.003$], but it had no effect on operant break points, and it did not affect either endpoint in the saline-treated control rats. Naltrexone did not significantly alter either mechanical thresholds or operant break points in paclitaxel-treated rats or their saline-treated controls. In IP paclitaxel-treated rats, mechanical sensitivity recovered to baseline levels by 12 weeks after paclitaxel treatment, but naltrexone still failed to reinstate mechanical hypersensitivity or produce depression of operant responding at this time (data not shown).

Figure 4 shows the effects of U69593 on operant responding. The high dose of 1.0 mg/kg U69593 significantly decreased break points in both the CFA-treated rats and their saline-treated controls [main effect of U69593 dose, $F(3,27) = 62.30$, $p < 0.001$], but there was no difference between groups. Similarly, 1.0 mg/kg U69593 significantly decreased break points in both paclitaxel treatment groups and in the saline-treated controls [main effect of U69593 dose, $F(3,39) = 59.13$, $p < 0.001$]. In this study, the main effect of treatment approached but did not reach the criterion for significance [$F(2,13) = 6.242$, $p = 0.072$], and there was no treatment × U69593 interaction. Thus, while IV paclitaxel-treated rats tended to have lower breakpoints across all U59593 doses, there was no significant difference in U69593 effects across groups. **Table 1** shows the effects of morphine determined 42–45 days after treatment with high-dose IV paclitaxel. Break points after morphine vehicle (i.e. saline) were still significantly lower than baseline ($t = 2.403$, $p = 0.037$); however, morphine did not alleviate this operant depression, and instead tended to reduce PR break points further.

Figure 5 shows CFA effects on operant responding under two additional conditions. First, suspension of access to operant responding did not delay recovery of operant responding. Break points on Days 7 and 14 after CFA were similar regardless of whether rats engaged in operant responding on Days 1–6 or not. Second, the time course of CFA-induced decreases in operant break points was similar regardless of whether rats were tested in the high-lever/NOX-plate context or the low-lever/bar-floor context. There was a significant interaction between test context and time [$F(5,45) = 2.746$, $p = 0.030$]; however, post hoc analysis did not reveal a difference between groups on any day.

DISCUSSION

This study examined the degree to which mechanical stimulation to the hindpaws might elicit the "affective/motivational" component of chronic pain as indicated by pain-related punishment of operant responding maintained under a PR schedule of food delivery in rats treated with either Ipl CFA (to produce paw inflammation) or repeated paclitaxel (to produce polyneuropathy). Mechanical stimulation to the hindpaws was achieved by requiring rats to rear on a textured metal "NOX" plate to reach the operant response lever. There were three main findings. First, the requirement to rear on the NOX plate did not function as a punisher in the absence of putative pain manipulations. PR break points during the three phases of initial training were identical regardless of whether rats had to rear on the NOX plate or not. Second, both CFA and paclitaxel produced significant depression of operant responding when rats were required to rear on the NOX plate; however, as discussed further below, these decreases in operant responding did not appear to reflect pain-related punishment. Lastly, studies with naltrexone, U69593, and delayed operant access suggested that

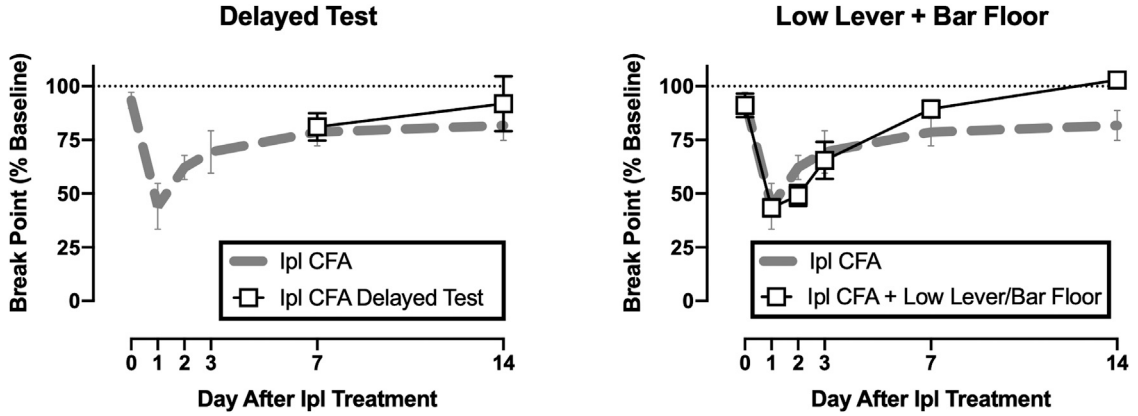

FIGURE 5 | Effects of delayed operant testing **(left panel)** or operant testing with a low lever and bar floor **(right panel)** in CFA-treated rats. Abscissae: days after CFA treatment. Ordinates: PR break point expressed as a % of the pre-CFA baseline. All points show mean ± SEM from *N* = 5 rats. There were no differences between operant break points in rats tested under these altered parameters (squares) in comparison to data collected under the original parameters (gray dashed line, from **Figure 2**).

rapid recovery of operant responding did not reflect processes of latent sensitization, altered KOR signaling, or learned compensatory behaviors. Overall, these results extend the range of conditions under which operant responding in rats is resistant to behavioral depression by putative chronic pain treatments.

Clinically relevant chronic pain states are often accompanied by functional impairment and decreases in activities of daily living, and a major goal of pain treatment is to restore normal function. Insofar as one goal of preclinical research is to model clinically relevant pain behaviors and predict effectiveness of candidate treatments, one theme of preclinical research has been to evaluate the degree to which acute, inflammatory, and neuropathic pain models produce analgesic-reversible decreases in unconditioned or operant conditioned behaviors in laboratory animals as a measure of the "affective/motivational" component of pain (Negus, 2019; Tappe-Theodor et al., 2019; Gonzalez-Cano et al., 2020). As one example, intraperitoneal injection of dilute acid (IP acid) can serve as an acute chemical noxious stimulus in rats and mice to decrease a range of unconditioned behaviors (e.g. nesting, locomotion, wheel running, consumption of palatable food) and operant behaviors (e.g. lever pressing for food or electrical brain stimulation) (Stevenson et al., 2006; Stevenson et al., 2009; Miller et al., 2011; Negus, 2013; Negus et al., 2015; Cone et al., 2018). Moreover, in many cases, these IP acid-induced decreases in behavior can be blocked by clinically effective analgesics (e.g. nonsteroidal anti-inflammatory drugs, MOR agonists) but not by many types of nonanalgesics (e.g. KOR agonists, cannabinoid 1 receptor agonists, neurokinin receptor antagonists) that often produce false-positive effects in conventional assays of reflexive pain behaviors (Negus, 2019). These types of results have been interpreted to suggest that preclinical assays of pain-related behavioral depression could have both face validity as models of clinically relevant functional impairment and predictive validity for evaluation of candidate analgesics.

Pain-related and analgesic-reversible behavioral depression has also been produced in laboratory animals by other noxious stimuli, but in many cases, behavioral depression is weak or transient in comparison to the time course of more commonly used pain behaviors in preclinical pain research. In particular, many procedures have been developed to model chronic inflammatory and neuropathic pain states in laboratory animals, and the chronicity of the pain state is typically claimed on the basis of sustained hypersensitivity of withdrawal reflexes to mechanical or thermal stimuli (e.g. sustained decreases in mechanical thresholds for paw withdrawal from von Frey filaments) (Le Bars et al., 2010). For example, Ipl CFA to model inflammatory pain routinely produces mechanical hypersensitivity for days to weeks, whereas chemotherapy treatments to model chemotherapy-induced neuropathic pain produce mechanical hypersensitivity for weeks to months (Stein et al., 1988; Polomano et al., 2001; Leitl et al., 2014b; Legakis et al., 2018). However, in contrast to the sustained functional impairment that drives many chronic pain patients to seek medical care, Ipl CFA and chemotherapy treatments typically reduce operant conditioned behaviors for only a few days if at all (Leitl et al., 2014b; Legakis et al., 2018; Legakis et al., 2019). This transience of behavioral depression reduces both face validity of the preclinical procedures and utility of these procedures for evaluating chronic-pain treatments. The present study tested the hypothesis that contingent exposure to hindpaw mechanical stimulation might punish operant responding in CFA- and paclitaxel-treated rats, augment the magnitude and duration of pain-related behavioral depression produced by these chronic pain models, and provide a baseline of chronic pain-related behavioral depression that could be used to evaluate candidate analgesics.

Hindpaw mechanical stimulation was achieved by requiring rats to rear on a textured NOX plate developed to excite nociceptors rendered hypersensitive by a nerve-ligation model of neuropathic pain, and the stimulus associated with locomotion

on this plate was found to punish locomotor activity in nerve-injured rats but not in sham controls (Boada et al., 2016). Consistent with these previous findings, rearing on the NOX plate did not punish operant responding in rats prior to treatment with CFA, paclitaxel, or their respective controls, nor did rearing on the NOX plate punish responding after control treatments. These findings suggest that mechanical stimulation associated with rearing on the NOX plate was not sufficient by itself to produce pain-related punishment of operant responding.

Responding was significantly reduced after CFA and high-dose IV paclitaxel treatment; however, three findings suggest that this reduction in operant responding was not a consequence of pain-related punishment. First, depression of operant responding was usually weaker or more transient than concurrently assessed hypersensitivity of paw-withdrawal responses to mechanical stimulation with von Frey probes. After Ipl CFA or low-dose IP paclitaxel, mechanical hypersensitivity was observed for at least three weeks as described previously, but depression of operant responding had resolved by three days after CFA and was never significant after IP paclitaxel. Second, high-dose IV paclitaxel did produce a more sustained depression of operant responding; however, this effect was small, and morphine doses shown previously to alleviate mechanical hypersensitivity in paclitaxel-treated rats (Legakis and Negus, 2018) did not increase operant responding, suggesting that the depression of operant responding was not related to pain. Lastly, the time course of operant depression after Ipl CFA was identical whether rats were required to rear on the NOX plate or not, further suggesting that the mechanical stimulation associated with rearing on the NOX plate did not augment depression produced by the CFA treatment alone. Similarly, the weak effects of low-dose IP paclitaxel on operant responding in the present study were similar to the weak and morphine-resistant effects observed previously in rats responding for food or electrical brain stimulation on a low lever with no NOX plate (Legakis et al., 2018; Legakis et al., 2019).

As one manipulation intended to increase the potential for detecting sustained pain-related behavioral depression, the present study used a high-dose IV paclitaxel treatment regimen that more closely mimics the IV doses used clinically in humans and that produces leukopenia similar to that produced by clinically effective paclitaxel dosing regimens (Hamity et al., 2017). This high-dose IV paclitaxel regimen did produce stronger effects than the low-dose IP regimen on several endpoints including 1) weight loss (sufficient to require euthanasia in one rat despite the supplemental feeding), 2) mechanical hypersensitivity after 1 and 3 weeks, and 3) significant depression of operant responding throughout the study. In contrast to results with the low-dose IP regimen in the present study or with the high-dose IV regimen in the original study (Hamity et al., 2017), the mechanical hypersensitivity was no longer significant after four weeks. Reasons for this discrepancy are not clear and may be related to methodological differences that included use of male rather than female rats, use of IV catheters rather than tail-vein injections for IV paclitaxel delivery, or repeated exposure to the operant behavioral procedure and NOX plate. Operant responding was decreased throughout the study in these rats, and this may be consistent with the previously reported effectiveness of mechanical stimulation in IV PTX-treated rats to punish locomotor behavior in the PEAP procedure (Hamity et al., 2017); however, as noted above, morphine was not effective to alleviate this effect, suggesting that the behavioral depression was not related to pain.

In addition to producing changes in behavior, inflammatory and neuropathic pain manipulations have also been reported to produce changes in endogenous opioid signaling, and two of those were examined here. First, "latent sensitization" has been described as a phenomenon in which inflammation- or neuropathy-induced mechanical hypersensitivity resolves due in part to the emergence of sustained constitutive MOR activity in the spinal dorsal horn (Corder et al., 2013; Marvizon et al., 2015). This "latent sensitization" can be revealed by the administration of naltrexone or other MOR antagonists to block antinociception associated with constitutive MOR activity and reinstate expression of mechanical hypersensitivity. In the present study, naltrexone-induced reinstatement of mechanical hypersensitivity in CFA-treated rats is consistent with latent sensitization; however, naltrexone did not reinstate depression of operant responding, suggesting that rapid recovery of operant responding did not reflect constitutive MOR activity in neural circuits mediating CFA-induced depression of operant responding. Additionally, naltrexone did not alter either mechanical sensitivity or operant responding in paclitaxel treated rats, suggesting that constitutive MOR activity did not contribute to resolution of either paclitaxel effect. Second, inflammation and neuropathy in rodents have also been reported to promote dynorphin signaling and KOR activation in the mesolimbic dopamine system, and this enhanced KOR signaling has been implicated in some signs of pain-related behavioral depression (Liu et al., 2019; Meade et al., 2020). We have not found evidence for this type of pain-related activation of KOR signaling in previous studies using both acute and chronic pain manipulations (Leitl et al., 2014a; Leitl et al., 2014b; Negus et al., 2015; Bagdas et al., 2016; Legakis et al., 2020), and the present study is consistent with our own previous findings. Thus, CFA and paclitaxel treatment produced only transient and/or weak evidence for depression of operant responding, so there was little evidence to suggest a KOR-mediated depressant effect in this study. It was possible that CFA- or paclitaxel-induced increases in dynorphin release may have occurred, but they were either insufficient to depress behavior or their impact was attenuated by compensatory KOR downregulation. To evaluate this possibility, we determined effects of the exogenous KOR agonist U69593, but dose-effect curves for U69593-induced depression of operant responding were not affected by either CFA or paclitaxel treatment. Thus, these results also do not provide evidence for either latent increases in dynorphin (which might have been additive with U69593 and shifted U69593 dose-effect curves to the left) or KOR downregulation (which might have shifted U69593 dose-effect curves to the right). Overall, our results provide no evidence for pain-related alterations in KOR signaling.

A final experiment in this study evaluated the possibility that rapid recovery of operant responding in CFA-treated rats may

have reflected behavioral tolerance (Schuster et al., 1966; Sannerud and Young, 1986; Foltin, 2015), which can be defined as tolerance to drug effects due to learning. For example, rats might have learned new postures that minimized contact between the CFA-injected paw and the NOX plate to reduce exposure to mechanical stimulation associated with that contact. To test for this possibility, a group of CFA-treated rats was given access to operant responding only one week after CFA injection, and behavioral tolerance would have been indicated by delayed recovery due to delayed opportunity for learning. However, recovery in this group was identical to recovery in the rats with daily access to operant responding, suggesting that learned compensatory behaviors analogous to behavioral tolerance did not contribute to rapid recovery.

In summary, the present results provide little evidence for sustained depression of food-maintained operant responding as a sign of the "affective/motivational" dimension of pain in rats treated with CFA or paclitaxel. The rapid recovery of operant responding in CFA- and IP PTX-treated rats cannot be attributed to latent sensitization associated with constitutive MOR activity, to altered KOR signaling, or to behavioral tolerance. Only the IV PTX-treated rats showed sustained operant depression, but even here, the effect was small and resistant to treatment with the opioid analgesic morphine, suggesting that it was not related to pain. The use of mild food restriction in the present study may have increased food motivation and rendered operant responding resistant to depression by pain manipulations; however, we recently found that mild food deprivation like that used here has no significant effect of food motivation measured with a between-day progressive-ratio procedure (unpublished data). Overall, these data add further evidence to suggest that putative chronic-pain manipulations in rats may be a poor model for research on the expression, mechanisms, and treatment of the functional impairment and behavioral depression commonly observed in human chronic-pain patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Virginia Commonwealth University Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

All authors contributed to the conduct of experiments and approved the final manuscript. SN was responsible for experimental design, data analysis, and writing an initial draft of the manuscript.

FUNDING

Supported by National Institutes of Health Grant P30DA033934 and F32DA047026.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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MINI REVIEW
published: 01 February 2021
doi: 10.3389/fphar.2020.620485

Modeling Complex Orthopedic Trauma in Rodents: Bone, Muscle and Nerve Injury and Healing

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Orthopedic injury can occur from a variety of causes including motor vehicle collision, battlefield injuries or even falls from standing. Persistent limb pain is common after orthopedic injury or surgery and presents a unique challenge, as the initiating event may result in polytrauma to bone, muscle, and peripheral nerves. It is imperative that we understand the tissue-specific and multicellular response to this unique type of injury in order to best develop targeted treatments that improve healing and regeneration. In this Mini Review we will first discuss current rodent models of orthopedic trauma/complex orthotrauma. In the second section, we will focus on bone-specific outcomes including imaging modalities, biomechanical testing and immunostaining for markers of bone healing/turnover. In the third section, we will discuss muscle-related pathology including outcome measures of fibrosis, muscle regeneration and tensile strength measurements. In the fourth section, we will discuss nervous system-related pathology including outcome measures of pain-like responses, both reflexive and non-reflexive. In all sections we will consider parallels between preclinical outcome measures and the functional and mechanistic findings of the human condition.

Keywords: pain, chronic pain, translation, regeneration, preclinical

OPEN ACCESS

Edited by:
E. Alfonso Romero-Sandoval, Wake Forest School of Medicine, United States

Reviewed by:
Steve Davidson, University of Cincinnati, United States
Zhifeng Yu, Shanghai Jiao Tong University, China

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Specialty section:
This article was submitted to Translational Pharmacology, a section of the journal Frontiers in Pharmacology

Received: *23 October 2020*
Accepted: *21 December 2020*
Published: *01 February 2021*

Citation:
Shen H, Gardner AM, Vyas J, Ishida R and Tawfik VL (2021) Modeling Complex Orthopedic Trauma in Rodents: Bone, Muscle and Nerve Injury and Healing. Front. Pharmacol. 11:620485. doi: 10.3389/fphar.2020.620485

INTRODUCTION

High energy trauma is a major public health concern as it is often associated with complex muscle, bone, nerve and connective tissue damage. Military injuries to extremities, including those with extensive soft tissue and bone destruction, are on the rise (Stojadinovic et al., 2006) with the most frequent injury in the Iraq and Afghanistan wars being blast wounds (Belmont et al., 2016). In a cohort study of battle injuries, combat-related extremity injuries required longer hospital stays and were responsible for 65% of total inpatient resource utilization (Masini et al., 2009). These injuries cause pain and long-lasting functional deterioration which demand intensive medical intervention and physical therapy. The lifetime medical cost of non-fatal crash injuries, for example, was estimated to be $18.4 billion in 2012 (Bergen et al., 2014). It is therefore imperative that we develop better treatments to alleviate acute and chronic trauma-related pain and functional deficits to facilitate patient rehabilitation. Here, we review preclinical rodent orthopedic trauma/injury models, including discussion of clinical relevance and face validity of these models with respect to the human condition. We further discuss outcomes to evaluate bone, muscle and nerve healing that go beyond simple reflexive measures of nociception. While a substantial number of studies have

demonstrated benefits of analgesic drugs in preclinical pain models, failure to translate these findings into clinically successful medications has resulted in shuttering analgesic drug development programs at several major companies (Percie du Sert and Rice, 2014). The reasons for these translational failures have been extensively reviewed elsewhere (Woolf, 2010; Barrett, 2015; Clark, 2016) and include poor animal models, poor pain measures and poor reporting practices. We therefore encourage investigators to consider clinically-informed models and outcomes as a means to bridge the gap between preclinical and clinical research efforts, particularly in the search for novel analgesics.

PRECLINICAL MODELS OF ORTHOPEDIC TRAUMA

Preclinical animal research is key to uncovering mechanisms underlying traumatic injury. Although the small size of rodents makes standardized orthopedic injury models and outcome measurements quite challenging, the possibility of genetic manipulation renders rodent models, especially mice, the ideal species for many studies, but ultimately depends on the exact questions being asked (Jacenko and Olsen, 1995; Houdebine, 2007).

One of the first models of orthopedic trauma was developed by Bonnarens and Einhorn and involved closed femur fracture with intramedullary (IM) pinning in the rat (Bonnarens and Einhorn, 1984). Subsequently, Manigrasso and O'Connor (2004) described a mouse model for further molecular and genetic analysis. To more closely approximate human bone fixation, modifications have been reported by Holstein et al. (2007), with the use of a locking nail or compression screw (Holstein et al., 2009) to fix rotational and axial movement of the IM pin to avoid secondary injury from micromotion of the pin within the marrow space. In order to develop a model with better reproducibility and less tissue damage, closed tibial fracture models were also established (Hiltunen et al., 1993; Otto et al., 1995; Handool et al., 2018). External fixation was also utilized in rats by Mark et al. (2003) and further enhanced using customized fixators for studies focused on implanted osteoconductive materials (Kaspar et al., 2007). Additionally, different non-union rodent models were developed to study delayed healing or non-unions (Garcia et al., 2013). For example, atrophic non-union models that result from periosteal injury (Garcia et al., 2008) or segmental defect (Garcia et al., 2011), critical size defect models that cause reliable non-unions with proper fixation (Zwingenberger et al., 2013), and osteosynthesis-associated infections models (Wong et al., 2020) have all been adopted for mechanistic investigations.

When animal models accurately mimic the human condition, conclusions derived from their use have the potential to be effectively translated to clinical care. Orthopedic trauma is not only limited to bone injury, but also results in destruction of muscle, soft tissue and peripheral nerve damage that contribute to healing complications (Haffner-Luntzer et al., 2019). As a result, we developed a complex orthopedic trauma mouse model (Tawfik et al., 2020b), consisting of open tibial fracture with pin fixation and muscle injury, to reflect nociceptive sensitization, muscle fibrosis, and muscle fiber loss, as well as bone injury. Other examples of models that seek to more closely emulate the human condition include trauma-hemorrhage mouse models combining external femur fixator with pressure-controlled hemorrhagic shock on femoral arteries. Such models demonstrate that hemorrhagic shock can cause delayed fracture healing with decreased callus strength (Gascho et al., 1989; Neunaber et al., 2013; Bundkirchen et al., 2017). Similarly, femoral artery resection (Lu et al., 2007; Kalogeris et al., 2012) and diabetic ischemia (Follak et al., 2004; Kayal et al., 2009) have been combined with fracture models to mimic bleeding and ischemia in trauma patients (Miclau et al., 2017). Finally, the effects of osteoporosis have been mimicked in fracture models using ovariectomized female (Yousefzadeh et al., 2020), aged (Meyer et al., 2006) or transgenic mice with decreased bone density (Watanabe and Hishiya, 2005) to match clinical scenarios of postmenopausal and age-related osteoporosis, respectively.

PRE-CLINICAL MEASUREMENTS IN ORTHOPEDIC TRAUMA: CONNECTION TO THE CLINIC

Most important to the success of such models is the use of appropriate and translationally-relevant outcome measures with face validity with respect to the human condition. We describe the most widely used paradigms for evaluating bone and muscle healing as well as pain-like behaviors in rodent models of trauma with discussion of the clinical correlates that we believe enhance the likelihood of translation (**Figure 1**).

Measurement of Bone Healing

Structural Analysis

Plain radiography (x-ray) is the most straightforward way to confirm bone bridge formation and post-operative displacement of implants or fixed bones (Schindeler et al., 2008; Manigrasso and O'Connor, 2010). However, detection of proper alignment alone without quantitative determination, does not provide full information about healing. In contrast to x-rays, micro-computed tomography (CT) is the most established imaging technique in rodent studies to examine bone structure, density, as well as fracture non-union (Schmidhammer et al., 2006). CT can be used to longitudinally monitor fracture healing at high resolution in living mice (Jiang et al., 2000). Using this highly accurate and consistent technique, researchers have reported differing geometric characteristics of long bones between sexes, strains, and ages of mice (Halloran et al., 2002; Ferguson et al., 2003; Willinghamm et al., 2010).

Although newly generated callus is a heterogeneous and three-dimensional structure, longitudinal or transverse bone sections are commonly stained with various dyes, including hematoxylin and eosin, safranin O/fast green and Masson's trichrome stain etc., to differentiate cartilage, collagen and bone from smooth muscle (Miller et al., 2007; Hu et al., 2020). Besides qualitative evaluation, standardized histomorphometry can be used to assess bone healing quantitatively (Gerstenfeld et al., 2005).

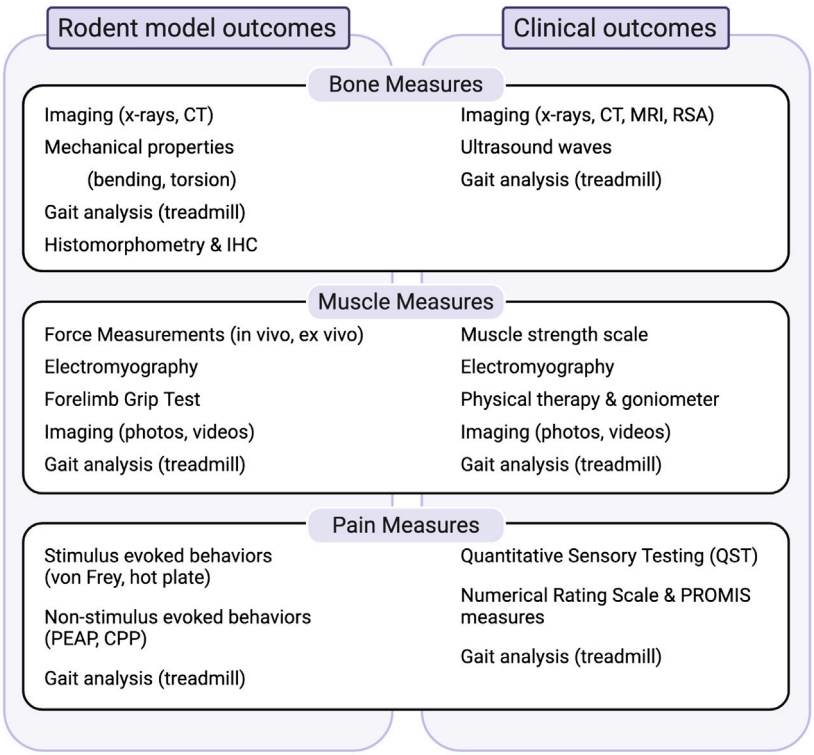

FIGURE 1 | Summary of measures to evaluate bone, muscle and pain-related outcomes in preclinical models and their relationship to clinical outcomes. Abbrevations: CPP, conditioned pain preference; CT, computed tomography; IHC, immunohistochemistry; MRI, magnetic resonance imaging; PEAP, place escape/avoidance paradigms; PROMIS, patient reported outcomes measurement information systems; RSA, radiostereometric analysis.

Additionally, immunohistochemistry can be used to detect changes of osteoblasts and osteoclasts, by alkaline phosphatase (ALP) and tartrate resistant acid phosphatase (TRAP) staining, respectively. Bone proteins, such as osteocalcin, bone morphogenetic proteins (BMPs), osteoprotegerin and vascular endothelial growth factor (VEGF), can be followed during the healing process from callus formation to bone resorption (Fedchenko and Reifenrath, 2014; Li et al., 2019; Li and Helms, 2021).

Physicians also rely heavily on imaging studies, including x-ray, CT, ultrasonography, and MRI (Lichtor et al., 1991; Cunningham et al., 2017), to evaluate anatomic bone healing. Specialized techniques, including radiostereometric analysis (RSA), further allow for precise radiographic measurement of fracture micromotion or deformation (Solomon et al., 2010).

Mechanical Properties

Most rodent studies use bending and torsion on long bones to mimic the typical loading modes in patients (Fritton et al., 2000). Monotonic bending is a major whole-bone measurement of bone ductility and can be performed in three-point or four-point bending (Liodaki et al., 2017; Zhang et al., 2017; Mumtaz et al., 2020; Zhang et al., 2020). While torsion tests are often used to evaluate fracture healing, fracture toughness tests (Poundarik et al., 2012), time-dependent tests (Lynch and Silva, 2008) and viscoelastic tests (Maruyama et al., 2015) are also common in fracture studies, as they are more sensitive to bone matrix changes. To measure the local properties of bone, nanoindentation is utilized to measure the moduli and hardness in different regions of the leg among different strains (Pathak et al., 2012; Pepe et al., 2020). Not only can this technique be applied to measure small biomaterial or callus tissue in rodent studies, but may also measure intrinsic properties of bone tissue in clinical studies by means of *trans*-iliac biopsy specimens (Vennin et al., 2017). Ultrasound waves can be used to non-invasively detect precise changes in both material density and structural integrity via velocity and attenuation in humans. For example, Sakai et al. (2008) used an echo-tracking system to evaluate patients' bone strength under three-point bending tests. Factors affecting long bone fractures including body size (Jepsen et al., 2015), location (Schriefer et al., 2005), sex (Glatt et al., 2007), age (Brodt and Silva, 2010), and strain (Fritton et al., 2000) have been systematically summarized by Jepsen et al. (2015), along with practical guidelines on establishing biomechanical mechanisms in different situations.

Gait Analysis

Besides pain control and proper tissue healing, the ultimate goal of treatment for orthopedic injury is to restore the function of fractured limbs. For lower extremity injury, gait analysis has been frequently used as a measure of function in large animals (Muybridge, 1979; Seebeck et al.,

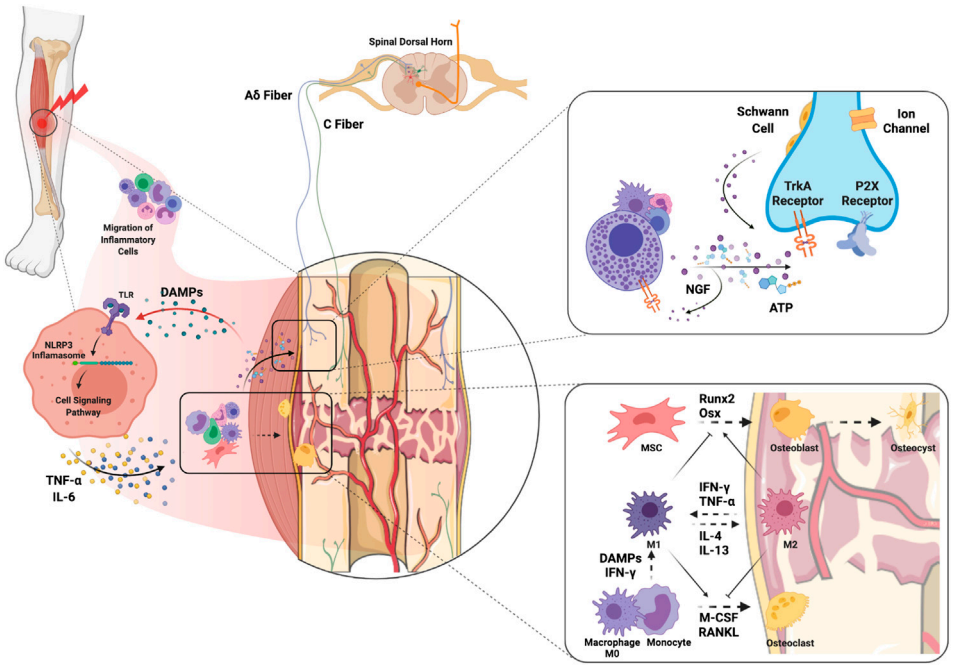

FIGURE 2 | Molecular and cellular mechanisms of complex orthopedic trauma and recovery. Initial trauma results in inflammatory cell migration to the site of injury as a result of cytokine and chemokine release from injured cells. DAMPs released from injured muscle bind TLRs on immune cells which leads to NLRP3 inflammasome-mediated release of inflammatory cytokines such as NGF, TNF-α and IL-6. ATP released from immune cells and injured nerves themselves can activate P2X receptors on sensory nerve terminals. In addition, release of NGF from inflammatory cells stimulates TrkA receptors on Aδ and C fibers which can maintain hyperexcitability. NGF-TrkA complexes are transferred to the DRG where primary afferent cell bodies reside, and these complexes can further maintain pain. Macrophages interfacing with bone are polarized to the M1 phenotype by DAMPs and pro-inflammatory cytokines to clean necrotic tissue and any pathogens. The transition from M1 to M2 would encourage activation of MSCs and differentiation of osteoblasts to promote osteogenesis. The balance between osteoblastic and osteoclastic activity plays a key role in the bone remodeling that results in fracture healing. Abbreviations: DAMPs, damage-associated molecular patterns; IFN-γ, interferon-gamma; IL, interleukin; M-CSF, monocyte-colony stimulating factor; MSC, muscle stem cells; NGF, nerve growth factor; Osx, osterix; RANKL, receptor activator of nuclear factor kappa-B ligand; TLR, toll-like receptor; TNF-α, tumor necrosis factor alpha.

2005), and in rodent models of nerve injury (Wang et al., 2008) or osteoarthritis (Williams et al., 1993), and in our own studies using the fracture-pin model of orthopedic trauma (Tawfik et al., 2020a).

Gait analysis using a treadmill can evaluate both static and dynamic gait patterns in mice by placing the rodent on a treadmill with a camera recording from below. This can be conducted with DigiGait or CatWalk (Kappos et al., 2017) analysis systems as described previously (Deuis et al., 2017). Based on Chen's modified systematic method (Chen et al., 2017), Hofman et al. (2020) evaluated five gait parameters including intensity, print area, swing speed, stand duration, duty cycle after intramedullary stabilized femoral fracture at multiple time points. Gait analysis can also be measured via a simple footprint test with paws covered in non-toxic paint and the pattern evaluated (Deuis et al., 2017).

As for clinical outcomes, gait analysis is an essential aspect of rehabilitation, especially for patients with lower extremity fractures (Rosenbaum et al., 2014). Macri et al. (Macri et al., 2012) proposed a standardized gait pattern score system in tibial fracture patients treated with an intramedullary nail that enables the classification stages of fracture consolidation. In the clinical setting, physicians and physical therapists can observe patients walking on the floor or on a treadmill to monitor gait, favoring of one limb over the other, or any changes in foot placement or asymmetry (Higginson, 2009).

Cellular Mechanisms of Bone Regeneration and Repair

The mechanisms of bone healing have been widely studied and recently comprehensively reviewed by Loi et al. (2016). It is important to highlight that inflammatory cells and their mediators both contribute to bone healing in the early stages but can also delay fracture union if inflammation becomes persistent (**Figure 2**). In particular, macrophages participate in bone fracture healing immediately after injury when the M1 "pro-inflammatory" subtype predominates and encourages greater mineralization through interactions with osteoprogenitors and mesenchymal stem cells (MSCs, **Figure 2**) (Loi et al., 2016). Yet persistent macrophage activation and proinflammatory cytokine expression is detrimental to proper bone healing by inhibiting the differentiation of MSCs to osteoblasts (Lin et al., 2017), and can be a trigger for peripheral neuron sensitization (Shepherd et al., 2018). Polarization of M1 macrophages to the M2 "anti-inflammatory" phenotype using IL-4 infusion or MSC

injection in the subacute period favors bone regeneration and presents an attractive approach to enhance fracture healing (Lin et al., 2018; Lin et al., 2019). In addition, osteocytes have important roles in every phase of fracture healing as they can sense both physical and biochemical signals to regulate bone metabolism regeneration and remodeling (Bonewald, 2011). For an in-depth review of the important role of osteocytes in bone healing see Choy et al. (2020). Bone healing occurs in the context of revascularization and reinnervation which are integral to the process. For example, with implants containing spatiotemporally released angiogenic factors, (Freeman et al. 2020) accelerated bone healing in a large bone defect mouse model. Moreover, Li et al. (2019) demonstrated that the neuronal NGF receptor, TrkA, is a key upstream mediator in ulnar stress fracture healing, clearly connecting neuronal responses with bone repair (also see *Molecular Mechanisms of Pain After Orthopedic Trauma* section).

Measurement of Muscle Healing

Muscle Force

Measurement of muscle force is important to determine the extent of strength regained after injury. Viscoelastic force relaxation, twitch dynamics, and max tetanic force measurements are performed by exposing the sciatic nerve and tibialis anterior (TA) and attaching them to a force transducer (Quarta et al., 2018). *Ex-vivo* measurements of muscle dynamics are performed by removing the TA muscle and attaching it to a force transducer lever and the testing apparatus (Quarta et al., 2017). The contraction of the TA is electrically induced through immersion in a culture bath, while force production was measured (Quarta et al., 2017). In the same way, isokinetic muscle functioning testing can record the forces applied by muscle groups in humans (Osternig, 1986).

Muscle Innervation/Electrical Conduction

In both mouse models and human injuries, electromyography (EMG) is used to diagnose and evaluate the prognosis of myopathy (Daube and Rubin, 2009) while also examining muscle innervation. Researchers surgically put the EMG electrodes within the muscle of interest in mice, then electrically stimulate the treated muscle and record the induced peak-to-peak voltage response (Sicari et al., 2014). Similarly, clinicians place needle electrodes in the belly of injured muscle and motor nerve conduction amplitude pre- and post-treatment can be recorded (Dziki et al., 2016).

Muscle Strength/Function

Several testing paradigms have been developed to evaluate muscle strength and function in rodents with clear clinical correlates. The forelimb grip test evaluates a mouse's forelimb and/or hindlimb skeletal muscle strength (Bonetto et al., 2015). These compare to the 1–5 scale from the British Medical Research Council for muscle strength testing in humans (Compston, 2010). Physical therapists also use a goniometer to collect range of motion measurements on the injured limb (Grogan et al., 2011). Lastly, photos and videos of injured and surrounding muscles are used for mice models and humans to evaluate functional movements and to record the wounds and atrophy of the muscle (Grogan et al., 2011).

Exercise with either wheel or treadmill access is an additional way to measure motor function and provide rehabilitation after injury in mice. Shi et al. (2018) demonstrated that 4 weeks of exercise resolved allodynia, warmth, swelling, and unweighting compared to unexercized mice in a fracture-casting model. Our laboratory has also shown the effectiveness of delaying exercise. In the tibial fracture-pin model of orthopedic trauma, mice that had access to a running wheel immediately after injury had worse muscle fibrosis compared to non-exercized mice, however, mice with delayed access to exercise had improved muscle, bone and pain outcomes (Tawfik et al., 2020b). Routine exercise can prove to be extremely beneficial for patients by improving function and decreasing pain (Merkle et al., 2020).

Monitoring mice throughout their daily activity using systems such as HomeCageScan which utilize automated video can give a better sense of spontaneous pain behaviors and movement (Deuis et al., 2017). Similarly, tracking daily movement using a body-fixed sensor can facilitate monitoring of activities of daily living (ADL) and post-op rehabilitation in patients (Brandes et al., 2011; Benzinger et al., 2014). Luna et al. (2019) used actigraphy-based assessments to evaluate early postoperative physical function. They correlated intensity of activity to individual recovery trajectories and found that early stratified physiotherapy interventions are needed for patients with reduced activity.

Cellular Mechanisms of Muscle Regeneration and Repair

After a muscle injury, the skeletal muscle regenerates via activation, proliferation, migration, and differentiation of muscle stem cells in a conducive microenvironment (Sicari et al., 2014). It has also been shown that perivascular stem cells, $CD146^+$ endothelial cells, and $NG2^+$ (neurogenin 2-positive) polydendrocytes, and M1 and M2 macrophages have a crucial role in muscle regeneration (Sicari et al., 2014; Hurtgen et al., 2016). Without correct muscle repair, volumetric muscle loss (VML) can ensure with associated chronic muscle pain, therefore there is a need to develop new therapies to ensure proper muscle regeneration (**Figure 2**).

Quarta et al. (2017) used bioconstructs of muscle stem cells and other muscle resident cells to restore structure and function after an acute VML. To further support muscle regeneration, Sicari et al. (2014) implanted a porcine urinary bladder extracellular matrix (ECM) into VML injured muscle to create a supportive microenvironmental niche. Moreover, they performed successful proof-of-concept studies in five patients with VML injury (Sicari et al., 2014) and a subsequent trial with thirteen patients (Dziki et al., 2016) also showed strength increase, *de novo* muscle formation, and muscle innervation. Clear limitations include the small number of participants, possible placebo effect, lack of a control group, and not being able to correlate *de novo* muscle formation to functional improvement, however, these are steps toward the clinical use of such bioconstructs and ECM transplantation for VML.

Measurement of Pain-like Behaviors

Reflexive Behaviors

Reflexive stimulus-evoked measures with varying modalities (touch, temperature, pressure) are commonly used approaches to assess pain-like behaviors in preclinical models. Tests used for mechanically evoked pain-like behaviors include manual or electronic von Frey test, and Randall-Selitto test (Deuis et al., 2017). Tests used to evaluate heat responses include the hot-plate, Hargreaves, and thermal probe tests (Deuis et al., 2017). In humans, mechanical allodynia and hyperalgesia can be assessed using a pinprick or monofilament, or through the application of pressure while heat sensitivity can be assessed by placing a metal probe on the skin (Deuis et al., 2017). Quantitative Sensory Testing (QST), dynamic or static, can be used to evaluate stimulus-evoked pain. Dynamic QST assesses response to multiple stimuli and allows for examination of central processing of nociception, whereas static QST assesses the response to a single stimulus (Mackey et al., 2017). Dynamic QST includes temporal summation (TS) and conditioned pain modulation (CPM). In TS, continual probing by noxious stimuli causes increased perception of pain, whereas in CPM the perception of pain caused by a noxious stimulus can be decreased with the presence of a second noxious stimulus (Mackey et al., 2017).

Non-Reflexive Behaviors

Methods used to assess non-reflexive pain-related behaviors include gait analysis (see above), and place escape/avoidance paradigms (PEAP) and conditioned place preference (CPP) (Navratilova and Porreca, 2014). PEAP assesses the affective and sensory components of pain while CPP facilitates understanding of reward and aversion behavior motivated by pain-relief, or pain avoidance (Navratilova and Porreca, 2014; Kuhn et al., 2019). For example, a change in escape latency in the Mechanical Conflict Avoidance test (MCA)–a voluntary, non-reflexive behavioral assay–could indicate spontaneous pain after spared nerve injury or Complete Freund's adjuvant injection (Shepherd and Mohapatra, 2018). The mouse grimace scale (MGS) is another measure of spontaneous pain. The MGS scores the movements of individual facial muscles on a three-point rating scale. It accounts for orbital tightening, cheek bulge, nose bulge, whisker position, and ear position (Mogil et al., 2020).

The Numerical Rating Scale (NRS) is frequently used to assess pain in humans but does not provide much depth of information. It allows patients to rate pain on a scale of 0–10 (or 0–100), with the lower limit indicating no pain and the higher limit denoting the worst pain (Krebs et al., 2007). Other more detailed evaluations were subsequently developed to more fully evaluate patients presenting with chronic pain. The McGill Pain Questionnaire, for example, is a multidimensional framework that measures the sensory, affective, cognitive, and behavioral aspects that comprise pain by evaluating pain location, intensity, quality, and pattern (Ngamkham et al., 2012). More recently, several pain clinics including our own (Bhandari et al., 2016), have integrated patient-reported outcome (PROMIS) measures that evaluate physical, psychological and social functioning, fatigue and sleep, among other parameters (Sturgeon et al., 2015a; Sturgeon et al., 2015b).

Molecular Mechanisms of Pain After Orthopedic Trauma

The bone periosteum, made up of outer fibrous and inner cambium layers, appears to have the densest sensory and sympathetic innervation (Mantyh, 2014). Pain following bone fracture is attributed to the nerve fibers in the periosteum, including A-delta and C-fibers which get sensitized after injury (Mantyh, 2014). Initial activation of neuronal P2X receptors by ATP released from injured keratinocytes and infiltrating inflammatory cells facilitates the transmission of nociceptive information through membrane depolarization and calcium entry (Bernier et al., 2018). The inflammatory response to injury further results in the release of nerve growth factor (NGF) by immune and Schwann cells, which binds to TrkA (Mantyh et al., 2011). NGF-TrkA form a complex which is internalized and transported to cell bodies in the DRG where feedforward hypersensitivity of neurons triggers pain (Mantyh et al., 2011). Since the NGF-TrkA complex can generate and maintain pain, the use of anti-NGF, which prevents the binding of NGF to TrkA, can reduce pain-related behaviors in a fracture model without negatively affecting bone healing (Jimenez-Andrade et al., 2007; Koewler et al., 2007). In clinical trials, the administration of tanezumab, a monoclonal antibody directed against NGF, was reported to exhibit efficacy in low back pain (Chang et al., 2016).

When a peripheral injury occurs, endogenous danger-associated molecular patterns (DAMPs) are released from damaged muscle fibers to facilitate repair (Hurtgen et al., 2016; Kelley et al., 2019). DAMPs trigger an innate immune response to injury by binding to pattern recognition receptors, such as toll-like receptors (TLRs) (Bianchi, 2007) with downstream activation of the NLRP3 inflammasome and production of inflammatory mediators such as TNF-alpha and IL-6, among others (Bianchi, 2007) (**Figure 2**). TNF-alpha and IL-6 have both been shown to cause hyperalgesia when intramuscularly injected into rodents likely through induction of additional sensitizing mediators such as PGE_2, NGF, and CGRP (Schafers et al., 2003; Manjavachi et al., 2010). Importantly, commonly used clinical medications such as dexamethasone and morphine can prevent the IL-6-induced reduction in pain threshold (Manjavachi et al., 2010). While these drugs can provide some relief for post-injury pain, there is still a need to identify better treatment options (Manjavachi et al., 2010).

Imaging Pain Generators

One way to identify pain generators is through the use of positron emission tomography (PET), a noninvasive imaging tool that allows spatiotemporal visualization of cellular responses to injury. PET ligands that specifically target different cell types and varying cellular activation states can be utilized to monitor disease progression or measure treatment success (Jain et al., 2020). We previously used longitudinal ^{18}F-TSPO-PET imaging in the tibial fracture mouse model to track the activation of peripheral and central myeloid cells during the acute-to-chronic pain transition (Cropper et al., 2019). In patients, we have also used PET/MRI imaging with the novel radiotracer ^{18}F-FTC-146 to image the sigma-1 receptor, a pro-nociceptive receptor which is upregulated in inflamed tissue. Imaging identified a previously unappreciated mass with high focal uptake of ^{18}F-FTC-146 in the intercondylar notch, removal

of which resulted in resolution of the patient's knee pain (Cipriano et al., 2018).

CONCLUSIONS AND FUTURE DIRECTIONS

Mouse models of orthopedic trauma that mimic multi-tissue injury, including bone, muscle and nerve, are most representative of human complex extremity trauma. Using such models combined with outcome measures that have clear human correlates, will improve translation of basic science findings to clinically useful treatments. We encourage all preclinical researchers to consider these approaches in designing their studies.

AUTHOR CONTRIBUTIONS

All authors (HS, AG, JV, RI, and VT) contributed to the formulation of the ideas for this review and to the writing and editing of the manuscript and figures. All authors approved the final version.

FUNDING

This work was supported by grant funds from the Department of Anesthesiology, Perioperative and Pain Medicine Stanford University School of Medicine (VT) and National Institutes of Health (NIH) Grant No: R35 GM137906 (VT). Individual fellowship support was provided by the Department of Orthopaedic Surgery, First Affiliated Hospital of Soochow University (HS) and the Department of Anesthesiology, Shimane University (RI).

ACKNOWLEDGMENTS

All figures were created with BioRender.com.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

PHARMACOLOGY
published: 03 February 2021
doi: 10.3389/fphar.2020.628438

Characterization of Mechanical Allodynia and Skin Innervation in a Mouse Model of Type-2 Diabetes Induced by Cafeteria-Style Diet and Low-Doses of Streptozotocin

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OPEN ACCESS

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Specialty section:
This article was submitted to Translational Pharmacology, a section of the journal Frontiers in Pharmacology

Received: *11 November 2020*
Accepted: *31 December 2020*
Published: *03 February 2021*

Citation:
Castañeda-Corral G, Velázquez-Salazar NB, Martínez-Martínez A, Taboada-Serrano JN, Núñez-Aragón PN, González-Palomares L, Acosta-González RI, Petricevich VL, Acevedo-Fernández JJ, Montes S and Jiménez-Andrade JM (2021) Characterization of Mechanical Allodynia and Skin Innervation in a Mouse Model of Type-2 Diabetes Induced by Cafeteria-Style Diet and Low-Doses of Streptozotocin. Front. Pharmacol. 11:628438. doi: 10.3389/fphar.2020.628438

Background: Painful distal symmetrical polyneuropathy (DPN) is a frequent complication of type-2 diabetes mellitus (T2DM) that commonly presents as neuropathic pain and loss of skin nerve fibers. However, there are limited therapies to effectively treat DPN and many of the current animal models of T2DM-induced DPN do not appear to mirror the human disease. Thus, we validated a DPN mouse model induced by a cafeteria-style diet plus low-doses of streptozotocin (STZ).

Methods: Female C57BL/6J mice were fed either standard (STD) diet or obesogenic cafeteria (CAF) diet for 32 weeks, starting at 8 weeks old. Eight weeks after starting diets, CAF or STD mice received either four low-doses of STZ or vehicle. Changes in body weight, blood glucose and insulin levels, as well as oral glucose- and insulin-tolerance tests (OGTT and ITT) were determined. The development of mechanical hypersensitivity of the hindpaws was determined using von Frey filaments. Moreover, the effect of the most common neuropathic pain drugs was evaluated on T2DM-induced mechanical allodynia. Finally, the density of PGP -9.5$^+$ (a pan-neuronal marker) axons in the *epidermis* from the hindpaw glabrous skin was quantified.

Results: At 22–24 weeks after STZ injections, CAF + STZ mice had significantly higher glucose and insulin levels compared to CAF + VEH, STD + STZ, and STD + VEH mice, and developed glucose tolerance and insulin resistance. Skin mechanical sensitivity was detected as early as 12 weeks post-STZ injections and it was significantly attenuated by intraperitoneal acute treatment with amitriptyline, gabapentin, tramadol, duloxetine, or carbamazepine but not by diclofenac. The density of PGP-9.5$^+$ nerve fibers was reduced in CAF + STZ mice compared to other groups.

Conclusion: This reverse translational study provides a painful DPN mouse model which may help in developing a better understanding of the factors that generate and maintain

neuropathic pain and denervation of skin under T2DM and to identify mechanism-based new treatments.

Keywords: painful distal polyneuropathy, type 2 diabetes mellitus, skin hypersensitivity, mechanical allodynia, skin nerve fibers, cafeteria diet.

INTRODUCTION

Diabetes mellitus (DM) is a highly prevalent heterogeneous group of metabolic disorders characterized by hyperglycemia due to an absolute or relative deficit in insulin production or action. Currently, DM represents a severe public health problem in the world; 463 million people worldwide were estimated to have DM in 2019, and is expected that this number will increase to 700 million by 2045 (IDF, 2019). The most common type of diabetes is type-2 diabetes mellitus (T2DM) accounting for 90–95% of DM patients (ADA, 2019). T2DM results mainly from low physical activity and a high caloric diet leading to obesity (West and Kalbfleisch, 1971; Kolb and Martin, 2017). Obesity-induced T2DM is characterized mainly by chronic hyperglycemia and insulin resistance in peripheral tissues.

Among the T2DM long-term complications, diabetic neuropathies are one of the most common chronic complications, and distal symmetrical polyneuropathy (DPN) is the most prevalent type of diabetic neuropathy, with 30–50% of T2DM patients developing DPN (Candrilli et al., 2007). Although a large number of patients with DPN may be asymptomatic, approximately a quarter of people with T2DM suffer from painful DPN as a result of damage to or dysfunction of the sensory nerve fibers, along with a loss of the nerve fiber axons within the skin (Abbott et al., 2011). The affected patients with painful DPN often describe unpleasant sensory symptoms, burning pain, shooting pains down the legs; lancinating, contact pain often with day-time clothes and bedclothes (allodynia); pain when walking; sensations of heat or cold in the feet; persistent achy feeling in the feet and cramp-like sensations in the legs (Tesfaye, 2003). Although there has been significant progress in the understanding of the mechanisms underlying the development and maintenance of T2DM-induced painful DPN, there is still a limited repertoire of efficacious therapies to successfully treat it.

Preclinical models have been highly valuable tools to investigate the pathophysiology of T2DM-induced painful DPN. Current T2DM animal models are mainly based on genetic, dietary, chemical, surgical approaches, or a combination of chemicals administration with diet alterations (Srinivasan and Ramarao, 2007; Badole et al., 2013; Stenkamp-Strahm et al., 2015). However, genetic models might be relatively expensive and uneasily accessible for some investigators (Islam and Wilson, 2012). Furthermore, it has been pointed out that some of these preclinical models of T2DM do not mimic the pattern of the DPN development and progress that occurs in humans (Sullivan et al., 2008). Animal models resembling the human diabetic state have recently been developed; a miniature swine model of T2DM induced by a cafeteria-style diet (CAF) followed by streptozotocin (STZ), effectively mimics the natural history of the disease in humans, with diabetes and insulin resistance following a period of obesity (Coelho et al., 2018). The CAF diet consists of highly palatable and energy-dense foods which are very common in Western society (Sclafani and Springer, 1976; Sampey et al., 2011). The CAF diet along with the administration of STZ in Göttingen minipigs resulted in hyperphagia, ketonuria, and hyperglycemia (Coelho et al., 2018).

While this model of T2DM consisting of CAF followed by multiple low-doses of STZ has been characterized from a metabolic perspective in minipigs (Coelho et al., 2018), to our knowledge, there is no study that has adapted and characterized this model of T2DM in rodents to study the pathogenesis of painful DPN. Thus, we aimed to perform a reverse translational study to characterize the T2DM-induced long-term neuropathic pain-like behaviors, the effect of different analgesics on these behavioral pain-like responses, and the loss of nerve fiber axons within the glabrous skin of the mouse hindpaw.

MATERIALS AND METHODS

Animals

Female C57BL/6J mice, aged initially six weeks old, were obtained from our breeding facilities. They were weight-matched and housed in groups of 4, in a temperature and humidity-controlled room (21 ± 2°C) under a 12:12 light/dark cycle (lights on at 07:00 h), with free access to food and tap water. All experimental procedures were performed following the Mexican Official Norm of Animal Care and Handling (NOM-062-ZOO-1999, 1999), the international guidelines on ethical standards for investigation in animals, and the international guidelines for the study of pain (Zimmermann, 1983). Moreover, the protocol was approved by the Institutional Animal Care and Use Committee of the Facultad de Medicina, Universidad Autónoma del Estado de Morelos. Efforts were made to minimize the number of animals used.

Drugs

The following drugs/chemicals were used: STZ (lot number WXBC8740V), amitriptyline hydrochloride (lot number LRAA9172), duloxetine hydrochloride (lot number LRAB0283), carbamazepine (lot number MKCF7846), and gabapentin (lot number LRAA5714) were purchased from Sigma-Aldrich (St. Louis, MO). NPH insulin (lot number S19V698) and Tramadol hydrochloride parenteral solution were obtained from AMSA (Mexico City, Mexico) and Pisa Biotec (Guadalajara, Mexico), respectively. Diclofenac was donated by Senosiain S.A de C.V (Mexico City, Mexico). All drugs were dissolved in 0.9% sterile saline solution and prepared the same day they were tested.

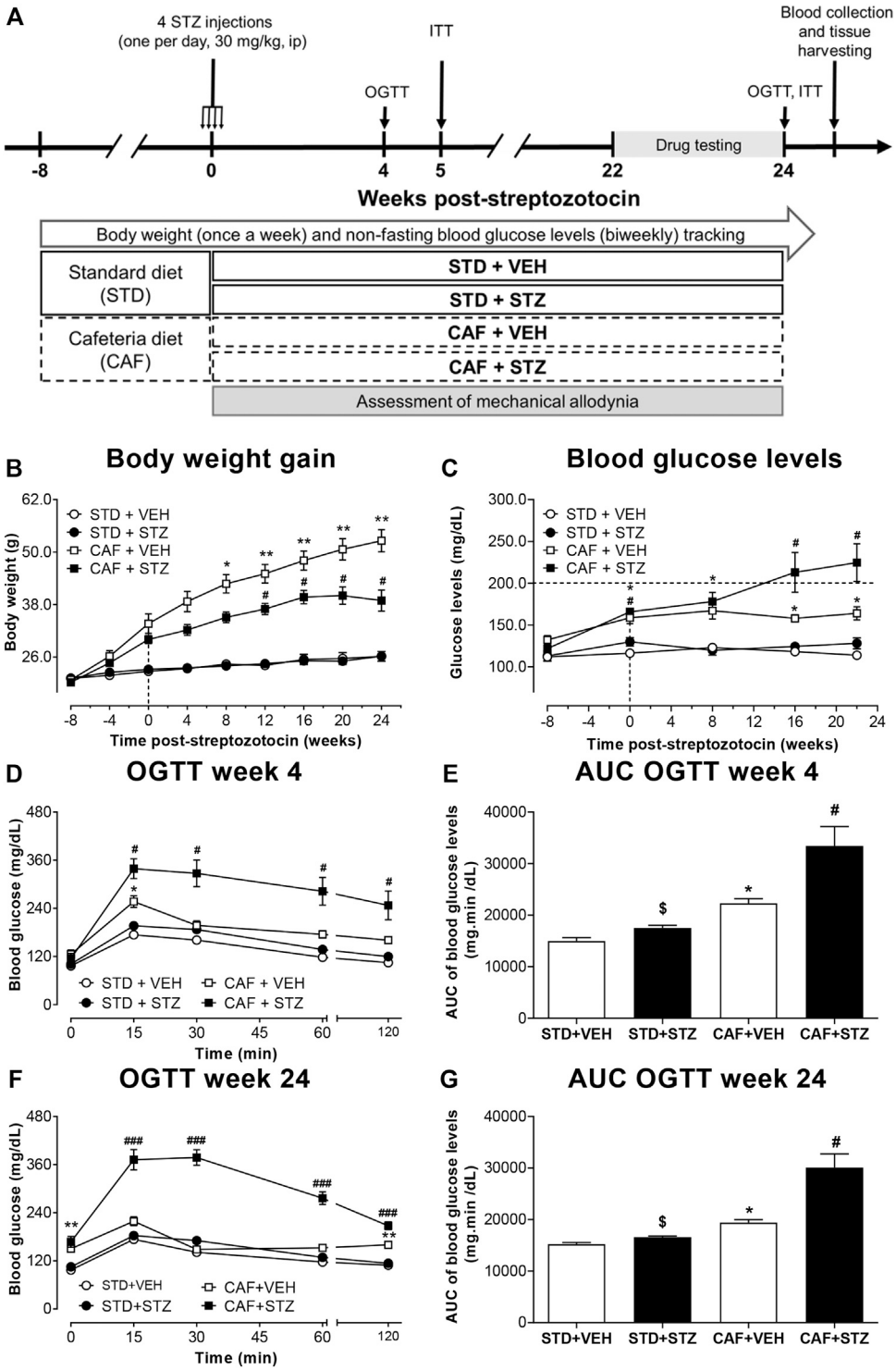

FIGURE 1 | Outline of the experimental design **(A)** and changes in body weight **(B)**, blood glucose levels **(C)**, and glucose tolerance **(D–G)** in mice. Female C57BL/6J mice at 8 weeks-old were fed either cafeteria (CAF) or standard (STD) diet and injected with vehicle (VEH) or streptozotocin (STZ). Eight weeks later, CAF or STD mice were administered either with four low-doses of STZ (30 mg/kg, i.p., one per day) or vehicle. Values are means ± SEM (n = 10). In **(B)** $*p < 0.05$, $**p < 0.01$ for CAF [$F_{(1,36)} = 109.5$]. *, #$p < 0.05$ for the interaction CAF × STZ [$F_{(1,36)} = 7.94$]. In **(C)** $*p < 0.05$ for CAF [$F_{(1,36)} = 74.11$] #$p < 0.05$ for the interaction CAF × STZ × Time [$F_{(1,36)} = 3.10$]. In **(D)** $*p < 0.05$ for CAF [$F_{(1,36)} = 33.84$], #$p < 0.05$ for the interaction CAF × STZ × Time [$F_{(1,36)} = 6.82$]. In **(F)** $*p < 0.05$ for CAF [$F_{(1,36)} = 150.6$], ###$p < 0.001$ for the interaction CAF × STZ × Time [$F_{(1,36)} = 22.16$]. Notably, the interaction CAF × STZ showed an important effect on the variable ($F = 48.8$, $p < 0.05$). In **(E)** $*p < 0.05$ for CAF [$F_{(1,36)} = 35.12$], \$ ($p < 0.05$) for STZ [$F_{(1,36)} = 12.17$], # ($p < 0.05$) for the interaction CAF × STZ [$F_{(1,36)} = 4.75$]. In **(G)** $*p < 0.05$ for CAF [$F_{(1,36)} = 40.65$], \$ ($p < 0.05$) for STZ [$F_{(1,36)} = 18.78$], # ($p < 0.05$) for the interaction CAF × STZ [$F_{(1,36)} = 11.73$]. Data in **(B–F)** were analyzed by Repeated-Measures Three-Way ANOVA, followed by Bonferroni's. Data in **(E)** and **(G)** were analyzed by Two-Way ANOVA. OGTT: oral glucose tolerance test.

Type-2 Diabetes Model Induced by CAF-Style Diet and Low-Doses of STZ

After a two-week acclimation period, mice were randomly assigned to one of the two following dietary groups: 1) standard diet (STD) or 2) CAF diet. The STD diet provided an average of 19.49 Kcal/day, from which 10.7% is fat, 63% are carbohydrates and 26.1% is protein. On another hand, the CAF-diet used in this study was adapted from a CAF diet previously described by Leffa and coworkers (Leffa et al., 2015). This CAF-diet is rich in fat and carbohydrates (fat 36.5%, carbohydrates 42.2%, and protein 22.3%) and on average provides approximately 40.27 Kcal/day to each mouse. It consists of a choice of highly palatable, unhealthy human foods with known energy content and soft drinks such as orange-flavored and cola. Animals could select and freely consume the CAF items which were given in excess and daily replaced with fresh food (weekly menus and total energy value are outlined in **Supplementary Table S1**). To induce partial insulin deficiency after 8 weeks of feeding, the mice were subdivided into the following four groups: 1) STD + VEH, 2) STD + STZ, 3) CAF + VEH, and 4) CAF + STZ. Groups 1 and 3 were injected during four consecutive days with distilled water (0.1 ml/10 g body weight (BW), i.p.; vehicle), while groups 2 and 4 received four consecutive injections (i.p., one per day) of STZ at 30 mg/kg of BW (freshly dissolved in distilled water) (Zhang et al., 2008). After the last vehicle- or STZ-injection, all groups continually received their respective CAF or STD diets for the rest of the experiment. During the entire experimental period (32 weeks), the animals were weighed once a week (**Figure 1A**). Finally, at the 24th-week post-streptozotocin, the body mass index (BMI) was calculated as the ratio between body weight and square surface area according to the formula previously reported by Gargiulo et al. (Gargiulo et al., 2014). In all the experiments the number of experimental units per assay was 8–10. In this study a total of 62 mice were used as follows: STD + VEH (n = 10); STD + STZ (n = 10); CAF + VEH (n = 14); and CAF + STZ (n = 28). The number of animals from the CAF + STZ group was increased since they were used for assessing the analgesic drugs. In addition, from the CAF + STZ group, two mice were euthanized at week 18 post-STZ injections because they had lost more than 25% baseline BW and showed blood glucose levels above 400 mg/dL. Also, two other mice from this group were excluded because they had blood glucose levels below 200 mg/dL and normal glucose tolerance. Thus, the success rate of the model was higher than 80%.

Blood Glucose Levels, Oral Glucose Tolerance Test, and Insulin Tolerance Test

Non-fasting blood glucose levels were monitored biweekly during the entire experimental period. Oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) were performed at 4 and 24 weeks, and 5 and 24 weeks post-STZ, respectively (**Figure 1A**). In week 24 post-STZ, ITT was performed 4 days after the OGTT. In all cases, glucose quantification was performed in blood samples obtained from the tail vein blood using the calibrated glucometer system Accu-Chek Performa (Roche Diagnostic, Germany). For OGTT, mice fasted for 12 h received a bolus of aqueous glucose solution (2 g/kg) that was delivered into the stomach by a gavage probe (20-gauge, 38 mm long curved, with a 21/4 mm ball end). Blood glucose quantification was performed at 0 (basal), 15, 30, 60, and 120 min after the glucose load (Nagy and Einwallner, 2018). For ITT, mice with a 2 hours-fasting period were intraperitoneally injected with NPH insulin, which was dissolved in saline at a dose of 0.75 UI/kg BW (Nagy and Einwallner, 2018), and blood glucose levels were measured at the same time points as in the OGTT. For both, OGTT and ITT, the area under the curve (AUC) values for plasma glucose were calculated using the trapezoidal rule. It is important to mention that from the group CAF + STZ only mice that showed glucose levels above 200 mg/dL between 16 and 24 weeks post-STZ injections were used to perform OGTT (at 24 weeks), ITT (at 24 weeks), drug evaluation (weeks 22–24) and immunohistochemistry (24 weeks). Mice used to measure insulin or for immunohistochemistry did not receive any analgesic drug.

Insulin Levels and Sensitivity

Insulin levels were measured by using the ELISA method in blood samples obtained 24 weeks post-STZ injections (**Figure 1A**). Blood samples were collected around 4:00–6:00 PM, by cardiac puncture and allowed to clot for 30 min at room temperature. Then, samples were centrifuged at 3,000 rpm/4°C for 15 min to separate the serum. The supernatant was aliquoted and frozen at −70°C until further processing. Insulin was measured by using a mouse insulin ELISA kit (Millipore, Cat. # EZRMI-13K) according to the manufacture's specifications. The assay range for insulin was 0.1–10 ng/ml, and intraassay precision CV% < 10% and inter-assay precision CV% < 12%. All measurements were performed in duplicate and the mean of two measurements was considered. Insulin sensitivity was assessed by using the homeostasis model assessment-2 (HOMA2) index using an online-based calculator on the Diabetes Trials Unit of the University of Oxford website (https://www.dtu.ox.ac.uk/homacalculator) (Avtanski et al., 2019). To perform this, glucose levels were measured around 7:00–8:00 AM after a 12-h fasting period.

Quantitative Assessment of Mechanical Allodynia

Mechanical allodynia, as a feature of painful DPN, was determined using von Frey filaments (Stoelting, Wood Dale, IL, United States). Briefly, 30 min before the behavioral evaluation, the mice were placed in individual acrylic boxes with a wire mesh grid to allow them to acclimatize to their surroundings. Then, the filaments were presented perpendicular to the mid-plantar surface of the left hind limb with enough force to cause slight buckling against the paw and held for 3–5 s. A positive response was noted if the paw was sharply withdrawn, flinching, or licking immediately. Nociceptive response for mechanical sensitivity was expressed as the 50% paw withdrawal threshold (g), and was calculated using the up-down

method as described in Chaplan et al. (Chaplan et al., 1994). 50% paw withdrawal thresholds were assessed in all animals at 0, 4, 8, 12, 14, 16, 17, 18, 19, 20, 21, 22, 23, and 24 weeks after the last STZ injection. In all behavioral experiments, the investigator performing the evaluation was blinded to the experimental condition of the mice.

Drug Testing

The antiallodynic effect of drugs, used as the first or second line of treatment for neuropathic pain, was evaluated when CAF + STZ mice displayed established allodynia (50% withdrawal thresholds ≤ 0.4 g; observed between 22 and 24 weeks post-STZ injection). To decrease the number of mice used for this aim, this experiment was performed in a total of 16 CAF + STZ mice using a crossover design (drug-vehicle-drug-vehicle). To do this the 50% withdrawal threshold was measured to confirm mechanical allodynia. Next, the 16 mice were numbered and then sorted into two groups (A and B, n = 8 each) by randomization using the GraphPad Prism software version 8 (GraphPad Software, Inc.). Posteriorly, mice in group (A) were administered with one of the analgesic drugs and the animals in group (B) received saline and then the anti-allodynic effect was assessed by measuring the paw withdrawal threshold at the multiple time-points indicated after the administration of each drug. After the experiment was finished, the animals rested for a washout period of at least seven elimination drug-half lives to ensure that 99% of the drug had been eliminated, and then, animals that were used as controls in the previous assay (B) were treated with the analgesic drug and the other group (A) served as control receiving saline. This procedure was repeated until complete the evaluation of the six analgesic drugs. Drugs and doses were chosen based on previous studies where they were effective in reducing neuropathic pain. The order in which the drugs were evaluated was gabapentin (100 mg/kg) (Wagner et al., 2014), tramadol (30 mg/kg) (Codd et al., 2008), duloxetine (30 mg/kg) (Kremer et al., 2018), carbamazepine (100 mg/kg) (Kiguchi et al., 2004), diclofenac (50 mg/kg, negative control) (Grim et al., 2014), and amitriptyline (30 mg/kg) (Kremer et al., 2018).

Tissue Harvesting and Immunohistochemistry

Animals used for immunohistochemistry (IHC) were sacrificed 24 weeks post-STZ injections. Mice were deeply anesthetized with a mixture of ketamine and xylazine (100/10 mg/kg) followed by transcardiac perfusion first with phosphate-buffered saline (PBS, 0.1 M, pH 7.4, 4°C) and followed by 4% paraformaldehyde in PBS. Immediately after perfusion, the visceral adipose tissue, defined as the sum of epididymal, perirenal, mesenteric, and inguinal subcutaneous adipose tissue, was removed and weighed (Loredo-Pérez et al., 2016; Bagchi and MacDougald, 2019). Moreover, both posterior hindpaws were harvested, post-fixed for 24 h in the same fixative, and stored in PBS. Then, the skin of the hindpaw was cryoprotected in 30% sucrose solution at 4°C for further processing by IHC. Serial frozen sections of the glabrous skin from the hindpaws (30 μm) were cut with a cryostat (Leica CM1900) and thaw-mounted on gelatin-coated slides for processing. Posteriorly, the skin sections were washed in 0.1 M PBS, three times for 10 min each, incubated for 1 h with a blocking solution consisting of 3% Normal Donkey Serum and 0.3% Triton X100 in 0.1 M PBS, and then incubated for 12 h with a PGP-9.5 primary antibody (Protein gene product 1:3,000; Cedarlane; catalog number CL7756AP). Subsequently, preparations were washed in PBS and then incubated for 3 h with the secondary antibody (Cy3 monoclonal donkey anti-rabbit 1:600; Jackson ImmunoResearch; Catalog number 711-165-152). Later, skin sections were washed in PBS, dehydrated through an alcohol gradient (70, 80, 90, and 100%), cleared in xylene, and coverslipped with DPX mounting medium. For the quantification of the intraepidermal nerve fiber density (IENF), initially, at least 10 separate skin sections were scanned at low magnification (10x) to identify the areas with the best integrity thorough an epifluorescence microscope (Axio Scope. A1, Carl Zeiss, Jena, Germany). Then, an image of this area, of a least five different skin sections, was obtained at 20x magnification using a Carl Zeiss scanning confocal laser microscope (model LSM 800, Jena, Germany). The Z-stack images were analyzed using ImageJ software (National Institutes of Health) to determine the total length of nerve fibers. To do this, the nerve fibers innervating the *epidermis* were manually traced using the freehand line tool. The determination of the area of evaluation was obtained by manually tracing the area of the *epidermis*. IENF data are presented as the mean of the total length of nerve fibers (μm) per 100 μm^2 of the *epidermis* (Pham et al., 2018).

Statistical Analysis

All data are expressed as mean ± standard error of the mean (SEM). Sample sizes are displayed in the figure legend of each figure. Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, Inc.). To determine the main effects of treatments (Diet and STZ) and their interaction on variables, Two-Way ANOVA was used. In the case of time as an extra factor contributing to outcome, we used Repeated-Measures Three-Way ANOVA (main effects and interactions) followed by Tukey or Bonferroni post-hoc test. In the case of the time-drive analgesia assessment of drugs in the neuropathic pain induced by cafeteria diet and STZ, we used Repeated Measures Two-Way ANOVA followed by Bonferroni comparison. The area under the curve of the analgesic effect was compared with independent samples *t*-test. From previous pilot studies regarding the 50% withdrawal threshold assay, we had determined that 8–10 animals per group were adequate for an 80% statistical power and alpha cut-off at 0.05. Therefore, all along the study, $p < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

Establishment of a T2DM Mouse Model

In this study, female C57BL/6J mice were fed with a CAF-style diet followed by low-doses of STZ to establish a T2DM mouse model. The success of the establishment of T2DM was evaluated by changes in body composition (body weight, BMI, and the amount of visceral adipose tissue), blood glucose levels, OGTT, ITT, and levels of circulating insulin. Changes in body weight during the entire experimental period are illustrated in **Figure 1B**. At the beginning of the experiment, all the mice exhibited similar

body weight (20.9 ± 0.2 g). After 8 weeks of consumption of different diets, body weight was significantly increased in the CAF + VEH (30 ± 0.9 g) and CAF + STZ (31.9 ± 1.6 g) groups compared with the STD + VEH (22 ± 0.5 g) and STD + STZ (22.7 ± 0.6 g) groups. After the STZ or VEH injections, no significant changes in body weight were observed between STD + VEH and STD + STZ mice, which reached a final body weight of 26.2 ± 1.2 g and 26.1 ± 0.8 g, respectively. For the CAF + VEH group, these mice gained weight rapidly and linearly until reaching a final body weight of 52.6 ± 2.5 g. Finally, mice from the CAF + STZ group (final BW 38.9 ± 2.4 g) significantly gained weight in comparison with the mice in the STD + VEH and STD + STZ groups, but weight gain was slower and to a lesser extent than mice from the CAF + VEH group. The statistical analysis showed that the CAF diet was the factor with the highest effect on body weight [$F_{(1,36)} = 109.5$, $p < 0.001$], the interaction was also statistically significant [$F_{(1,36)} = 7.9$, $p < 0.01$] in the Three-Way ANOVA. Similarly, at 24th-week post-STZ, the amount of visceral adipose tissue or the BMI from mice of the STD + VEH group were not significantly different compared to those values in mice from the group STD + STZ. In contrast, mice from the CAF + VEH group showed the highest amount of intraperitoneal adipose tissue [$F_{(1,36)} = 208.68$, $p < 0.001$] and the highest BMI [$F_{(1,36)} = 227.17$, $p < 0.001$] compared to mice in the STD + VEH and STD + STZ groups. The mice from the CAF + STZ group also showed an increase in intraperitoneal adipose tissue [Interaction CAF × STZ, $F_{(1,36)} = 8.8$, $p < 0.01$] and BMI [Interaction CAF × STZ, $F_{(1,36)} = 15.1$, $p < 0.01$] compared to the STD + VEH and STD + CAF mice, but to a lesser extent than the mice from the CAF + VEH group (**Supplementary Table S2**).

Non-fasting glucose measurements showed that baseline blood glucose levels were 121 ± 3 mg/dL. Mice from the STD + VEH and STD + STZ groups showed blood glucose levels close to 120 mg/dL throughout the entire experimental period. In contrast, mice from the CAF + VEH group had significantly higher glucose levels (162 ± 7.2 mg/dL) compared to those from STD + VEH and STD + STZ groups. Mice from the CAF + STZ group displayed glucose levels similar in magnitude compared to those from the CAF + VEH group until week eight post-STZ injections. However, at week 16 post-STZ injections, the glucose levels in CAF + STZ mice, but not in CAF + VEH mice, were above 200 mg/dL, indicating the establishment of hyperglycemia (**Figure 1C**). The three-way ANOVA statistical analysis showed that the CAF diet was the factor with the most influence on blood glucose levels [$F_{(1,36)} = 74.1$], and it was potentiated by STZ after 16 and 24 weeks post-STZ as revealed by the significant interaction at these time points in the statistical analysis.

To identify whether the mice of the different experimental groups had impaired glucose tolerance, an OGTT was performed at 4 or 24 weeks post-STZ injections (**Figures 1D–G**). In both time points, the results showed that blood glucose levels were maximum at 15 min after the oral load of the glucose solution and returned to baseline values by 60 min (**Figures 1D, F**) in mice from STD + VEH, STD + STZ, and CAF + VEH groups. In contrast, the glucose levels from the CAF + STZ group were maintained above 300 mg/dL for 30 min after glucose load, and even after 120 min, blood glucose levels were still above 200 mg/dL. The OGTT results were also illustrated with the AUC analysis, where mice from CAF + STZ group showed the highest AUC values at 4 (**Figure 1E**) and 24 (**Figure 1G**) weeks post-STZ injections [$F_{(1,36)} = 12.17$; $F_{(1,36)} = 11.73$ respectively, $p < 0.05$ for the interaction CAF × STZ, Two-Way ANOVA].

Similarly, to identify whether mice displayed insulin resistance, an ITT was performed 5 or 24 weeks post-STZ injections (**Figures 2A–D**). At week 5, it was observed that exogenous insulin lowered the blood glucose levels from mice of the four experimental groups (**Figures 2A, B**). However, at 24 weeks after STZ injections, the ability of insulin to reduce blood glucose was dramatically impaired in the CAF + VEH and CAF + STZ groups (**Figure 2C**). However, significantly higher insulin resistance was observed in the CAF + STZ group as compared to the CAF + VEH group (**Figure 2D**). Likewise, it was observed that serum insulin levels were significantly greater in the CAF + VEH (2.2 ± 0.7 ng/ml) and CAF + STZ (1.4 ± 0.1 ng/ml) groups compared to the STD + VEH and STD + STZ groups (both 0.4 ± 0.1 ng/ml) (**Figure 2E**). Finally, the calculated HOMA-IR values of the mice in CAF + VEH and CAF + STZ groups were significantly higher compared to the STD + VEH and STD + STZ groups (**Figure 2F**).

Mice With T2DM Developed Long-Lasting Hindpaw Mechanical Allodynia

To determine whether diabetic mice developed tactile allodynia as a sign of painful DPN, 50% withdrawal thresholds were measured. Before the STZ injections, no significant 50% withdrawal threshold differences were found among the four experimental groups. In contrast, after STZ injections, only mice from CAF + STZ group developed a slow and progressive mechanical hypersensitivity in the left hindpaw. This mechanical hypersensitivity was characterized by a significant reduction of 50% withdrawal threshold (PWT) that was detected as early as week 12 and maintained until week 24 post-STZ injections (**Figure 3A**) The statistical analysis revealed an important effect of the interaction between CAF diet and STZ [$F_{(1,36)} = 42.4$] for overall experiment. During weeks 22–24 post-STZ, the reduction on PWT reached values of 0.35 ± 0.03 g for the left hindpaw. In contrast, no statistically significant changes in mechanical sensitivity from mice of the STD + VEH, STD + STZ, or CAF + VEH were observed at week 24 compared to their respective values before STZ-injections (**Figure 3B**).

Evaluation of Different Neuropathic Pain Drugs and Diclofenac on Mechanical Allodynia

In this study, the effect of different drugs used as first-line to treat diabetic DPN in human was evaluated on T2DM-induced tactile allodynia. The results showed that the 50% withdrawal threshold of both hindpaws was significantly increased in diabetic mice that received one i.p. injection of amitriptyline (**Figure 4A**),

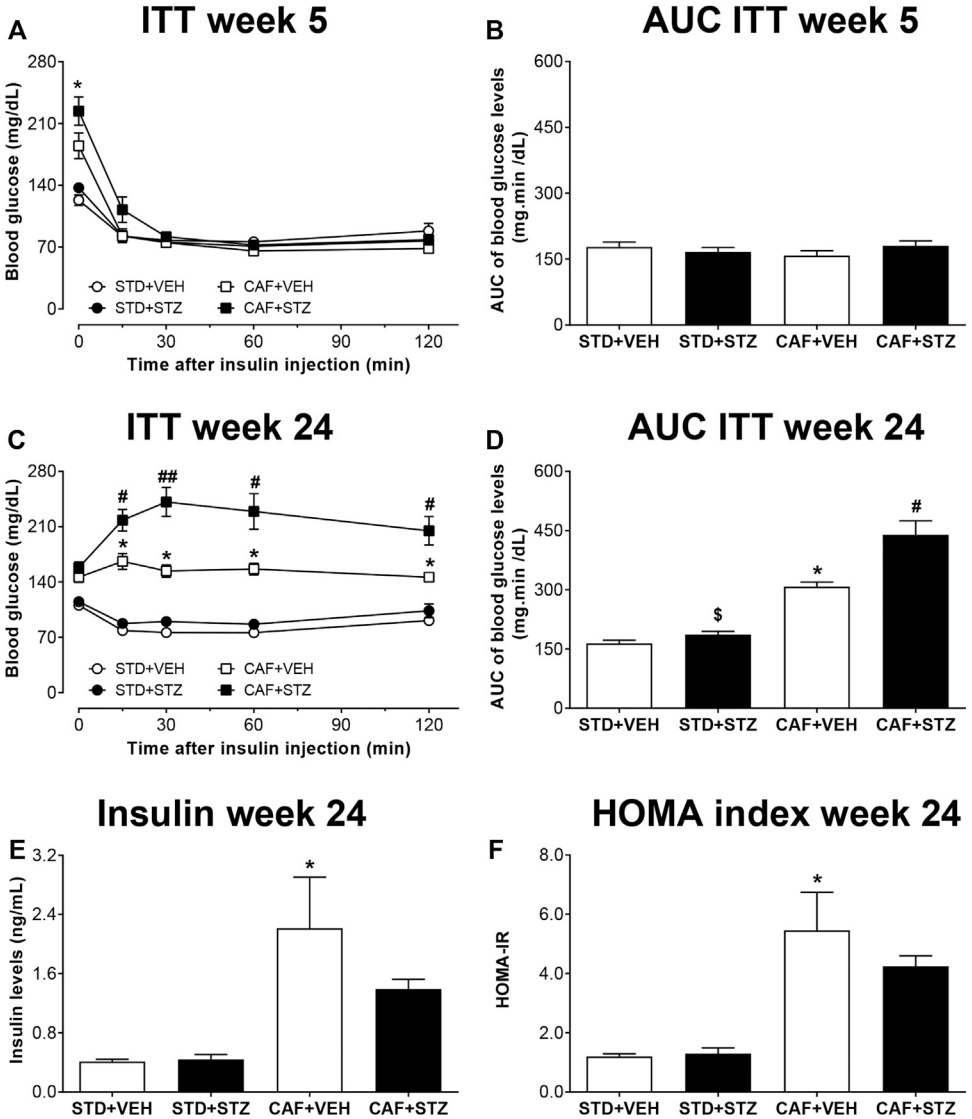

FIGURE 2 | Insulin tolerance test (ITT), insulin levels, and HOMA-index in C57BL/6J female mice with type-2 diabetes induced by CAF diet and low doses of STZ. Panels **(A)** and **(C)** show the blood glucose levels during an ITT at five or 24 weeks after STZ-injections, respectively. Panels **(B)** and **(D)** are showing the area under the curve (AUC) calculated from the ITT. **(E)** Serum insulin levels and **(F)** HOMA2 index calculated 24 weeks post-STZ. Values are means ± SEM (n = 8–10). In **(A)** $^*p < 0.05$ for CAF [$F_{(1,36)} = 7.01$]. No statistical differences were observed in **(B)**. In **(C)** $^*p < 0.05$ for CAF [$F_{(1,36)} = 107.9$]; # ($p < 0.05$) ## ($p < 0.01$) for the interaction CAF × STZ [$F_{(1,36)} = 5.5$]. In **(D)** $^*p < 0.05$ for CAF [$F_{(1,30)} = 76.81$], $ ($p < 0.05$) for STZ [$F_{(1,30)} = 11.55$], # ($p < 0.05$) for the interaction CAF × STZ [$F_{(1,30)} = 5.9$]. In E, $^*p < 0.05$ for CAF [$F_{(1,25)} = 30.71$]. No statistical differences were observed for STZ or CAF × STZ interaction ($p = 0.09$). In **(F)** $p < 0.05$ for CAF [$F_{(1,25)} = 52.82$]. No statistical differences were observed for STZ or CAF × STZ interaction. Data in **(A)** and **(C)** were analyzed by Repeated-Measures Three-Way ANOVA followed by Bonferroni multiple comparison. Data in **(B–F)** were analyzed by Two-Way ANOVA. STD: Standard diet; CAF: Cafeteria diet; VEH: Vehicle; STZ: Streptozotocin.

gabapentin (**Figure 4B**), tramadol (**Figure 4C**), duloxetine (**Figure 4D**), and carbamazepine (**Figure 4E**). The anti-allodynic effect started 30 min after the treatment injection of all drugs. This pharmacological effect lasted at least 2.5 h in the mice treated with amitriptyline, duloxetine, or tramadol, 5.5 h in the mice treated with gabapentin, and at least 7 h in the group treated with carbamazepine. In contrast, diclofenac did not affect the 50% withdrawal threshold values of either hindpaw up to 4 h post-injection compared with either the pre-injection withdrawal threshold or with vehicle-treated animals at the corresponding time points. (**Figure 4F**).

T2DM Mice Showed a Reduction of Intraepidermal Nerve Fibers in the Glabrous Skin of the Hindpaw

To evaluate the IENF density in skin sections from the hindpaw, skin sections were processed by IHC and labeled with a primary

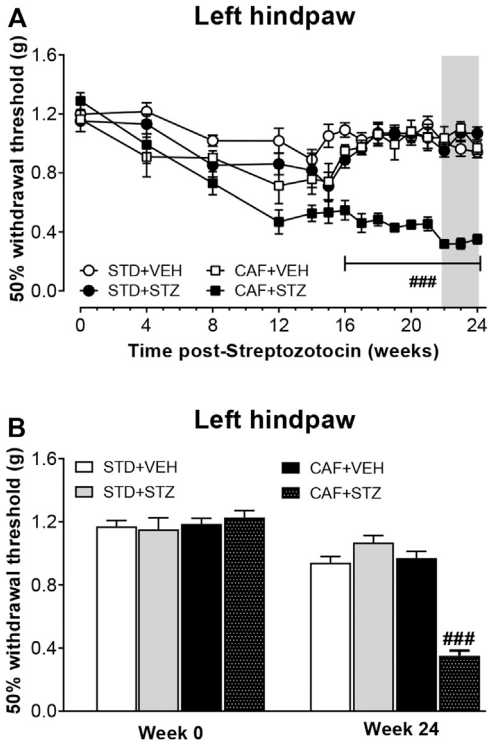

FIGURE 3 | Development of mechanical skin hypersensitivity in female mice with T2DM. **(A)** is showing the time course of the development of hindpaw mechanical hypersensitivity. **(B)** is showing the 50% withdrawal threshold values before STZ injection and 24 weeks-post STZ, respectively. Values are mean ± SEM (n = 10). In **(A)**, ###$p < 0.001$ for the interaction CAF × STZ [$F_{(1, 36)} = 42.4$]. In **(B)** ###$p < 0.001$ for the interaction CAF × STZ [$F_{(1, 36)} = 86.6$]. Data in **(A)** were analyzed by Repeated-Measures Three-Way ANOVA followed by Bonferroni multiple comparison. Data in **(B)** were analyzed by Two-Way ANOVA. STD: Standard diet; CAF: Cafeteria diet; VEH: vehicle; STZ: streptozotocin. The gray rectangle is indicating the period in which the analgesic drugs were tested.

antibody against PGP-9.5 (a pan-neuronal marker). Quantitative analysis of the density of PGP-9.5 immunopositive nerve fibers axons revealed that mice from the STD + STZ and CAF + VEH groups had reduced values of length of IENF compared to the control group (STD + VEH) as evaluated in the Two-Way ANOVA. Mice from the CAF + STZ group showed the loss of density of PGP-9.5 immunoreactive axons innervating the *epidermis* from the hindpaw skin as the arithmetical sum of the effects elicited by CAF and STZ non-implying potentiation, however, the effect observed was not negligent from a biological point of view, as it concerns a nerve terminal (**Figure 5**).

DISCUSSION

The Combination CAF Diet Plus Multiple Low-Doses of STZ Induced T2DM in Mice

T2DM is the most common type of diabetes and is often associated with obesity. This disease is increasing in pandemic proportions largely driven by a sedentary living and high-energy dietary intakes (Kolb and Martin, 2017; IDF, 2019). Thus, there is an urgent need to develop effective mechanism-based therapies to treat this disease and/or its complications. Preclinical models of T2DM play a pivotal role for the study of its pathophysiology and complications, as well as the rational screening of effective anti-diabetic drugs (Sullivan et al., 2008; Islam and Wilson, 2012; King, 2012; Kleinert et al., 2018). However, translation of findings from these models to human is often challenging due to most likely that many of the current preclinical models of T2DM do not appear to reproduce the main clinical features of patients and/or these models are relatively expensive and not easily available for a large number of researchers (King, 2012; Kleinert et al., 2018). This study reports, for the first time, the characterization of a translational non-genetic mouse model of painful DPN, in which T2DM was induced by a CAF diet followed by low-doses of STZ. The CAF diet was chosen because it closely mimics the unhealthy diet common to humans, which in turn contributes to weight gain and obesity, two frequent features of T2DM (Sclafani and Springer, 1976; Sampey et al., 2011). However, high-fat diets, such as the CAF diet, alone does not lead to diabetes in C57BL/6J mice (Kleinert et al., 2018). Thus, β-cell impairment was induced by the injection of four low-doses of STZ. This model in female C57BL/6J mice resulted in changes in body composition, evidenced by significant weight gain, and an increase in BMI and the amount of visceral adipose tissue, long-term hyperglycemia, hyperinsulinemia, resistance to insulin, and impaired glucose tolerance. Additionally, CAF + STZ mice slowly developed mechanical pain hypersensitivity and showed a significant reduction of epidermal PGP -9.5^{+} nerve fibers of the hindpaw skin. Altogether, this reverse translational study suggests that this model of painful DPN is easily accessible, fairly economical, and with a high success rate that closely parallels the common course of the human disease where obesity and β-cell dysfunction contribute to the development of T2DM.

Our results showed that CAF-diet fed mice exhibited rapid and significant weight gain. This is consistent with the results of several studies showing that an increase in dietary fat and carbohydrate content has been shown to produce obesity in various strains of mice and rats (Collins et al., 2004; Sampey et al., 2011). However, paralleling the human T2DM where weight loss can occur in patients with longstanding disease (Yang et al., 2016), CAF-diet fed mice who received low doses of STZ also experienced eventual weight loss.

Hyperglycemia is one of the main features of diabetes and determination of blood glucose level is widely used as a marker for diagnosis and disease progression (Stolar, 2010; ADA, 2019). In this study, mice with the CAF diet + STZ showed non-fasting blood glucose levels above 200 mg/dL from week 16 until the end of the study. These mice also displayed a glucose-intolerant phenotype at 4, and 24 weeks after STZ injections the combination potentiated the effects in term of body weight, blood glucose, and insulin resistance as is evident by blood glucose levels above 200 mg/dL even at 120 min after a glucose load during an OGTT. Our results also showed that CAF + STZ mice developed insulin resistance only at 24 weeks post-STZ injections, as revealed by the potentiated interaction (CAF ×

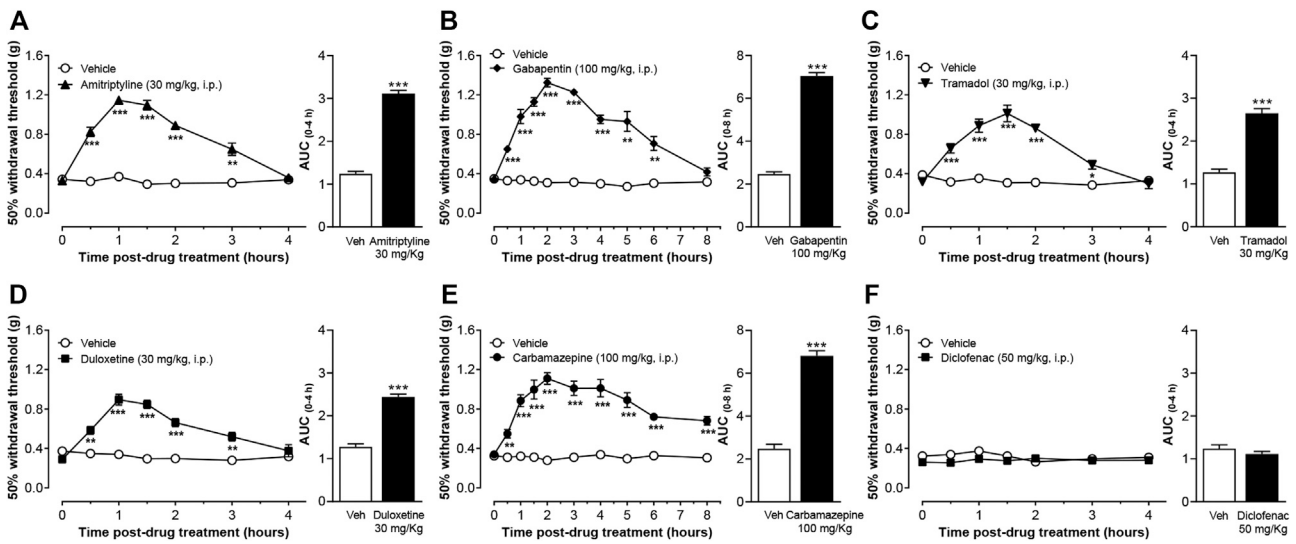

FIGURE 4 | Effect of the most common drugs used in the treatment of neuropathic pain, and diclofenac on mechanical skin hypersensitivity on the left hindpaw of CAF + STZ mice. Values are means ± SEM (n = 8). $^{**}p < 0.01$, $^{***}p < 0.001$; significantly different from the vehicle group by Repeated Measures Two-Way ANOVA, followed by Bonferroni's test. Attached the AUC of the effect is also shown, $^{***}p < 0.001$, *t*-student test.

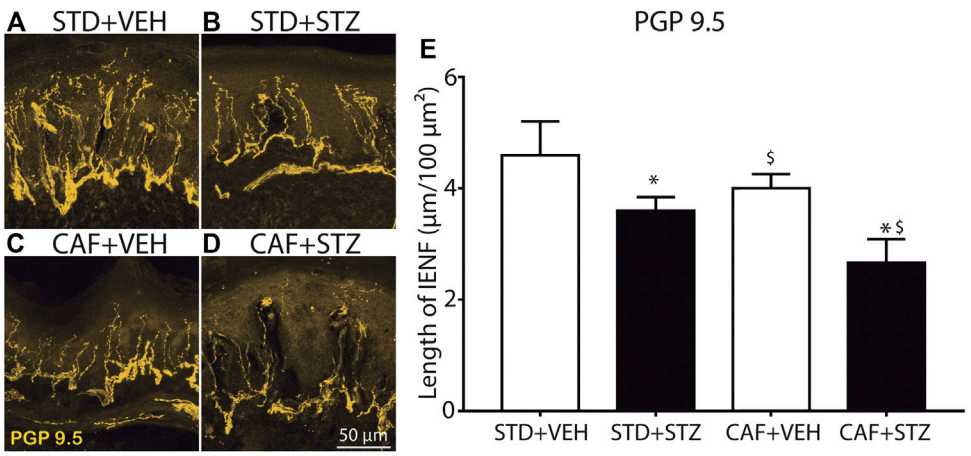

FIGURE 5 | Loss of epidermal nerve fibers in footpads of female mice with diabetes induced by cafeteria diet and low doses of STZ. Representative confocal images of intraepidermal PGP-9.5 immunopositive nerve fibers from the glabrous skin of the hindpaw, obtained from STD + VEH mice **(A)** STD + STZ mice **(B)** CAF + VEH **(C)**, and CAF + STZ **(D)**. **(E)** Quantification of the IENF in each experimental condition. Values are means ± SEM (n = 6). *$p < 0.05$ for CAF [$F_{(1,18)} = 5.45$], $ ($p < 0.05$) for STZ [$F_{(1,18)} = 9.14$], the interaction CAF × STZ did not reach statistical significance, * and $ on the CAF × STZ column represent the separate effect of each factor on the PGP-9.5 nerve length. Two-Way ANOVA.

STZ) in the statistical analysis. In contrast, mice with CAF + VEH showed glucose levels closer to 150 mg/dL, a modest impairment in glucose tolerance, and slight insulin resistance and hyperinsulinemia, features of a prediabetic state. These results agree with human data showing that not all obese patients develop diabetes (Ogden et al., 2015; Malone and Hansen, 2019). Altogether these results showed that the consumption of a cafeteria diet for long periods in conjunction with dysfunction of β-pancreatic cells leads to the development of T2DM. To our knowledge, we report for the first time the validation and characterization of T2DM using CAF diet along with low-doses of STZ in mice. However, we should recognize that previous studies performed in rodents have demonstrated that the combination of low-doses of STZ with a high-fat diet can recapitulate certain features of diet-induced T2DM seen in humans (Srinivasan et al., 2005; Zhang et al., 2008; Nath et al., 2017). However, it has been shown that the CAF diet is a more robust model of human metabolic syndrome compared to high-fat diet both in rats and

mice (Sampey et al., 2011; Higa et al., 2014). Finally, another advantage of using the CAF diet is that this diet more accurately reflects the variety of inexpensive highly palatable, energy-dense foods that is easily available in Western society and associated with the current obesity epidemic (West and Kalbfleisch, 1971; Sclafani and Springer, 1976; Sampey et al., 2011).

Diabetic Mice Slowly Developed Mechanical Allodynia

Nociceptors of the dorsal root ganglia and dorsal horn can become hyperexcitable in response to pathological conditions such as diabetes, which in turn may lead to the development of painful DPN (Todorovic, 2016). DPN is one of the most common and disabling complications with 30–50% of T2DM patients developing PDN (Candrilli et al., 2007; Singh et al., 2014; Juster-Switlyk and Smith, 2016). It commonly presents with progressive distal dysesthesias, pain, and/or sensory loss. Histologically, DPN is associated with loss of the nerve fiber axons within the skin (Rosenberger et al., 2020). In this study, we found that mice with CAF diet plus STZ treatment, but not mice with CAF diet or STZ treatment given alongside a normal diet, developed long-term mechanical allodynia in both hindpaws, which was detected as early as 12 weeks post-STZ treatment. Our study agrees with a previous report that the CAF diet alone given for 12 weeks does not alter the tactile sensitivity assessed by von-Frey monofilaments in rats (Hossain et al., 2020). However, our results differ from other studies in that mechanical allodynia has been reported in mice after STZ treatment alone (Wright et al., 2004; Christianson et al., 2007; Johnson et al., 2008; McGuire et al., 2009; Urban et al., 2010). While the reasons behind these contrasting results are unknown, it possible to suggest that the dose of STZ used by others (180–210 mg/kg, which usually induced T1DM, vs. four consecutive injections of 30 mg/kg in our study) and method of behavioral evaluation partially explain these behavioral differences. It is worthy to mention that this study reports for the first time a longitudinal assessment of neuropathic pain-like behaviors in mice with CAF diet plus STZ treatment. This longitudinal approach revealed that the mechanical allodynia was not evident at early time points of the disease stages. Our result is consistent with previous studies in which the development of mechanical allodynia was evident only at late stages in rats with T2DM induced by high-fat/high-fructose diet and low-doses of STZ (Barriere et al., 2018) and/or with humans studies showing damage to peripheral nerves at late stages of the disease (Said, 2007).

Mechanical Allodynia Induced by T2DM is Decreased by the Conventional Drugs Used to Treat Neuropathic Pain in Humans.

One goal of this study was to validate a mouse model of painful DPN that not only would closely mirrors the natural history of the human disease but also a reliable model that allows a rational screening of new analgesic drugs to treat painful DPN. In the present study, five different analgesics significantly decreased mechanical allodynia induced by T2DM in mice. These findings agree with clinical observations as these drugs have been included in the guidelines by the American Diabetes Association and European Federation of Neurological Societies Task Force to treat painful neuropathy in diabetic patients (Attal et al., 2010; Pop-Busui et al., 2017; Iqbal et al., 2018). Conversely, we found that acute administration of a clinically relevant dose of diclofenac lacked anti-allodynic effect. This result agrees with previous observations where diclofenac has no efficacy in treating painful diabetic neuropathy in humans (Tinoco-Samos et al., 2013) or mechanical allodynia in rats with STZ-induced T1DM (Yamamoto et al., 2009). Finally, this model provides a long window of evaluation time (at least 8 weeks) which might allow the analgesic evaluation of the different drugs given acutely, using the minimum number of experimental animals, as long as there is enough time for a washout period of at least seven drug-half-lives. Moreover, it also offers the possibility of evaluating chronic treatments in order to determine if the effect is maintained for long periods of time or to identify drug-related negative side effects. These results together suggest that this model has translational utility due to it may represent a reliable platform for predicting the clinical efficacy of drugs to treat painful DPN associated with T2DM.

Mice Fed with CAF Diet and Injected with Multiple Low-Doses of STZ Showed a Reduced IENF Density

Intraepidermal nerve fibers (IENF) are directly associated with functional innervation of the skin, and the reduction of IENF density constitutes one of the main histological features in T2DM patients (Ekman et al., 2020). In fact, quantification of epidermal innervation is considered as a reliable and minimally invasive methods of diagnosing and staging diabetic neuropathy (Beiswenger et al., 2008). In the current study, we observed that STZ treatment by itself caused a significant reduction in epidermal PGP -9.5$^+$ nerve fibers in agreement with other studies (Stavniichuk et al., 2014; Rojas et al., 2019). Animals receiving long-term CAF diet also showed a discrete, but significant effect on PGP -9.5$^+$ nerve fibers. The combination of CAF diet with STZ treatment was observed as the arithmetical sum of both factors by themselves on the density of epidermal PGP -9.5$^+$ nerve fibers, but without observing statistical potentiation (in the Two-Way ANOVA), thus suggesting that both factors could be acting by a similar mechanism non-implying potentiation, however, the effect observed was not negligent from a biological point of view, as it concerns a nerve sensitive terminal.

Limitations

The present study has some limitations. Firstly, just female mice were used in this study. However, due that T2DM has a similar prevalence in female and male patients (IDF, 2019), future studies are needed to reproduce these results in male mice. Secondly, we have found that there is a reduction PGP -9.5-immunoreactive intraepidermal of nerve fibers in the hindpaw skin of mice with T2DM which is consistent with the loss of cutaneous innervation in human patients with diabetes (Kennedy et al., 1996; Herrmann et al., 1999; Christianson et al., 2003; Christianson et al., 2007). However, we

should recognize that this parameter was determined only at 6 months after STZ injections. It is possible that the reduction of cutaneous innervation may occurs at earlier time points as it has been reported in models of T1DM (Christianson et al., 2003; Christianson et al., 2007; Johnson et al., 2008). Thirdly, while we used PGP -9.5 as widely pan-neuronal marker, this antibody does not allow to distinguish what subtype of C-fiber nociceptors (peptidergic vs. non-peptidergic) are predominantly affected in the present T2DM model. Given that it has been shown that peptidergic and non-peptidergic nociceptive neurons are differentially damaged under T1DM (Johnson et al., 2008), future studies are needed to identify the biochemical phenotype of nerve fibers affected in this model of T2DM. Fourthly, we have characterized the time-course of the development of mechanical allodynia in this T2DM as a neuropathy sign. While this behavioral endpoint is widely used as a surrogate of pain in humans with diabetic peripheral neuropathy (Perkins et al., 2001), further studies are needed to evaluate other pain-like behaviors that would allow determining the presence of ongoing pain or sensory deficits in this model.

In conclusion, we have shown that the CAF diet along with low-doses of STZ in mice may be effectively used to generate an easily accessible, fairly economical, and reliable model of peripheral neuropathy associated with T2DM after inducement of obesity and β-cell impairment in female C57BL/6J mice. This model mimics some of the metabolic features, mechanical allodynia, and a reduction of cutaneous innervation reported in humans with T2DM. Thus, this preclinical mouse model could be a useful and translational tool in evaluating the efficacy of new drugs to treat painful diabetic neuropathy and improve translation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of the Facultad de Medicina, Universidad Autónoma del Estado de Morelos.

AUTHOR CONTRIBUTIONS

GC, JA, VP, RA, and JJ contributed to the conception and design of the study. NV, JT, LG, PN, AM, JJ, and GC conducted experiments. SM performed and interpreted the statistical analysis. SM, RA, VP, and JA wrote sections of the manuscript. GC and JJ wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

FUNDING

This work was partially supported by the Universidad Autónoma de Tamaulipas (30UATINVEST20) for RIAG.

ACKNOWLEDGMENTS

This work is part of the master thesis dissertation of NV (CONACyT fellowship No. 961279)

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.628438/full#supplementary-material.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

REVIEW
published: 08 February 2021
doi: 10.3389/fphar.2020.614990

Preclinical Neuropathic Pain Assessment; the Importance of Translatability and Bidirectional Research

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For patients suffering with chronic neuropathic pain the need for suitable novel therapies is imperative. Over recent years a contributing factor for the lack of development of new analgesics for neuropathic pain has been the mismatch of primary neuropathic pain assessment endpoints in preclinical vs. clinical trials. Despite continuous forward translation failures across diverse mechanisms, reflexive quantitative sensory testing remains the primary assessment endpoint for neuropathic pain and analgesia in animals. Restricting preclinical evaluation of pain and analgesia to exclusively reflexive outcomes is over simplified and can be argued not clinically relevant due to the continued lack of forward translation and failures in the clinic. The key to developing new analgesic treatments for neuropathic pain therefore lies in the development of clinically relevant endpoints that can translate preclinical animal results to human clinical trials. In this review we discuss this mismatch of primary neuropathic pain assessment endpoints, together with clinical and preclinical evidence that supports how bidirectional research is helping to validate new clinically relevant neuropathic pain assessment endpoints. Ethological behavioral endpoints such as burrowing and facial grimacing and objective measures such as electroencephalography provide improved translatability potential together with currently used quantitative sensory testing endpoints. By tailoring objective and subjective measures of neuropathic pain the translatability of new medicines for patients suffering with neuropathic pain will hopefully be improved.

Keywords: neuropathic pain, electroencephalography, translatability, preclinical, clinical, burrowing, endpoints, quantitative sensory testing

OPEN ACCESS

Edited by:
Maree Therese Smith,
The University of Queensland, Australia

Reviewed by:
Irina Vetter,
The University of Queensland, Australia
Peter Michael Grace,
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Specialty section:
This article was submitted to Translational Pharmacology, a section of the journal Frontiers in Pharmacology

Received: *07 October 2020*
Accepted: *10 December 2020*
Published: *08 February 2021*

Citation:
Fisher AS, Lanigan MT, Upton N and Lione LA (2021) Preclinical Neuropathic Pain Assessment; the Importance of Translatability and Bidirectional Research. Front. Pharmacol. 11:614990. doi: 10.3389/fphar.2020.614990

INTRODUCTION

Neuropathic pain arises from lesions or diseases affecting the somatosensory component of the nervous system at any level of the peripheral or central nervous system (Jensen et al., 2011). Neuropathic pain is a distinct clinical description based on common neurologic signs and symptoms despite a large variety of etiologies (Baron et al., 2010). Sleep disturbances, anxiety and depression are frequent and severe in patients with neuropathic pain, whilst quality of life (QoL) is more impaired in patients with chronic neuropathic pain than in those with chronic non-neuropathic pain that does not come from damaged or irritated nerves (Colloca et al., 2017). Reducing QoL poses a huge economic burden to the health system and society (Feldman et al., 2017).

TABLE 1 | Summary of clinical and preclinical primary efficacy assessment endpoints for translation of current licenced medicines for painful diabetic peripheral polyneuropathy.

Licenced/recommended medicine year of approval in Europe daily dose (mg)	RCT Primary and secondary endpoints	Preclinical neuropathic pain model	Preclinical efficacy MED	Primary stimulus evoked sensory endpoints	Translation
[a]Pregabalin Lyrica®, (2004) 300–600 mg/day	↓ Pain intensity/quality (weekly SF-MPQ VAS score) ↓ sleep interference score Freynhagen et al. (2005), Rosenstock et al. (2005), Richter et al. (2005)	STZ (50 mg kg ip rat)	3 mg kg po 10 mg kg po	↓VFH (static allodynia) ↓VFH (Dynamic allodynia) Field et al. (1999)	[a]Forward
Gabapentin Neurontin®, (2002) 900–3,600 mg/day	↓ Pain intensity (weekly 11-point Likert score) ↓QoL, ↓sleep interference score Backonja (1998), Morello et al. (1999), Simpson et al. (2001)	STZ (50 mg kg ip rat) CCI (rat) SNL L5/6 (rat)	10 mg kg po, 10 μg i.t. 30 mg kg po, 1 μg i.t. 30 mg kg po, 100 mg kg ip 100 mg kg ip	↓VFH (static allodynia) ↓VFH (Dynamic allodynia) ↓VFH (static allodynia) ↓PWL (cold water allodynia) ↓VFH (static allodynia) Hunter et al. (1997), Field et al. (1999), Field et al., (2002)	Back/Forward (Bidirectional)
Amitripyline 25–100 mg/day (recommended)	↓Pain intensity (weekly 11-point Likert score, VAS) ↓sleep interference score/daily activities Max et al. (1992), Biesbrock et al. (1995), Morello et al. (1999)	STZ (50 mg kg ip rat) CCI (rat) SNL L5/6 (rat)	0.5 mg kg po 1.5 mg kg sc 10 mg kg ip, 60 μg i.t., 100 nmol ipl	↓VFH (static allodynia) No effect (Dynamic allodynia) ↓tonic pain score ↓PWL (Thermal hyperalgesia) No effect (static allodynia) Ardid and Guilbaud (1992), Field et al. (1999), Esser and Sawynok (1999)	Back/Forward (Bidirectional)
Duloxetine Cymbalta® (2005) 60–120 mg/day	↓Pain intensity (weekly 11-point Likert score) ↓QoL No change in dynamic allodynia Wernicke et al. (2006), Goldstein et al. (2005), Raskin et al. (2006)	STZ (50 mg kg ip rat) STZ (200 mg kg ip mouse) SNL L5/6 (rat)	20 mg kg po 20 μg i.t. 20 mg kg po 20 mg kg po	↓VFH (static allodynia), ND (Dynamic.allodynia) ↓PWL (Thermal hyperalgesia) ↓VFH (static allodynia) Iyengar et al. (2004), Mixcoatl-Zecuatl and Jolivalt (2011), Kuhad (2009)	Back/Forward (Bidirectional)

[a]Recommended first-line treatment, RCT (randomised clinical trial), MED (minimum effective dose), STZ (streptozocin), CCI (chronic constriction injury), SNL (spinal nerve ligation), VFH (Von Frey hair), PWL (paw withdrawal latency), ND (not determined), i.t. (intrathecal), po (oral), ip (intraperitoneal), visual analogue scale (VAS), ipl (intraplantar) of the Short-Form McGill Pain Questionnaire (SF-MPQ), QoL (measures of quality of life (Short Form–36 Quality of Life Questionnaire and Profile of Mood States)), Equivalent therapeutic human doses are not determined in preclinical studies as drug plasma concentrations are not reported.

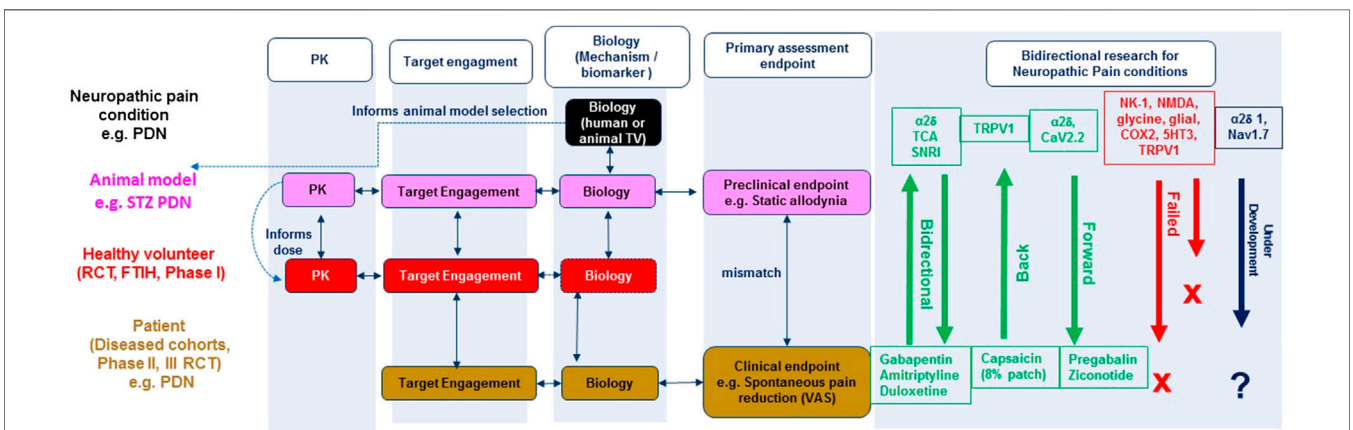

FIGURE 1 | Integrated bidirectional research approach for neuropathic pain: Linking animal and human biology data. RCT (randomised clinical trial), FTIH (First time in human), TV (target validation), STZ (Streptozocin), PDN (painful diabetic neuropathy), PK (Pharmacokinetic), VAS (Visual analogue scale), TCA (tricyclic antidepressant), SNRI (serotoninnorepinephrine reuptake inhibitor). Bidirectional research illustrates the targets/mechanisms that have successfully translated forward (from preclinical research), back (from clinical research), under development (e.g. α2δ1, blue) and failed (e.g., Neurokinin 1 (NK-1), red) novel targets/mechanisms identified from preclinical research.

Neuropathic pain is mechanistically heterogenous encompassing degrees of neurogenic sensitization, deafferentation and/or neurogenic inflammation. Signs and the symptoms of neuropathic pain include allodynia, hyperalgesia and paresthesia. One mechanism can underlie many different symptoms, the same symptom in two patients may be caused by different mechanisms, more than one mechanism can operate in a single patient, and these mechanisms may change with time (Woolf and Mannion, 1999). Many mechanisms are also still to be elucidated. Notably, without biomarkers that predict neuropathic pain or identification of the underlying mechanism(s), the optimum treatment strategy for the patient's neuropathic pain cannot easily be selected or identified preclinically for successful translation.

Severity of neuropathic pain is a primary predictor of the negative health impact on patients (Doth et al., 2010). Hence the goals of therapy include improvement in pain control, coping skills and restoration of functional status. Clinically meaningful chronic neuropathic pain relief is measured in randomised clinical trials (RCTs) as a significant reduction in reported pain intensity numerical rating score (NRS) encompassing spontaneous and stimulus evoked pain (**Table 1**) (Finnerup et al., 2015). Pharmacological treatment represents the main option for managing chronic neuropathic pain with moderate efficacy based upon number needed to treat (Colloca et al., 2017). The anticonvulsant drug, pregabalin (Lyrica®) is the most extensively studied drug by far, with clinical studies evaluating almost 12,000 participants across eight different neuropathic pain conditions (Derry et al., 2019). Pregabalin at daily oral doses of 300–600 mg can provide at least 50% pain intensity reduction in around 3 to 4 out of 10 people compared with 1–2 out of 10 for placebo in postherpetic neuralgia and painful diabetic neuropathy patients. Given that half of those treated with pregabalin will not achieve worthwhile pain relief indicates there is significant scope for improvement (Derry et al., 2019).

Current systemic and topical pharmacological treatments of neuropathic pain have substantial limitations in terms of the level of efficacy provided and/or the side effect profile. This means the management of neuropathic pain is unsatisfactory in both preventing its development and in halting or modifying its progression (Finnerup et al., 2015). A key to developing much needed new treatments to better manage neuropathic pain is to understand the pharmacology of novel molecules to aid their translation from preclinical species to efficacy and safety in patients. Given that there has been no translation of a new medicine for neuropathic pain since Qutenza (capsaicin 8% patch) in 2009 (Baranidharan et al., 2013) one consideration is to look more closely at the way in which neuropathic pain is modeled and measured preclinically compared to human studies (Percie du Sert and Rice, 2014; Mogil, 2019a).

The primary focus of this review is to examine the industry standard measures of neuropathic pain and analgesia in animal models and patients in combination with how bidirectional research is implementing ways to measure spontaneous ongoing chronic pain and associated QoL in animals to further improve forward translation. As will be discussed, currently industry standard markers of neuropathic pain in humans and animals are distinct and subjective. In humans this is not a problem as pain is an individual experience and subjective markers such as questionnaires accurately measure a human's pain level, although can lead to significant variability. However industry standard stimulus evoked pain markers in animals, such as static allodynia (von Frey) are often a poor representation of the animal's spontaneous neuropathic pain and rely on human interpretation. Translatability between these distinct and subjective industry standard markers is poor as shown by the lack of current translational success. An improvement would be to compliment simple stimulus evoked markers with an objective marker in animals such as burrowing which removes human subjectivity, but cannot be directly translated to a marker in humans. An ideal marker for neuropathic pain (and chronic pain per se) is likely one that is objective in both humans and animals and can be directly translated between the two, such as data from Electroencephalography (EEG) recordings, which can be conducted in both species with no human subjectivity involved.

BI-DIRECTIONAL TRANSLATION OF CURRENT TREATMENTS FOR NEUROPATHIC PAIN

Most neuropathic pain conditions are clinically managed with a choice of four licenced or recommended medicines, amitriptyline, duloxetine, gabapentin, or pregabalin whilst carbamazepine is specifically licenced for trigeminal neuralgia (National Institute for Health and Care Excellence, 2013). These medicines (except pregabalin) were originally licenced for other therapeutic indications (gabapentin for epilepsy, duloxetine and amitriptyline for depression) and successfully back translated (this term is used interchangeably with reverse translated) from anecdotal clinical use in RCTs in neuropathic pain patients followed by preclinical efficacy in animal models.

Painful peripheral diabetic neuropathy is a widely studied condition in RCTs to assess neuropathic pain therapies as it is a leading cause of chronic peripheral neuropathic pain affecting between 25 and 50% of patients (Abbott et al., 2011; van Hecke et al., 2014). To date, there are 39 phase 2/3 RCTs in the United States/United Kingdom in patients with painful peripheral diabetic neuropathy filed on clinicaltrials.org which represents 24% of the total number of RCTs for investigating peripheral neuropathic pain. Pre-diabetes and diabetes now affect 316 million and 387 million people worldwide, respectively, and it is estimated that at least 60–70% will develop associated neuropathy complications, with prevalence increasing with duration of diabetes (Feldman et al., 2017). Furthermore, there is a positive correlation between diabetic neuropathy severity, poor glycaemic control with risk and intensity of neuropathic pain (Themistocleous et al., 2016).

We have summarized in **Table 1** pivotal clinical (RCTs) and preclinical studies supporting the approval of these four licenced medicines for painful diabetic peripheral neuropathy, highlighting successful bidirectional translation of duloxetine, amitriptyline and gabapentin and forward translation of pregabalin. Duloxetine is a selective norepinephrine and serotonin reuptake inhibitor that in 2004 was the first medicine approved for painful diabetic neuropathy in the United States. This was based upon reducing spontaneous

pain intensity at 60 mg once or twice per day and reversal of nociception and static mechanical allodynia in rodent face validity models of peripheral neuropathic pain. A few RCTs reported significant improvement in spontaneous neuropathic pain with amitriptyline treatment supported by the reversal of thermal hyperalgesia in a rodent spinal nerve ligation and subsequent back translation reversing static mechanical allodynia in a rodent STZ type-1 diabetes model. Amitriptyline has since been the most prescribed of the tricyclic agents for diabetic neuropathic pain for the past two decades. The gabapentinoid medicines, gabapentin and pregabalin are calcium channel α2δ-1 and α2δ-2 subunit ligands that were first approved in Europe in 2002 and 2004, respectively. Preclinical efficacy of gabapentin in rodent models of mono-neuropathic pain supported the subsequent clinical observations (**Table 1**). Following this, preclinical studies demonstrated a superior preclinical profile reversing static and dynamic (light moving stimuli) mechanical allodynia in a rodent STZ type-1 diabetes model, compared with amitriptyline (**Table 1**). Furthermore, intrathecal administration of gabapentin was 10-fold more potent against dynamic vs. static mechanical allodynia. Given that dynamic allodynia is the most troublesome evoked sign in subgroups of neuropathic pain patients it was considered to be an important and differentiating stimulus evoked sensory endpoint in preclinical poly-neuropathic pain models. However, the fact that gabapentin and amitriptyline have equivalent clinical efficacy in reducing diabetic neuropathic pain (Morello et al., 1999) indicates this superior gabapentin preclinical efficacy does not translate in the clinic. Another aspect where back translation is not straightforward is the delayed-onset analgesia observed clinically, for example a titration phase of 1–2 weeks is required with gabapentin, while acute gabapentin analgesia is typically observed in rodents (Backonja, 1998; Field et al., 1999). Whiteside's group compared drug exposure in humans with exposure in a rat spinal nerve ligation model demonstrating this mono-neuropathic model, despite lacking face validity (patients with mono-neuropathies represent only 9% of trials (Finnerup et al., 2005)), back translates and predicts efficacious exposure in humans for gabapentin and duloxetine (Whiteside et al., 2008). The proven forward and back translation of these clinically effective drugs demonstrated confidence in the validity of induced animal models of neuropathic pain, similarity between rat and human pain biology and relevance of stimulus evoked sensory measures providing clinically relevant data for diverse mechanisms.

The gold standard treatment pregabalin, is sometimes portrayed as an exemplar forward translational success story for the use of animal models in the neuropathic pain field. As seen in **Table 1**, rodent STZ type-1 diabetic models were employed in the preclinical development of pregabalin, providing key decision-making allodynia efficacy data for subsequent clinical trials. Pregabalin requires lower doses preclinically and clinically, in contrast to gabapentin (**Table 1**), as it has a linear, dose proportional absorption in the therapeutic dose range (Freeman et al., 2008). Of note, both drugs require no titration (single dose) preclinically. However, an area of potential bias is that the findings from these preclinical studies only started to appear (Field et al., 1999) at approximately the same time as the pivotal clinical trials of gabapentin (Backonja, 1998; Morello et al., 1999). Irrespective of whether pregabalin is viewed as an exemplar of forward and/or back translation, it is undoubtedly a success story that reinforced the clinical precedence of a novel target α2δ, much needed by the neuropathic pain field. What makes this story even more intriguing is the discovery of the novel mechanism (α2δ-1 and α2δ-2) only one year earlier (Gee et al., 1996) and the subsequent discovery that the analgesic efficacy of pregabalin and gabapentin is mediated by the α2δ-1 sub-unit of voltage gated calcium channels (Field et al., 2006). Mirogabalin, is a potent and selective α2δ-1 ligand with a wider safety margin and superior long lasting efficacy reversing static mechanical allodynia in a rat STZ type-1 diabetes model, compared with pregabalin (Domon et al., 2018). Mirogabalin has also shown promising results on reducing daily pain scores and sleep interference in RCTs for the treatment of diabetic peripheral neuropathic pain (Vinik et al., 2014; Merante et al., 2017; Baba et al., 2019) indicating that a more selective approach may well offer patients a safer and more efficacious option in the future.

The chemical ingredient in chilli pepper, capsaicin, has been available since the 1980s in various formulations as lotions, creams or patches in low concentrations of 0.025–0.075% over the counter to treat neuropathic pain, such as diabetic neuropathy. Clinical efficacy therefore was recognized long before identification of its molecular target in 1997 (Caterina et al., 1997). Capsaicin selectively and potently activates the transient receptor potential cation channel subfamily V member 1 (TRPV1) ligand gated channels on nociceptive fibers leading to TRPV1 desensitization (Caterina et al., 1997). Derry and Moore (2012) concluded that low-concentration topical capsaicin had no clinical efficacy beyond that of placebo but a single application of a prescription strength high concentration capsaicin patch (Qutenza, 8%) is clinically effective in postherpetic neuralgia and diabetic painful neuropathies (Burness and McCormack, 2016; Vinik et al., 2016) with additional QoL improvements (Derry et al., 2017). However, compliance can be low due to the erythema and burning sensation experienced on topical application.

Forward Translation (Bench to Bedside) of Neuropathic Pain (Figure 1)

For the past two decades rational drug discovery efforts have been mechanistically driven addressing targets arising from a better understanding of the mechanism of existing analgesic drugs e.g. TRPV1 (capsaicin patch), α2δ-1 (gabapentinoids) or novel mechanisms arising from biological, human pathophysiological, or genomic studies e.g. Nav1.7, Neurokinin 1 (NK-1) (**Figure 1**). Despite common neuropathic pain symptoms patients are generally recruited for trials on disease stratification (Finnerup et al., 2015). Similarly, disease or mechanism based animal models that more closely recapitulate the human neuropathic pain clinical condition (face, construct and predictive) are preferred for bidirectional translation. For example, in the last two decades diabetes research has focused on glucose and the STZ type-1

Preclinical endpoints	Human RCT endpoints
***Stimulus evoked sensory endpoints** (thermal, mechanical, chemical withdrawal e.g. von Frey filaments)	Reflex Quantitative Sensory Threshold (QST) (thermal, mechanical, chemical withdrawal e.g. von Frey filaments)
X	***Reported pain intensity and Daily living (QoL)** (e.g. VAS numerical rating pain)
X	**Affective / Cognitive state** depression, anxiety, motivation, sleep anticipation of pain (preference/avoidance)
X	**Objective markers** EEG

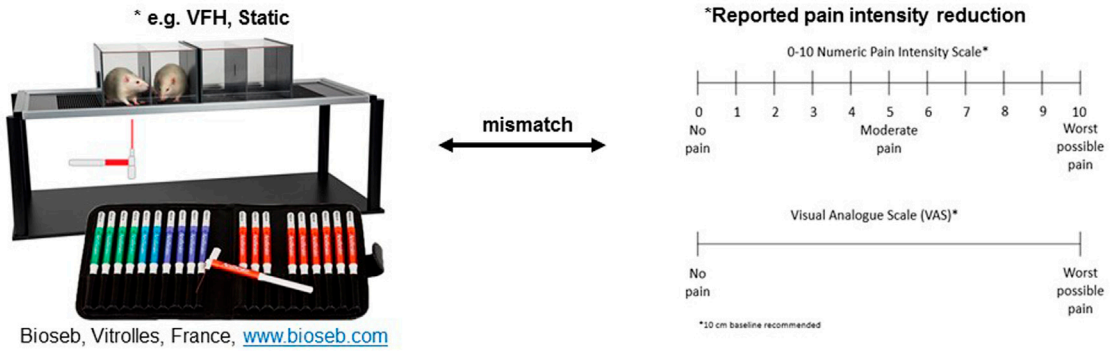

FIGURE 2 | Mismatch of primary neuropathic pain assessment endpoints in preclinical vs. clinical trials. *Primary endpoint, RCT (randomised clinical trial), EEG (electroencephalography), QoL (quality of life), VFH (von Frey hair), Static (static mechanical allodynia).

diabetic rat as the preclinical model to understand diabetes disease pathogenesis including painful peripheral polyneuropathy (Lenzen et al., 2008; **Table 1**; **Figure 1**), despite evidence that different mechanisms underly type-1 and type-2 forms (Callaghan et al., 2012).

Figure 1 illustrates the integrated bidirectional research approach of animal and human biology for rational target/mechanism identification, drug discovery and development for neuropathic pain conditions. Knowledge related to the anatomy, physiology, pharmacology, molecular biology, and genetics of pain conditions in experimental animals (typically rodents) or humans (e.g. erythromelalgia) informs pain model selection for target validation, engagement and evaluation of efficacy using clinically relevant endpoints. Bidirectional research in **Figure 1** illustrates the target mechanisms that have successfully translated both forward (from preclinical research) and back (from clinical research).

There have been few forward translation successes (see **Figure 1**). Ziconotide (Prialt, licenced in 2005) is the synthetic form of an ω-conotoxin peptide derived from *Conus magus*, a cone snail SNX-111 that blocks the N-type (CaV2.2) neuronal voltage gated calcium channel. Ziconotide produced striking analgesia in reflexive pain animal models and RCTs for cancer and AIDS related neuropathic pain when administered intrathecally (Malmberg and Yaksh, 1995; Staats et al., 2004). Despite clinical precedence for this target there has unfortunately been limited progress in the development of selective small molecule orally bioavailable N-type calcium channel blockers (Jurkovicova-Tarabova and Lacinova, 2019).

There is potential for translation of novel neuropathic pain mechanisms from human biology target validation (**Figure 2**, e.g., Nav1.7) although the number of mechanisms validated in this way is likely to be extremely limited. The sodium channel (Nav1.7) is a neuropathic pain molecular mechanism with conceivable success because of the rare mutations in the Nav1.7 channel identified in patients with inherited erythromelalgia (Nassar et al., 2004; Cox et al., 2006; Minett et al., 2012; Geha et al., 2016), a subset of idiopathic small fiber neuropathy patients and is supported by target validation in genetic animal models of neuropathic pain (Grubinska et al., 2019). A state dependent non-selective Nav inhibitor, Raxatrigine (a.k.a. CNV1014802, BIB074, Deuis et al., 2016) has forward translated in a phase II clinical trial of patients with painful lumbosacral radiculopathy demonstrating that it is well tolerated and produces a remarkable reduction in pain compared to placebo (Versavel, 2015). Furthermore Nav1.7 gain of function mutations give rise to a diabetes induced increased sensitivity of dorsal root ganglion neurons (Hoeijmakers et al., 2014), more severe burning pain and greater sensitivity to pressure stimuli during QST (Blesneac et al., 2018). Hence, targeting this channel may offer hope for

reducing pain severity in these different patient groups. However, since Biogen, Xenon and Pfizer have all discontinued research on this target after failure in phase II clinical trials it would be wise to cautiously view Nav1.7 as a panacea to pain (McDonnell et al., 2018).

Despite rational drug discovery efforts identifying numerous novel mechanisms that are efficacious and tolerable in industry standard preclinical models of neuropathic pain, these have all subsequently failed in the clinic (**Figure 1**, NK-1 antagonists, NMDA antagonists, Glycine antagonists, Glial-modulators, COX-2 inhibitor, 5HT3 antagonist, TRPV1 antagonist, NMDA antagonist, cannabinoid agonist, for recent review see (Yezierski and Hansson, 2018)). Many of these target mechanisms failed in the clinic due to dose limiting toxicity (e.g., cannabinoid agonist, NMDA antagonist, TRPV1 antagonist) and hence should not necessarily be permanently abandoned, for example TRPV1. The clinical development of the highly selective TRPV1 antagonist, AMG-517 was halted by hyperthermic responses in healthy volunteers, believed to be modulated by a peripheral on target mechanism of action (Gavva, 2009). However, recent reports (DelloStritto et al., 2016) indicate that TRPV1 function is rapidly downregulated peripherally in diabetes and our findings have shown the hyperthermic side effects of the TRPV1 antagonist, ABT-102 are absent at an analgesic dose in a rat STZ type-1 diabetes neuropathic pain model (Pritchard et al., 2016). The next step will be to demonstrate this concept in a type-2 animal model of diabetes, opening the potential for the safe use of a TRPV1 antagonist to treat neuropathic pain in the wider diabetic population. Given the lack of forward translation of numerous targets (Pop-Busui et al., 2017) and the established clinical effectiveness of capsaicin patch for painful diabetic neuropathy (back translation, **Figure 1**), this cannot come soon enough.

In contrast other novel mechanisms, such as substance P (NK-1) antagonists have failed in the clinic despite convincing evidence of analgesic efficacy in animal models. NK-1 receptor antagonists, such as the antiemetic aprepitant at clinically safe and tolerable doses, occupying and engaging >90% of its central target are inefficacious in relieving ongoing pain in postherpetic neuralgia patients despite convincing analgesic efficacy in animals (Rice and Hill, 2006). The lack of forward translation from animal to patients may be explained by species differences in 1) the pathophysiology of substance P and 2) pain measurements of clinical spontaneous pain reduction vs. stimulus evoked sensory endpoints studied preclinically. Differences in the distribution and expression of NK-1 receptors between species is observed at supraspinal sites (pain perception and conscious sensation) and not the dorsal horn of the spinal cord (nociception) (Hill, 2000). An upregulation of supraspinal NK-1 receptors in certain disease states e.g. neuropathic pain, may imply that an even higher receptor occupancy (>90%) is needed for clinical efficacy compared with emesis. Further, clinical spontaneous pain is a conscious sensation that relies on supraspinal cortical processing (Borsook and Becerra, 2006) whilst preclinical stimulus evoked sensory withdrawal reflects a spinal cord reflex activation (Lascelles and Flecknell, 2010). This may imply that the site of pain perception from preclinical to clinical has been inadequately assessed for this mechanism. It is essential to consider whether this mismatch between preclinical and clinical primary pain assessment endpoints is a key contributor to the high number of false positives and lack of forward translation across mechanisms (**Figure 2**) (see next section). This points to a need to improve bidirectional research i.e. "bedside to bench" to further the clinical relevance of pain assessment in animals and improve "bench to bedside" forward translation.

INDUSTRY STANDARD MEASUREMENTS OF NEUROPATHIC PAIN FOR THE DEVELOPMENT OF ANALGESIC TREATMENTS (FIGURE 2)

Randomised Clinical Trials (RCTs)

Patients experiencing chronic neuropathic pain are frequently troubled by more than just their pain; comorbid conditions commonly accompany or are caused by the pain. Most notable are sleep disruption and depression/anxiety therefore, diagnosis relies on the patient's subjective rating of the unpleasant emotional *and* physical sensations (**Figure 2**). The predominant clinical feature of most neuropathic pain conditions is spontaneous pain (either continuous and/or paroxysmal) as opposed to evoked pain, hence RCTs focus on endpoints relating to verbal self-reporting (visual analogue scales (VAS)) of spontaneous pain intensity reduction (**Figure 2**; **Table 1**). Visual analogue or numerical pain scales used in human subjects are a self-reporting system (Edwards and Fillingim, 2007) that is individual, typically influenced by comorbidities and cannot be objectively verified (Farrar, 2010). Sensory gain (mechanical/thermal hyperalgesia and allodynia) is much less frequently reported in neuropathic pain conditions; ranging from 24–33% and is rarer than loss of function symptoms like numbness (42%) (Baron et al., 2017). Hence, reflexive endpoints are infrequently measured in RCTs (Baron et al., 2017), although several recent studies have profiled patients based on individual sensory characteristics (Rice et al., 2018). Self-reporting by its nature poses an inherent subjectivity. Therefore, within the clinical setting there is also a clear need to improve trial outcome measures. Patient stratification, based on sensory profiling and subgrouping, has been one such proposal (Baron et al., 2017) which seems reasonable based on the appearance of sensory gain vs. loss of function symptoms in neuropathic pain conditions. Since individual profiling of patients is labor intensive the ideal scenario would be an objective biomarker of neuropathic pain rather than a subjective reflexive endpoint.

Industry Standard Stimulus Evoked Sensory Endpoints Primary Pain Endpoints in Preclinical Research

The industry standard primary measure of pain preclinically is standardized evoking (heat, cold, or mechanical) stimuli delivered by an experimenter to assess loss and gain of

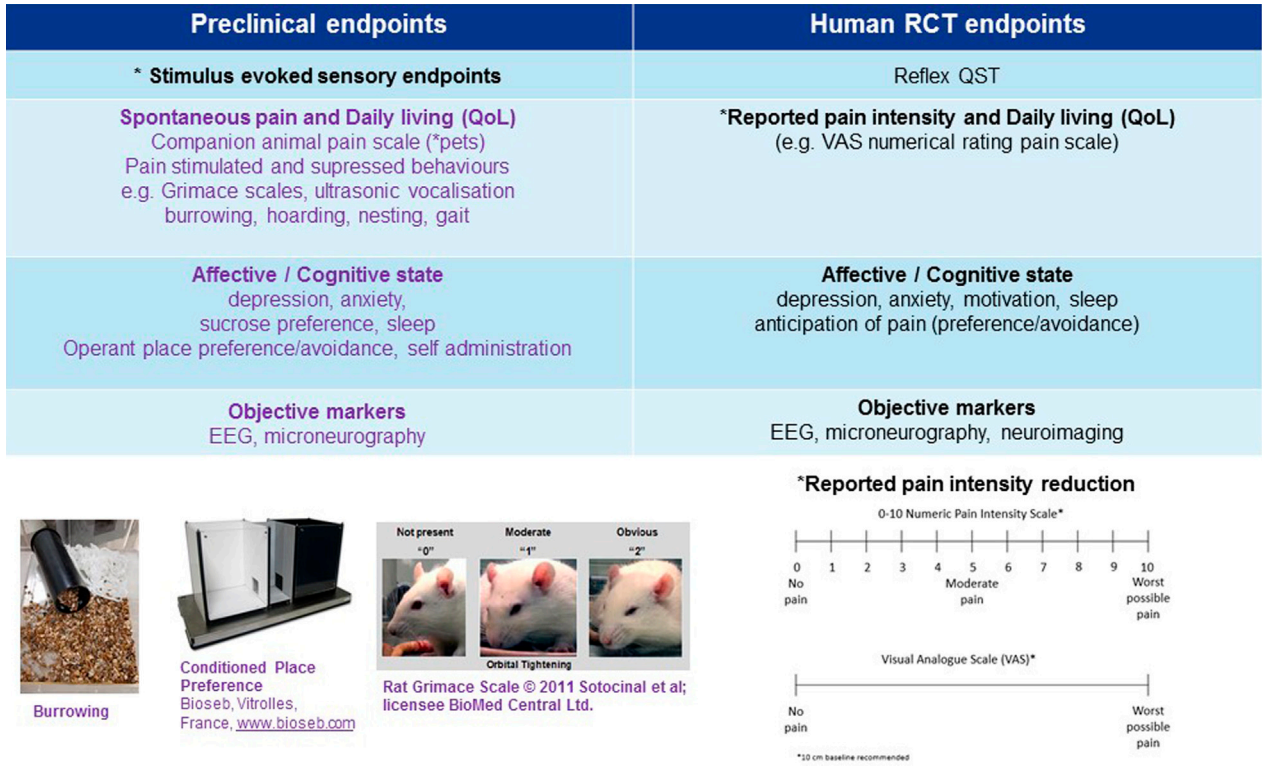

FIGURE 3 | Multiple preclinical neuropathic pain assessment endpoints in development to improve translatability of preclinical to clinical research. *Primary endpoint, RCT (randomised clinical trial), EEG (electroencephalogram), QoL (quality of life).

function of different afferent fiber classes (Aβ, Aδ, and C fibers) (Backonja et al., 2013) (**Figure 2**). Primarily analgesic efficacy is based upon the significant reduction of hyper-sensory phenomena (e.g., allodynia, hyperalgesia) whilst sensory loss (e.g., deafferentation, anesthesia dolorosa) rarely so (Rice et al., 2008). This is opposite to the clinical scenario where hypo-sensory usually dominate over hyper-sensory symptoms (e.g., painful diabetic neuropathy/radiculopathy) (Baron et al., 2017). Moreover, the hyper-sensory "pain" responder rate is greater than 70% in preclinical models e.g. STZ type-1 diabetes model (static mechanical allodynia) (Field et al., 1999; Fisher et al., 2015) in contrast to only 20–30% in patients (Baron et al., 2017). Of note, Field et al. (1999) demonstrated the prevalence of dynamic mechanical allodynia (60%) was more in line with the debilitating clinical complaint and differentiated gabapentinoid efficacy over amitriptyline which may be a more clinically relevant reflexive endpoint, although it is rarely measured preclinically or clinically.

Stimulus evoked sensory endpoints are robust, reproducible, high throughput measures, with face (although not in terms of prevalence) and predictive validity (back translation, **Table 1**). However, chronic neuropathic pain is a system based subjective experience that relies on cortical activation and motivational-affective aspects (Melzack and Casey, 1968; Borsook and Becerra, 2006), which is far more complex than a reflex in response to sensory stimulation (spinal or spinal–brainstem–spinal pathways). Restricting preclinical evaluation of pain and analgesia to exclusively subjective, reflexive outcomes is both oversimplified and can be argued not clinically relevant given the continuous lack of forward translation across diverse mechanisms (**Figure 1**, lack of face and predictive validity). Despite the unsustainable high forward translation attrition rate stimulus evoked sensory endpoints remain the primary decision-making method of neuropathic pain assessment in animals and highlights the necessity to address this clear mismatch of methodological assessment of neuropathic pain in animals and humans.

THE MISMATCH PROBLEM

Preclinical scientists and clinicians have become acutely aware of these methodological mismatch issues and consequently it is becoming more conventional to consider markers in patients that can help design more clinically relevant pain assessment tests in animals (bidirectional research). Since the presence or absence of analgesia in animals can only be inferred from observations made by humans (surrogate behaviors, objective measurements) and cannot be self-reported (Percie du Sert and Rice, 2014) this presents a challenge. Many research groups are now looking to improve the markers used preclinically and clinically with the goal of producing markers that can successfully forward translate preclinical candidates to human clinical trials.

ALTERNATIVE NEUROPATHIC PAIN ASSESSMENT ENDPOINTS TO IMPROVE TRANSLATABILITY BETWEEN ANIMALS AND HUMANS (BIDIRECTIONAL RESEARCH) (FIGURE 3)

Alternative preclinical assessments can be used to infer signs of not just the classical reflex hypersensitivity but also spontaneous pain measurements along with the other comorbidities such as anxiety, depression, sleep issues and cognitive deficits so often found associated with chronic pain patients (**Figure 3**). Here we examine evidence from preclinical and clinical studies focusing on how bidirectional research is addressing current research gaps and helping to develop greater translational, predictive, and more clinically relevant neuropathic pain assessment endpoints. We critique the validity of these alternative behavioral endpoints of spontaneous neuropathic pain and associated comorbidities (QoL, affective and cognitive measures) and how translational techniques, such as EEG, present potential developments in the field for measuring objective signatures of neuropathic pain.

Spontaneous Pain and Daily Living (QoL): Assessment Questionnaires (Patient and Companion Animals) Correlation to Efficacy Doses

Because pain is an internal, private experience, self-report remains the gold standard for its measurement in the clinic (Fillingim et al., 2016) and the development of easy to use questionnaires, based mainly on self-report of symptoms, has improved diagnosis and management. Two types of questionnaires (screening and assessment) have been validated, rapidly translated and revalidated in several languages (Attal et al., 2018). A number of pain scales are implemented in the clinic for the assessment of the different components of neuropathic pain (for reviews see (Cruccu et al., 2010; Hjermstad et al., 2011; Fillingim et al., 2016; Attal et al., 2018)).

An excellent example of cross-species use of assessment tools has been the use of the numerical rating scale (NRS) in the assessment of pregabalin efficacy in dogs. In human clinical trials, pregabalin efficacy in peripheral neuropathy has been evaluated with success using patients' daily pain scores (Jenkins et al., 2012). In the first RCT reporting the efficacy of pregabalin in dogs with neuropathic pain, Sanchis-Mora et al. (2019) used the owners "daily pain assessment" with a NRS as the primary efficacy endpoint and stimulus evoked sensory endpoints as the secondary endpoint. Owners assessed spontaneous vocalisations, phantom scratching episodes and exercise impairment (spontaneous behaviors previously validated using VAS (Plessas et al., 2012)) to score the pain severity daily (from 0 no pain to 10 worse pain). Owners' daily NRS scores were significantly lower during the pregabalin treatment phase compared to placebo. In this study, the daily owner assessment NRS appeared to be a reliable and reproducible assessment tool. An advantage of daily NRS scoring was that potential bias from assessing isolated timepoints was avoided (Colloca et al., 2016; Sanchis-Mora et al., 2019) and the endpoint is translatable across species. However, the disadvantage remains that the assessment of pain being owner dependent is anthropomorphic, therefore inferred by humans and consequently not objective.

Whilst subjective measures of pain in humans does not present the same challenges as subjective measures in preclinical species there is still room for improvement. Subjective measures in humans still introduce variability and limit the translational potential of markers used preclinically to clinical trials, through using different endpoints. Whilst an important improvement to forward translatability would be to use objective preclinical endpoints with the current subjective clinical ones. Using the same objective endpoint in both is the ideal goal for markers of neuropathic pain.

Pain Supressed Behavioral States: Hoarding, Grooming, Rearing, Nesting, Wheel Running, Burrowing, Gait

Rodents possess several naturalistic/innate characteristics within their behavioral repertoire and suppression of these characteristics can be observed in a similar way to the assessment of QoL measures in neuropathic pain patients. These include wheel running (Stevenson et al., 2011; Pitzer et al., 2016; Green-Fulgham et al., 2020), nesting (Jirkof, 2014), rearing/climbing/exploration (Piel et al., 2014; Pitzer et al., 2016; Deuis et al., 2017) and burrowing (Andrews et al., 2011; Huang et al., 2013; Fisher et al., 2015; Rutten et al., 2018; Sliepen et al., 2019). However, others have found no change in locomotion or rearing in the chronic constrictive injury (CCI) and spared nerve injury (SNI) mice models (Mogil et al., 2010) and that CCI and SNI mice had no change in markers of QoL (Urban et al., 2011). This may be due to nature of rodents as prey animals that will avoid displaying injury or pain to predators (Roughan and Flecknell, 2001).

Analysis of burrowing behaviors in rodent pain models has become increasingly popular over the last 10 years (Pubmed search on pain and burrowing revealed 5 publications in 2010 and 25 in 2019) and demonstrates reproducible results across laboratories (Wodarski et al., 2016). Rodent burrowing is reduced in mono-neuropathic pain states and reversed by clinical analgesics (gabapentin, pregabalin) at 10-fold lower doses (in line with therapeutic exposure in humans) than are needed to decrease hypersensitivity (Andrews et al., 2011; Lau et al., 2013; Rutten et al., 2018). This suggests its usefulness as an objective measure of pain with improved face and predictive validity over stimulus evoked measures of hypersensitivity. Experimental design should always include assessment of drug treatment on burrowing in naïve animals to ensure there is no false positive direct drug effect(s) on this natural behavior. One confound that occurs with all pain supressed spontaneous rodent behavioral tests is that any stimulus that disrupts wellbeing, including disruption of memory (Deacon et al., 2002; Deacon, 2009) can decrease rodent behaviors such as nesting and burrowing. For example, it has recently been demonstrated that a high dose of STZ (75 mg/kg) induces diabetic poly-neuropathic pain,

hypersensitivity and abolishes burrowing. However, in contrast to mono-neuropathic pain models burrowing is not reversed by pregabalin, which the authors suggest reflects diabetes associated alteration of the animals' welfare; and not spontaneous pain (Rutten et al., 2018). Previously, our group have reported a similar decline in burrowing following a high dose (65 mg/kg) of STZ that is resistant to pregabalin but can be reversed by social pairing, indicating pair housing of diabetic rats can improve their welfare and consequently burrowing behavior (Fisher et al., 2015).

This emotional contagion whereby burrowing behavior of STZ type-1 diabetic rats increased if they burrowed with their cage partner (rather than alone) and their individual burrowing increased if they were socially housed with a control partner not in pain (compared with an STZ partner, Fisher et al., 2015) has also been observed by other groups (Langford et al., 2006). The experiments in the laboratory of Mogil et al., demonstrated that mice display higher levels of pain behavior when tested alongside familiar (but not stranger) conspecifics, and featured synchronization of both level and timing of that behavior within the partnership (Langford et al., 2006). Therefore, when evaluating pain suppressed spontaneous behaviors, consideration of the use of appropriate controls is essential e.g. dose of STZ, consistent housing (pair housing at least) during pain testing (stimulus evoked sensory endpoints, burrowing) and testing with control groups is recommended to maximize the welfare and wellbeing of rodents.

As with many other pain supressed behaviors, gait analysis is applicable across a wide range of species and the animal literature is supported by many human studies examining the effect of neuropathic pain on gait (Lalli et al., 2013; Karmakar et al., 2014; Alam et al., 2017). The use of gait analysis to assess pain possesses several advantages most notably the measurements are performed in freely moving animals. Furthermore, when considering development of potential treatments in early drug discovery, only drugs that improve movement will pass screening. Compounds that produce sedative or motor impairments will not restore normal walking and therefore will fail due to side effect profiling. Furthermore, data from gait analysis studies tend to be reproducible across trials and animals within a specific condition (Tappe-Theodor et al., 2019). When considering the use of gait analysis in neuropathic pain models it is worthwhile to note that neuropathic conditions cause both pain and motor effects, either of which can alter gait. The lack of a correlation between the time course for neuropathy induced mechanical hypersensitivity and gait change, in combination with the lack of recovery of normal gait following treatment with standard analgesics would indicate a motor problem (Mogil et al., 2010; Lau et al., 2013; Shepherd and Mohapatra, 2018) which may confound results.

Pain suppressed spontaneous natural behaviors have some important limitations and can be affected by various factors such as caging and animal welfare. However, their aim to provide objective measures of neuropathic pain preclinically is important. If objective pain suppressed behaviors such as burrowing/gait behavior can be successfully refined and developed into robust high throughput markers (initially alongside mechanical hypersensitivity markers), these may improve the translatability potential of preclinical data.

Pain Stimulated Behavioral States

Facial Pain Scoring/Grimace Scale Assessment "Pain Face"

Changes in facial features of humans convey a wide range of states and emotions which have been extensively studied in the context of pain, particularly in neonates (Grunau and Craig, 1987; Maxwell et al., 2013; Chow et al., 2016; Jones et al., 2018; Kappesser et al., 2019) and non-verbal patients (Chow et al., 2016). The idea that facial expressions can reflect the affective ("emotional") component of the pain experience has been extrapolated to identify pain specific features ("pain face") using pain scales ("grimace-scales") for a number of non-human species including rodents (**Table 2**). The rodent grimace scale originally developed in the laboratory of Jeffrey Mogil by Langford et al., 2010 relies on photo analysis for scoring and consequently is a subjective non-evoked endpoint. Frame grabbing can be done manually, which is very time consuming, however with the development of the Rodent face finder software™ it can now be done automatically (Sotocinal et al., 2011). The system works by recognizing frames containing an eye or ear and tracts pixel movement to ensure extracted images are free from motion blur, thus improving accuracy.

Despite the extensive amount of literature (**Table 2**), the use of the grimace scale in rodent neuropathic conditions has been hampered by an apparent disparity between pain duration, expression of a painful grimace and mechanical sensitivity (Sotocinal et al., 2011; De Rantere et al., 2016). It is likely that the mismatch arises because of the difference in time course between evoked and spontaneous pain. Furthermore, a lack of grimacing does not mean that the animal is pain free, just that the pain no longer elicits a grimace (Tappe-Theodor and Kuner, 2014). As with ultrasonic vocalisation (USV) (described below) the selectivity of facial grimacing has also been called into question with changes in facial musculature observed in aggressive and fearful contexts (Defensor et al., 2012) and nausea (Yamamoto et al., 2017). Despite this, the use of the grimace scale has shown face validity in several neuropathic pain models including reduction of the grimace scale with fentanyl in the CCI of the infraorbital nerve (Akintola et al., 2017), with meloxicam in a rodent model of radiculopathy (Philips et al., 2017) and Schneider et al. (2017) have shown facial action units are increased following stimulation with acetone in a rat spinal cord injury (SCI) model. Therefore, the rodent grimace scale shows great promise as a valid non-evoked subjective marker of preclinical spontaneous neuropathic pain.

Ultrasonic Vocalisation

USV would seem an ideal pain stimulated behavior which can be detected using an inexpensive bat detector (as well as several other commercially available USV detection systems), recorded, and quantified (Sirotin et al., 2014) for review see (Tappe-Theodor and Kuner, 2014; Mogil, 2019b; Turner et al., 2019). The range of rodent sounds fall into the human audible (<20 kHz frequency) and ultrasonic vocalisation (USVs; > 20 kHz frequency) (Roberts, 1975). Many of the studies investigating USVs within the chronic pain space have looked at the "alarm" calls rats emit at approximately 22 kHz. Pain induced USVs have

TABLE 2 | Species in which pain assays producing a grimace scale have been used.

Species	References
Human	Ashraf et al. (2009), Lucey et al. (2011), Bartlett et al. (2014)
Rodent (rats and mice)	Langford et al. (2010), Sotocinal et al. (2011), Matsumiya et al. (2012), Leach et al. (2012), Oliver et al. (2014), Faller et al. (2015), Miller et al. (2016), De Rantere et al. (2016), Akintola et al. (2017), Philips et al. (2017), Schneider et al. (2017), Sperry et al. (2018), Dalla Costa et al. (2018), Cho et al. (2019), Klune et al. (2019), Leung et al. (2019), Ernst et al. (2020)
Pigs/Piglets	Di Giminiani et al. (2016), Viscardi et al. (2017)
Cat	Evangelista et al. (2019), Evangelista et al. (2020)
Sheep	Häger et al. (2017)
Rabbit	Banchi et al. (2020)
Ferrets	Reijgwart et al. (2017)
Horse	Dalla Costa et al. (2014), Dalla Costa et al. (2018)

For review see (Tappe-Theodor and Kuner, 2014; Deuis et al., 2017; Mogil, 2019b; Tappe-Theodor et al., 2019; Turner et al., 2019).

been observed in mice in the SNI model of neuropathic pain (Kurejova et al., 2010) and SCI model of neuropathic pain (Ko et al., 2018) where morphine reduced the SCI induced USVs. Kurejova et al. (2010) used an improved method which enabled the temporal recording of USVs in freely moving mice repeatedly over several weeks and demonstrated a reduction in the SNI induced USVs with gabapentin. The strength of their study, and perhaps the reason why USV effects were observed where others have not seen any (Wallace et al., 2005) lies in the reporting of USVs on a longitudinal timescale and the use of higher frequencies of 37 and 50 kHz, avoiding the 22 kHz frequency range so often used. The criticisms of this endpoint pertain to alarm calls as unselective for pain measurements, being initiated by human handling (Brudzynski and Ociepa, 1992) stress/anxiety (Naito et al., 2003), anticipation of negative events (Knutson et al., 2002) and tightly coupled to sniffing behavior (Sirotin et al., 2014). Furthermore, USVs are highly sensitive to background noise requiring further validation of this technique for use in pain measurements.

Affective/Cognitive State

Anxiety, Depression, Sucrose Preference

As already discussed, neuropathic pain in the clinic is characterized by disturbances of both sensory and affective components. These frequently encountered comorbidities which include appetite decrease, depression, anhedonia and disruptions to sleep cycles present behaviors that can influence pain directly within the preclinical setting (Kontinen et al., 1999). Anxiety/depression like behaviors have recently shown utility within the preclinical setting and the study of neuropathic pain (Narita et al., 2006; Yalcin et al., 2011, Yalcin et al., 2014; Alba-Delgado et al., 2013). Several studies have demonstrated the time dependent nature in the symptomatic development of these comorbidities (Seminowicz et al., 2009; Yalcin et al., 2011; Barthas et al., 2015; Sellmeijer et al., 2018), which have been shown to present long after the mechanical hypersensitivity has worn off (Sellmeijer et al., 2018). This highlights the importance of measuring more than one behavioral change whilst taking into consideration the time at which the measures are captured and analgesia is monitored.

Sucrose preference can measure anhedonia, a key feature of depression (Nestler and Hyman, 2010) which is frequently seen in chronic pain patients but rarely measured in behavioral animal pain studies, despite evidence that depression alters the threshold of pain (Ang et al., 2010). Sucrose preference has been used to explore the influence that chronic pain may have on otherwise rewarding behaviors and has been shown repeatedly to be supressed in chronic neuropathic pain models (Wang et al., 2011; Bura et al., 2013; Amorim et al., 2014; Thompson et al., 2018). We (Fisher et al., 2015) have found that STZ type-1 diabetic rats show a dramatic reduction in 2% sucrose preference within 48 h of STZ administration that is maintained for up to 8 days. This is in line with Wang et al. (2011) who also observed a reduced sucrose preference within 2 days following SNI surgery that persists for up to 2 months, but only in those animals that developed mechanical hypersensitivity, indicating that reduced sucrose preference offers an objective anhedonia marker of spontaneous neuropathic pain. In contrast to Wang et al. (2011), we have consistently observed that STZ type-1 diabetic rats switch back to a 90% sucrose preference (not significantly different from control rats) 9 days after STZ administration despite mechanical hypersensitivity allodynia persisting for at least 18 days (Fisher et al., 2015). This indicates mechanical hypersensitivity may not be a key driver of the acute or chronic anhedonia. It is likely that STZ-dosed animals drink significantly less 2% sucrose than control animals, not due to anhedonia, but as a result of their increased water consumption, due to the emerging diabetes hyperglycaemia and polydipsia phenotype (Lenzen et al., 2008). The development of polyphagia, polydipsia, hyperglycaemia and mechanical hypersensitivity all stabilize 8 days after STZ administration at the same time point that the now diabetic animals switch to a normalized sucrose preference, indicating anhedonia is not an endpoint that can be measured using sucrose preference in this STZ type-1 diabetic rat model of poly-neuropathic pain (Fisher et al., 2015). The fact that localized nerve ligation neuropathic pain models demonstrate persistent reduced sucrose preference alongside mechanical hypersensitivity over many weeks (Wang et al., 2011; Bura et al., 2013) indicates anhedonia offers a pharmacodynamic objective marker in mono-neuropathic pain models. A potential confound when measuring sucrose preference involves potential analgesic (or hyperalgesic) effects of the sweeteners themselves (Suri et al., 2010; Shahlaee et al., 2013) and therefore it is essential to correlate any objective

changes in sucrose preference alongside changes in other markers of neuropathic pain, such as stimulus evoked sensory endpoints when validating its suitablility for translatability.

Operant and Classical Conditioning (Place Avoidance, Place Preference)

Non-evoked ongoing (spontaneous) pain along with the motivational/affective component of spontaneous pain can be assessed using conditioned place avoidance (CPA), conditioned place preference (CPP) (King et al., 2009; Navratilova et al., 2013) and place escape/avoidance paradigm (PEAP) for reviews see (Sufka, 1994; Tzschentke, 2007; Navratilova et al., 2013; Tappe-Theodor and Kuner, 2014; Tappe-Theodor et al., 2019). CPA is induced by pairing a painful experience with a distinct context which subsequently results in avoidance of the same contextual cues (Johansen et al., 2001; Johansen and Fields, 2004), thereby utilizing the protective function of pain to motivate escape and avoid harm. CPP is the opposite of CPA and assumes that pain relief is rewarding. CPP works by pairing a rewarding experience with a distinctive environment resulting in an increase in the time spent in that environment (Navratilova and Porreca, 2014; Navratilova et al., 2015, Navratilova et al., 2016). The most popular protocol for inducing CPP or CPA as described in rats and mice uses two conditioning chambers distinguished by visual, textural and occasionally odor cues (**Figure 3**). CPA simply occurs by pairing a painful experience with a specific chamber. In PEAP testing an animal with a paw made hypersensitive is placed in a chamber with a dark (normally preferred) side and a bright side, on top of a wire mesh. At regular intervals one paw is mechanically stimulated with a stiff filament, the hypersensitive paw when the animal is on the dark side and the paw with normal sensitivity when the animal is on the bright side. The shift in the fraction of time the animals spends in the normally preferred dark side provides a measure of the aversiveness of the stimulation of the sensitive paw (Usdin and Dimitrov, 2016).

Synonymous with the time reliant changes observed with anxiety/depression-like behaviors the use of CPP has proved useful in demonstrating the temporal nature in the development of spontaneous ongoing neuropathic pain (Agarwal et al., 2018; Gao et al., 2019). Additionally, it has dissociated the spinal and supraspinal effects of gabapentin (Bannister et al., 2017) and the sensory from the affective/motivational and cognitive aspects of chronic pain (Tappe-Theodor and Kuner, 2014; Shiers et al., 2020). The ability to measure both the affective and non-evoked ongoing/spontaneous component of chronic pain is a major advantage of the CPP/CPA and PEAP paradigms. However, they are all relatively labor intensive and require complicated and time-consuming protocols. Another limitation of the technique is that it does not measure pain in real time; the existence of pain in the past must be inferred by the presence of a CPP in the present (Mogil, 2019a). Long term or chronic neuropathic pain states are not suitable for inducing CPA as they persist outside the context and are therefore not specific to the distinctive environment (Tappe-Theodor et al., 2019). Although there are many difficulties associated with CPP/CPA and PEAP they have and continue to play an influential role in understanding the motivational/affective component of chronic neuropathic pain in animals as well as aiming to improve forward translatability by assessing subjective comorbidities as indirect markers of neuropathic pain in animals, as is the case in RCTs.

Cognitive State

Chronic neuropathic pain patients can produce poor performance in tasks requiring cognitive flexibility even when taking commonly prescribed analgesics (Ryan et al., 1993; Povedano et al., 2007; Attal et al., 2014). Cognitive malfunction can be translated and measured in rodents (see Usdin and Dimitrov, 2016 for a review). Indeed, many preclinical studies have demonstrated pathological changes in the hippocampus following peripheral nerve injury (SNI or spinal nerve ligation) that may underlie cognitive deficits (Ren et al., 2011; Mutso et al., 2012; Moriarty et al., 2016; Liu et al., 2017) for example increased TNF-α (Ren et al., 2011; Liu et al., 2017) and altered hippocampal synaptic plasticity and neurogenesis (Mutso et al., 2012).

Studies investigating cognitive dysfunction that can occur during chronic neuropathic pain have been able to dissociate the effects of gabapentin from that of the anti-diabetic drug metformin on cognition (Shiers et al., 2018) and the effects of amitriptyline from those of lornoxicam (Hu et al., 2010). In the elegant studies by Shiers et al. (2018) gabapentin demonstrated a worsening effect in the attentional set shifting task (ASST) in the SNI model whereas metformin completely reversed the cognitive impairment, at doses that completely reversed static mechanical allodynic (Shiers et al., 2018). Further studies from this group using the MNK inhibitor tomivosertib (eFT508) have identified MNK-eIF4E as a novel pathway playing a crucial role in the development of spontaneous pain (measured using CPP) and executive functioning using ASST (Shiers et al., 2020). Whereas no effect was observed on the mechanical hypersensitivity, which the authors attribute to the afferent fiber type affected in the neuropathic pain model (Shiers et al., 2020). In a recent review from Shiers and Price they expand on this dissociation by highlighting the fact that pain relief using currently prescribed transient analgesics is insufficient to reverse cognitive impairments and therefore, there is a clear need to investigate treatment options that can target both pain and its prefrontal cortex driven indirect comorbidities (Shiers and Price, 2020) to improve patient outcome measures.

Using the same mono-neuropathic model, Higgins et al. (2015) have demonstrated that following SNI surgery rats show the equivalent behavioral response to sham controls for food reward under a progressive ratio schedule, thereby implying a similar level of motivation. In contrast, a performance deficit was observed in the 5-choice serial reaction time task (5-CSRTT, a test of attention and reaction time) in the SNI animals only. The deficit became apparent in the second month post-surgery, consistent with an attentional deficit (Higgins et al., 2015) again highlighting the importance of temporal profiling in chronic neuropathic pain models measured over long periods of time.

TECHNIQUES WITH THE POTENTIAL TO PRODUCE OBJECTIVE MEASURES OF NEUROPATHIC PAIN

Currently the most promising advances toward objective measures of neuropathic pain have been made using microneurography, neuroimaging and EEG. These techniques can be conducted on both preclinical species and on humans and thus data from these techniques can be directly translated between species providing a key opportunity for the development of objective markers for neuropathic pain.

Microneurography

Microneurography (minimally invasive recording of intact peripheral nerve fibers *in vivo*) provides the opportunity to study the pathophysiology of sensory and axonal abnormalities in pain processing; abnormalities which may underlay the phenomenon of spontaneous pain (Jørum and Schmelz, 2006; Cruccu et al., 2010; Serra et al., 2010) and can also be used as a powerful diagnostic tool for use in patients with neuropathic pain (Serra et al., 2004; Jørum and Schmelz, 2006; Cruccu et al., 2010). Microneurography studies have identified spontaneous activity (primarily in C fibers) that is related to pain, suggesting a potential peripheral mechanism for neuropathic pain (Kleggetveit et al., 2012; Serra, 2012). Importantly from a translational perspective, microneurography can also be used in the rat (Serra et al., 2010; Garcia-Perez et al., 2018) and pig (Jones et al., 2018) providing the opportunity for direct translation of *in vivo* evidence of efficacy, from the preclinical through to the clinical setting.

Microneurography can be time consuming and relies on a fully trained technical expert investigator. Furthermore, microneurography is currently performed in only a few centers around the world. For these reasons, it has only been used on very few occasions to study neuropathic pain patients (Cruccu et al., 2010) (only 21 publications over the last 10 years). These few extant studies have observed similar conductance velocities in rodents and humans (Handwerker et al., 1991; Cain et al., 2001). There is no published normative data for healthy subjects, and published reports are unblinded group comparisons only (Cruccu et al., 2010).

Neuroimaging

The brain neuroimaging technologies, including magnetic resonance imaging (MRI), positron emission tomography (PET), and magnetoencephalography (MEG), have contributed substantially to our understanding of the perception and processing of pain in humans. The use of PET and MRI for assessment of the brain response to pain, in man, particularly neuropathic pain have been reviewed previously (Peyron et al., 2000; Moisset and Bouhassira, 2007; Morton et al., 2016). Their applicability within rodents positions their use as a key translational biomarker in understanding the same processes from the clinic to the pre-clinical situation (Tracey and Mantyh, 2007; Thompson and Bushnell, 2012; Da Silva and Seminowicz, 2019; Tracey et al., 2019). In rodents, MRI is the most commonly used with PET rarely employed (Da Silva and Seminowicz, 2019). MRI has the advantage of providing better spatial and temporal resolution than PET. However, PET allows for the imaging of neurotransmitters and non-neuronal cells, e.g. astrocytes, in addition to functional imaging.

Functional magnetic resonance imaging (fMRI) is often viewed as the gold standard for longitudinal studies because it is non-invasive. This technique typically uses the blood oxygen level dependent (BOLD) methodology to identify changes in hemoglobin oxygenation which indicates alterations in neural metabolism over time. Although fMRI possesses good spatial resolution it provides only an indirect measure of neuronal activity associated with a task or stimulus and is associated with poor temporal resolution. PET is hampered by both poor spatial and temporal resolution but its advantage over fMRI is that via glucose metabolism, using fluodeoxyglucose (FDG)-PET, it is a more direct measurement of neuronal activity than the BOLD signal. Well-known major confounds associated with rodent brain imaging are the methods used to secure the subject during the scan. Unquestionably, anesthetic agents and restraint techniques impact the results obtained from rodent studies and must be taken into consideration in their design and interpretation (Lancelot and Zimmer 2010). Some PET methods do allow for tracer uptake before the animal is anesthetized however, imaging a moving animal is also not without drawbacks (Gold et al., 2018).

PET scanning using translocator protein (TSPO)-binding radioligands (e.g. [11C]PBR28) is a promising option for studies of neuroinflammation (Albrecht et al., 2016; VanElzakker et al., 2019). TSPO is an 18 kDa, five transmembrane domain protein, mainly situated in the outer membrane of mitochondria. TSPO is thought to be involved in a wide array of vital cellular functions, including steroidogenesis, mitochondrial respiration and cellular proliferation (Herrera-Rivero et al., 2015; Albrecht et al., 2016). It has recently become the molecule of choice for most PET imaging studies which are aimed at imaging glial activation and neuroinflammation (Albrecht et al., 2016). therefore it is ideal for imaging neuropathic pain studies where inflammation and mitochondrial activation represent a predominant feature e.g. diabetes induced neuropathy (Fernyhough, 2015) and traumatic neuropathy (Ellis and Bennett, 2013).

Under healthy baseline conditions TSPO is expressed constitutively at low levels by multiple cell types including neurones and glial cells (Cosenza-Nashat et al., 2009). During an inflammatory response, TSPO becomes substantially upregulated predominantly, if not exclusively, in glial cells in many animal models and human disorders (Chen and Guilarte, 2008; Rupprecht et al., 2010; Wei et al., 2013; Sandiego et al., 2015; Liu et al., 2016). For example, TSPO expression in spinal cord dorsal horn is upregulated in a rodent model of spinal nerve ligation and returns to baseline once the neuropathic pain has resolved or is reversed by the TSPO agonist, Ro5-4864; suggesting that the TSPO upregulation might act as a marker of neuropathic pain and its subsequent recovery.

Though, not all studies find a correlation between the rodent and human microglia/macrophage TSPO response to

lipopolysaccharide (LPS) inflammation (Owen et al., 2017). Owen et al. demonstrate no change in TSPO expression in primary human microglia/macrophages compared to a 9-fold increase in rodent primary microglia/macrophages following LPS stimulation (Owen et al., 2017). The authors suggest that TSPO expression (hence TSPO PET binding) may reflect changes in microglia density/proliferation rather than cell activation, which appears to be different from rodents. Whilst others have found an increase in TSPO PET binding possibly reflecting microglial activation in humans following intravenous LPS (Sandiego et al., 2015). This highlights a lack of correlation between activation and induction of TSPO expression. Differentiating whether TSPO PET binding reflects microglia proliferation/density and/or activation *in vivo* is a challenge and extrapolation of TSPO biology from rodent to human myeloid cells should be done with caution (Owen et al., 2017).

Despite the innate challenges associated with the various imaging techniques this technology is still commonly used in rodents and considerable progress is continuing to be made in this field. As such, this has led to the identification of a core pattern of nociceptive-evoked events and brain regions activated in human pain imaging studies (somatosensory cortex, cingulate cortex, thalamus) that are also activated in the majority of the rodent studies (Tracey and Mantyh, 2007; Thompson and Bushnell, 2012). Moreover, pharmacological imaging in rodents shows overlapping activation patterns with pain and opiate analgesics, similar to that found in humans. For example, many of the pain related regions in the brain possess mu opioid receptors e.g. the periaqueductal gray, amygdala and thalamus (Zubieta et al., 2001) and imaging studies in both rodents (Shah et al., 2005) and man (Casey et al., 2000) have elucidated the activity of opioids alone and following induction of pain on these specific brain regions. Following opioid administration these studies have demonstrated a clear reduction in the pain-evoked activation of the brain region demonstrating the translational nature of this methodology.

Electroencephalography (EEG)

EEG, first conducted in humans by Hans Berger in 1924, is the technique used to study the electrical currents produced in the brain (Berger, 1929). It is recorded using electrodes: in humans commonly placed on the scalp (Sazgar and Young, 2019); in rodents usually placed in direct contact with the dura mater (Lundt et al., 2016), to produce an EEG signal. The EEG signal represents synchronized electrical activity from populations of neurons (Binnie and Prior, 1994). EEG signal patterns may be useful as objective and translatable central markers of neuropathic pain as EEG signals can be recorded in both animals and humans without introducing observational bias, that current neuropathic pain stimulus evoked sensory endpoints may create (Bove, 2006; Leiser et al., 2011; Wilson et al., 2014; Sullivan et al., 2015).

Early evidence showed neuropathic pain patients had an increased theta (4–8 Hz) power, decreased alpha (8–12 Hz) power and increased rhythmicity of theta oscillations termed thalamocortical dysrhythmia (Llinas et al., 1999). Evidence from animal models indicated the ventral posterolateral nucleus of the thalamus as the cause of thalamocortical dysrhythmia, which transmits spinal cord signals to the somatosensory cortex (Gerke et al., 2003; Caylor et al., 2019). Thalamocortical dysrhythmia has continued to be a commonly referenced model for the changes in theta oscillations seen in neuropathic pain (LeBlanc et al., 2016b; Vuckovic et al., 2018a; Vanneste et al., 2018). However, in humans Stern et al. (2006) identified that although there were many similarities in EEG signal (such as increase theta power), differences in the extent of this increase in theta power were related to the neuropathic pain cause. Thus, further development of EEG may produce individual patterns of EEG changes for different neuropathic pain causes.

Findings in humans that spinal cord stimulation (SCS) reduces neuropathic pain have now been replicated in rodents through using EEG patterns as a marker. This procedure involves a surgically implanted device applying an electrical stimulation to the spinal cord of patients to alter neuronal activity (Caylor et al., 2019). SCS has been used in humans for many years to treat chronic and neuropathic pain, although the mechanism of action is still being investigated (Sivanesan et al., 2019). Human neuropathic pain patients successfully treated with SCS have shown a reduction in delta (0.5–3 Hz), theta (3–8 Hz) (Sufianov et al., 2014) and high theta (7–9 Hz) power (Schulman et al., 2005). Findings in the CCI rat model show that SCS successfully reversed thermal hyperalgesia and reduced the increased EEG power in the 3–4 Hz range (Koyama et al., 2018b). This provides supportive evidence that the increase in theta power seen in many neuropathic pain studies may be a reliable marker that can be used to screen drugs against. To this end Koyama et al. (2018a) have developed a rodent model of pain that uses increased theta (4–8 Hz) power as a marker. This model demonstrated a reversal of theta power and allodynia after treatment with pregabalin and EMA 401 (an angiotensin II type 2 receptor inhibitor with positive efficacy in a phase II postherpetic neuralgia trial, although clinical development has now been halted due to toxicological side effects) (Rice et al., 2014). However, Moreover, minocycline (glial cell inhibitor with poor results in human clinical trials) failed to reverse these markers (Vanelderen et al., 2015). Significantly, sub- and supra-optimal doses equivalent to human exposure were identified only when using theta power and not allodynia as an endpoint, providing strong evidence for the use of EEG signal patterns as a translational marker of neuropathic pain (Koyama et al., 2018a). This back translation of the effect of pregabalin is an important step in validating this method as a marker of neuropathic pain.

Other than the potential of data generated through EEG recordings as a translatable marker, it also has uses in neuropathic pain treatments that avoid the use of animals, by being applied directly to humans. One such method is neurofeedback modulation (NFB) which uses a real time EEG display, to allow patients to monitor and regulate their brain oscillations (Jensen et al., 2008). After training, patients are given targets such as to increase alpha (9–12 Hz), whilst decreasing theta (4–8 Hz) and high beta (20–30 Hz) oscillations. This method of NFB has been found to significantly reduce pain for some neuropathic pain patients (Hassan et al., 2015). Recently, patients have been able to practice NFB at home when it is most needed. In this study 12/15 patients achieved a statistically significant reduction in pain, with upregulation of alpha (9–12 Hz) oscillations being the most successfully achieved

(Vuckovic et al., 2019). Although placebo-controlled testing is difficult in NFB, pre-recorded EEG data has been examined during training session and patients reported no changes in pain scoring (Hassan et al., 2015). This provides additional evidence that modulating EEG oscillations can provide therapeutic benefit.

In SCI, EEG signals have been used to classify (Wydenkeller et al., 2009) and even predict patients that will develop neuropathic pain (Vuckovic et al., 2018b; Vuckovic et al., 2018a). Wydenkeller et al. (2009) found that reduced peak EEG frequency between 6 and 12 Hz was able to classify between neuropathic pain and non-neuropathic pain patients with 84% accuracy. Recently, EEG reactivity to eyes opening (Vuckovic et al., 2018b) and feature classification (Vuckovic et al., 2018a), have been used to predict neuropathic pain development in SCI. In 85% of cases on average these methods predicted non-neuropathic pain patients that would develop neuropathic pain. Interestingly this study found that the oscillations involved in the most accurate predictions included alpha (8–12 Hz) and beta (4–8 Hz). This contributes to the developing theory that theta changes occur progressively with neuropathic pain development and alpha and beta changes are seen before neuropathic pain onset (Vuckovic et al., 2018a). Our research is investigating whether these findings can be translated into an animal model such as the STZ type-1 diabetic rat to open an exciting avenue of research into drugs to slow or even prevent the development of neuropathic pain.

The use of data produced by EEG as a marker of neuropathic pain has solid potential; however, it does come with limitations. One key example is the different methods of EEG electrode placement in humans and rodents (Lundt et al., 2016; Sazgar and Young, 2019). The skull and skin have different conductivities which can alter EEG recordings and affect translatability between species (Drinkenburg et al., 2016; Vorwerk et al., 2019). One way to improve this is using epicranial screws in rodents that are placed into but not through the skull which is closer to the placement of electrodes in humans (LeBlanc et al., 2016a; Koyama et al., 2018a, Koyama et al., 2018b). Additionally, differences between rodent and human EEG signals may occur due to physiological differences in brain size and pathways, or procedural differences in EEG recordings (Leiser et al., 2011; Wilson et al., 2014). For example, coherence in the theta (4–9 Hz) band was increased in human neuropathic pain patients but decreased in rats (Llinas et al., 1999; Sarnthein and Jeanmonod, 2008; Leblanc et al., 2014).

An important consideration when using EEG to investigate neuropathic pain is whether the changes seen in the brain or spinal cord are most important. Whilst it is important to continue work in both areas, the current understanding that pain is a combination of both sensory and emotional aspects leads to the likelihood that EEG recordings focusing on changes in the brain will have a greater ability to analyze the true pain experience. However, the changes seen in the spinal cord may provide a more accessible target as shown by SCS where the effects of neuropathic pain are not clouded by additional factors such as mental state and previous experiences.

EEG is a technique that provides a promising way of producing data that can be used as a biomarker of neuropathic pain as it is both translatable and unbiased. Further development is needed to fully characterize the changes produced by individual neuropathic pain causes and identify specific EEG patterns that translate between animals and humans.

CONCLUSION

In this review, we have discussed how bidirectional research is attuning animal models closer to the human condition, by refining neuropathic pain assessment outcome measures to aid translation of preclinical results to the clinic. Alternative behavioral measures outlined in this review provide a means by which preclinical researchers can study not only the development of neuropathic pain but the analgesic response to clinical candidates in a more comprehensive manner. However, the very nature of moving away from simple reflex based measures does mean that behavioral measures may be more easily perturbed by subtle changes relating to environmental events e.g. housing conditions and minor protocol variations that can result in inter-laboratory and inter-group variability. For example, short term social isolation has been shown to suppress burrowing behavior in STZ type-1 diabetic but not control rats (Fisher et al., 2015). Given that observational pain behavioral measures are easily modifiable and subjective we advocate a need for further validation of objective pain markers such as data produced by EEG. Despite automation, objective markers of pain, by their very nature, are longer lasting and more labor intensive than the routinely favored stimulus evoked sensory measures. Although, results from such studies can be translated directly into and back from human studies. The more examples of successful back and forward translation that are documented using multiple neuropathic pain endpoints, the more evidence we will have as to whether these provide superior animal to human predictivity, compared to stimulus evoked sensory endpoints alone, and the more confidence we are likely to have in the success of clinical interventions derived from and/or supported by rational drug discovery.

AUTHOR CONTRIBUTIONS

AF, ML, and LL conceptualized the paper. LL drafted the Abstract, Introduction, Bi-directional translation of current treatments for neuropathic pain, Forward translation (bench to bedside) of neuropathic pain and conclusion sections. AF drafted the Industry standard measurements of neuropathic pain for the development of analgesic treatments, the mismatch problem, Alternative neuropathic pain assessment endpoints to improve translatability between animals and humans, Microneurography and Neuroimaging sections. ML drafted the Electroencephalography (EEG) and reference sections. AF, ML, NU, and LL critically revised the subsequent drafts.

FUNDING

ML is a Hertfordshire Knowledge Exchange Partnership PhD student in collaboration with the University of Hertfordshire and

Transpharmation Ltd. supported by the Hertfordshire Local Enterprise Partnership's Single Local Growth Fund, the European Union's European Regional Development Fund and Transpharmation Limited.

ACKNOWLEDGMENTS

We thank Andy Billinton and Sandor Kantor for their critical reading of the manuscript.

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Conflict of Interest: NU, ML and AF were employed by the company Transpharmation Ltd.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

REVIEW
published: 15 April 2021
doi: 10.3389/fphar.2021.603186

Bridging the Translational Divide in Pain Research: Biological, Psychological and Social Considerations

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A gap exists between translating basic science research into effective pain therapies in humans. While preclinical pain research has primarily used animal models to understand biological processes, a lesser focus has been toward using animal models to fully consider other components of the pain experience, such as psychological and social influences. Herein, we provide an overview of translational studies within pain research by breaking them down into purely biological, psychological and social influences using a framework derived from the biopsychosocial model. We draw from a wide landscape of studies to illustrate that the pain experience is highly intricate, and every attempt must be made to address its multiple components and interactors to aid in fully understanding its complexity. We highlight our work where we have developed animal models to assess the cognitive and social effects on pain modulation while conducting parallel experiments in people that provide proof-of-importance for human pain modulation. In some instances, human pain research has sparked the development of novel animal models, with these animal models used to better understand the complexity of phenomena considered to be uniquely human such as placebo responses and empathy.

Keywords: pain, translation, memory, mouse, social, biopsychosocial

OPEN ACCESS

Edited by:
Maree Therese Smith, The University of Queensland, Australia

Reviewed by:
Kevin A Keay, The University of Sydney, Australia
Christoph Stein, Charité–Universitätsmedizin Berlin, Germany

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Specialty section:
This article was submitted to Translational Pharmacology, a section of the journal Frontiers in Pharmacology

Received: *05 September 2020*
Accepted: *22 February 2021*
Published: *15 April 2021*

Citation:
Cho C, Deol HK and Martin LJ (2021) Bridging the Translational Divide in Pain Research: Biological, Psychological and Social Considerations. Front. Pharmacol. 12:603186. doi: 10.3389/fphar.2021.603186

INTRODUCTION

In order to completely understand complex conditions such as chronic pain, multiple factors that contribute to the personalized experience of pain must be considered. The biopsychosocial model, introduced by (Engel 1977), emphasizes the importance of considering biomedical evidence along with a patient's subjective psychological wellbeing and social environment in the manifestation and progression of an illness. Though biological components such as genetics may predispose individuals to certain chronic pain conditions, psychological and social variability often impact the time of onset, severity and the course of pain associated with multiple conditions (Engel 1981). Moreover, psychological and social disturbances can often influence overall health and exacerbate existing illness-related symptoms (Engel 1981). For example, we naturally assume that people with high levels of pain and disability always must have had more severe injuries than those who have less pain and disability. Of course, sometimes this is true, but in the majority of cases, psychological influences such as anxiety, fear and even social relationships can imbue either a positive or negative effect upon one's pain perception and experience (Salovey et al., 2000; Pressman and Cohen 2005; Pulvers and Hood 2013).

The biopsychosocial model encompasses biological, psychological, social and all other associated processes that include cognitive, affective and behavioral constructs (Meints and Edwards 2018). It is precisely these biopsychosocial influences that are rarely considered when novel drugs are screened using preclinical models and eventually assessed in clinical trials. By only evaluating biological contributors, we are missing a fundamental aspect of the pain experience and the lack of psychological and social considerations may hinder understanding chronic pain conditions in their entirety. Herein, we parse out the biopsychosocial framework into distinct lines of pain research that consider biological, cognitive, psychological and social influences. We begin by discussing common biological approaches and our own research that identified genes associated with the development of temporomandibular joint disorder pain in people and then used animal models to understand their involvement in pain processing and identify a biological mechanism (Martin et al., 2017). In separate lines of research, we have used unconventional approaches and formed novel collaborations with psychologists and social scientists to perform contemporaneous studies where virtually the same experiment is conducted in mouse and human subjects. To this end, we have investigated acute pain modulation as an aberrant memory and have developed novel models to examine the memory of pain in both mice and people (Martin et al., 2019). Our work has also highlighted the importance of the social context by studying empathy by using mouse and human subjects (Martin et al., 2015). The "pain memory" and "empathy" themes assume that a significant contributor to pain modulation and chronification is the context through which we experience pain.

BIOLOGICAL CONSIDERATIONS

Animal models–comprising the bulk of preclinical pain research–have received a lot of criticism for their lack of clinical validity. Yet, these same animal models provide the blueprints for drug development and ultimately failed clinical trials (Bennett and Xie 1988; Wallace et al., 2002; Mogil et al., 2010). Over the last few decades, only a handful of new and highly efficacious analgesics have been developed by the pharmaceutical industry despite identifying hundreds of novel molecular targets and investment of billions of dollars (Kissin 2010; Woolf 2010). However, the majority of analgesics developed within the past five years are mostly conjugates of existing drugs, reformulations or chance observations that have led to the repurposing of drugs designed to treat another disease (Smith et al., 2013; Moore et al., 2015; Sisignano et al., 2016). The failure of animal models has led many in the pain field to re-evaluate preclinical animal models and ask how the preclinical research process may be improved. Several labs now incorporate tissue samples from animal and human donors to understand where similarities and differences may exist (Ray et al., 2018; Sheahan et al., 2018; Dedek et al., 2019). The validation of new molecules by using human tissue is advantageous because confidence in targets increases before progressing toward much more time-intensive and costly clinical trials. However, this approach still does not adequately address what biological substrate–molecules, cells, circuits or systems–should be prioritized to produce the most effective pain therapies.

The development of anti-CGRP (calcitonin gene-related peptide) antibodies for the treatment of migraines is a recent example of translational success. The successful development of these drugs was based on a detailed understanding of CGRP signaling, key clinical evidence for the role of CGRP in migraine headaches and a precise role for CGRP in the trigeminovascular system in the pathophysiology of migraine (Edvinsson et al., 2018). The evidence was too overwhelming for anti-CGRP-based pharmaceuticals not to work as migraine therapeutics; however, this was not an overnight success as CGRP was discovered in 1982 and has been extensively studied ever since. In stark contrast, one of the most devastating failures of pharmaceutical translation was the neurokinin 1 (NK1) receptor antagonists from rodents to humans. Despite an overabundance of research focused on the role of substance P in pain transmission, the development of high affinity and selective NK1 receptor antagonists and the plethora of animal data supporting their utility as analgesics, the clinical trial data did not support the profile of these drugs as effective analgesics in a variety of pain states (Boyce and Hill 2004). In clinical trials, NK1 receptor antagonists failed to show any analgesic efficacy in patients suffering from migraines, visceral pain, osteoarthritis and fibromyalgia (Borsook et al., 2012). The differences observed between animals and humans may not only be explained by species differences in substance P/NK1 receptor but also by the lack of understanding of how NK1 receptors function and vary across species (Hill, 2000; Khan, 2015; Navratilova and Porreca, 2019). In the case of NK1 receptors, their activation induces a redistribution to endosomes that causes sustained excitation of spinal neurons and pain transmission, which is alleviated by targeting endosomal NK1 receptors. Thus, conventional NK1 receptor antagonists as chronic pain therapies may have failed due to their inability to inhibit internalized NK1 receptors (Jensen et al., 2017). The presumption that animal models recapitulate the disease being studied or the hypothesis being pursued can often constrain research. Such assumptions may cloud the interpretation of scientific findings which can often result in the failure of possible drug treatments and therapies in clinical trials.

To improve translation between humans and animal models, we and others have reversed this process. Specifically, we used human genetics to identify unique single nucleotide polymorphisms in genes associated with clinical pain and then used animal models to understand how they regulate pain and their involvement in chronic pain (Martin et al., 2017). Our human genetics studies identified polymorphisms in the genes encoding for epiregulin (EREG) and the epidermal growth factor receptor (EGFR) as among the top three "hits" associated with the development of clinical pain. EREG and EGFR form a ligand and receptor, respectively, that are well-studied in cancer research, yet their prominent role in pain processing remained unclear. Albeit, a few case reports had shown that EGFR inhibitors may provide pain relief in cancer patients (Moryl et al., 2006; Kersten and Cameron 2012) and neuropathic pain patients (Kersten et al.,

2015). There was also evidence that serum concentrations of epiregulin and other EGFR ligands are upregulated in rheumatoid arthritis patients. Local blockade of these growth factors suppressed the development of cytokine-induced arthritis in mice by inhibiting chemokines and interleukin-6 expression (Harada et al., 2015). These studies, combined with our genetic association results, gave us confidence in fully exploring the contribution of EGFR and EREG to nociception using animal models. We found that inhibiting EGFR at the tyrosine kinase site reduced nociceptive sensitivity in mouse models of inflammatory and neuropathic hypersensitivity, including spontaneous facial expressions (Martin et al., 2017). Further, epiregulin administration increased inflammatory nocifensive behavior through a mechanism involving the PI3K/AKT/mTOR pathway and matrix metalloproteinase 9 in sensory neurons (Martin et al., 2017).

Currently, available EGFR inhibitors are associated with unwanted side effects, including pruritus (i.e., itchy skin rash) and severe diarrhea[7]. These side effects would limit their utility as analgesics. This is where further work using animal models would be necessary and may help develop and refine EGFR-based therapies devoid of side effects that would preclude their use by pain patients. We recently showed that a monoclonal antibody targeting epiregulin reversed inflammatory nociception. Still, the timing of antibody administration was critically important in determining whether nociceptive reflexes were augmented or attenuated in mice (Verma et al., 2020). Together, our mouse studies combined with clinical observations and small-scale clinical trials showing the efficacy of EGFR inhibitors for the treatment of refractory pain conditions provide compelling evidence for their utility as a novel analgesic strategy. The aforementioned epiregulin antibody experiments used mice but were informed by clinical human genetic association data showing that patients with different polymorphisms of the EGFR recover differently. The next step would be to develop a humanized version of this antibody for small-scale testing in people. Although, the development of novel pain therapies such as EGFR inhibitors or antibodies should more comprehensively assess the pain experience through the incorporation of a wide spectrum of pain behavior including memory, conditioning, and affective consequences.

In our studies, EGFR polymorphisms were identified in the white blood cells of chronic pain patients. We used this finding as a proxy to investigate EGFR-related signaling in sensory neurons (i.e., dorsal root ganglion, DRG); however, this approach was not guaranteed to work, and EGFR-related pain modulation may have occurred within the skin or at some other site. These alternatives could have easily been investigated by directly comparing the same site of innervation (DRG, spinal cord, brain) using rodent models and human samples, typically taken from post-mortem donors (Ray et al., 2018; Sheahan et al., 2018; Dedek et al., 2019). While the upregulation of DRG-specific ion channels in mouse and rat models of neuropathic pain (LaCroix-Fralish et al., 2011; Zhang and Dougherty 2014) and human neuropathic pain (Li et al., 2018) has been observed, transcriptome analysis indicates the presence of stark sex differences for neuropathic pain in both species for the gene modules and signaling pathways associated with immune responses and neuronal plasticity (North et al., 2019; Stephens et al., 2019). Evidence toward sexually dimorphic mechanisms of ion channel regulation in pain has found higher expression of some ion channels including ANO8, GRIK5, GRIN1, HCN2, KCNAB2, KCNC1, KCNG1, KCNH2, KCNK3, and PANX2 in a male-pain cohort compared to a female-pain cohort. However, it is essential to recognize that baseline differences in gene expression between the sexes could account for a certain degree of differential expression (Stephens et al., 2019). Animal models are highly valuable in this respect because differential gene expression can be directly controlled through comparisons with naïve animals. Still, species considerations are much more difficult as the abundance of ion channels and receptors involved in pain processing may be vastly different between species (Shiers et al., 2020).

To completely understand the involvement of specific genes, follow-up studies using a candidate approach must be undertaken. A candidate approach could be in the form of a genetic knockout or knockdown study using animal models, potential transgenic "rescue" experiments and the incorporation of pharmacological tools to block proteins of interest. For instance, studies have confirmed sex differences in pain modulation, including the genetic variation of *OPRK1* (encoding κ-opioid receptor) in humans (Gear et al., 1996; Sato et al., 2013) and the efficacy of κ-opioids to induce analgesia in rats with female rats exhibiting quicker analgesic onset and magnitude (Bartok and Craft 1997). Strikingly, the sex-dependent response of females to *κ*-opioid agonists may not be directly related to OPRK1, but instead the melanocortin-1 receptor gene (*MC1R*). Mogil et al., 2003 used quantitative trait locus mapping, a candidate gene strategy and pharmacological tools to reveal that the *MC1R* mediates *κ*-opioid analgesia in female mice only. Their translational studies showed that women with variations of the *MC1R* allele, associated with red hair and fair skin, also display greater analgesia from the *κ*-opioid pentazocine. However, in humans, subjects' genetic characteristics must be measured rather than assumed. For example, resistance to subcutaneous lidocaine was measured in women with red hair that were assumed to have the mutated *MCR1* gene even though genetic mutations in *MCR1* were not directly measured (Liem et al., 2005). The same group has also shown that genetic mutations in *MCR1* are associated with an increased requirement for general anesthesia in redheads (Liem et al., 2004).

Both clinicians and experimentalists report that sensitivity to pain, propensity to develop painful pathology and response to pain-inhibiting (i.e., analgesic) strategies all feature large individual differences (Elmer et al., 1998; Nielsen et al., 2008; Nielsen et al., 2009). The genetic portion of such variability can be studied using inbred mouse strains and analogous twin studies in humans (Mogil et al., 1999; Lariviere et al., 2002; Smith et al., 2004; Fillingim et al., 2008). However, these types of heritability studies have made it clear that most of the observed variance–even in the laboratory environment–is not explained by genetic factors, but environmental influences and their interaction with genes (Mogil et al., 2004). In mice, a within-

cage "order-of-testing effect" in which the first mouse in a cage tested on the tail-withdrawal test displays higher withdrawal latencies (i.e., lower sensitivity) than subsequently tested mice from that cage (Chesler et al., 2002a; Chesler et al., 2002b). We have also shown that the gender of the experimenter impacts pain behavior in mice (Sorge et al., 2014), while others have observed a similar phenomenon for human pain testing (Wise et al., 2002; Gijsbers and Nicholson 2005). Several laboratory environmental factors are also known to influence baseline nociceptive responding in mice, including housing, diet, test conditions and experimental design (for a full review see Mogil 2017). Certain aspects of these phenomena may be driven by epigenetic factors, which are known to cause changes in memory (Chwang et al., 2007), nociceptive sensitization (Chiechio et al., 2010) and behavioral responses to opioid-based drugs (Liang, Li et al., 2013). Research in epigenetics of pain has revealed that changes within an individual's environment can lead to heritable changes in gene function through processes like histone modification, DNA methylation and chromatin remodeling (Denk and McMahon 2012; Crow et al., 2013), all of which may influence replication between laboratories and translation.

COGNITIVE AND PSYCHOLOGICAL CONSIDERATIONS

The perception of pain, whether acute or chronic is a subjective experience modulated by our history and expectations (Flor 2002). To complicate matters further, individual differences in the perception of the environment despite the same physical stimuli led to vastly different pain experiences (Tabor et al., 2013; Harvie et al., 2016). In a remarkable study, Moseley and Arntz (2007) placed a cold piece of metal on the hand of subjects for 500 ms and asked subjects to rate their pain when shown either a red or blue visual cue. These were healthy participants who were not told why they were being shown the light, it was just part of the context and coincided with the application of the cold stimulus. Amazingly, for some people, pain was rated as more intense when a red light was shown as opposed to a blue light even though the nociceptive stimulus in both conditions was identical—the reason being that the evaluative context was critical for modulating the pain experience. Throughout our lives, we have learned to associate the color red with hot and potentially dangerous situations, while blue is typically associated with cool, calm and less damaging stimuli. Observations such as these have led others to investigate the individualized expression of pain, which is, in part, influenced by the emotional context such as motivation, arousal, mood, and learning (Miron-Shatz et al., 2009; Murty et al., 2010; Mirandola and Toffalini 2016).

The seminal work by Fordyce (Fordyce et al., 1973) brought into light the significance of learning in chronification of pain and its treatment. Fordyce postulated that the pain response in chronic pain is a learned behavior that can be altered using learning mechanisms such as operant conditioning. In operant conditioning, an association is formed between a (pain) behavior (e.g., verbalizations, actions and facial expressions) and the consequence of that behavior in the form of positive or negative reinforcement. In line with this, patients with chronic musculoskeletal pain increased their pain behavior when it was reinforced with positive reinforcements (i.e., attention and empathy) by the caregivers (Romano et al., 2000). Conversely, operant conditioning was used to mitigate pain behavior of chronic pain patients with musculoskeletal pain and fibromyalgia by reinforcing positive behaviors and extinguishing negative behaviors (Fordyce et al., 1973; Thieme et al., 2003; Thieme et al., 2006). Operant conditioning was also effective in healthy individuals using pressure (blood-pressure cuff) (Jolliffe and Nicholas 2004), heat (Hölzl et al., 2005; Kunz et al., 2011) and electric stimuli (Lousberg et al., 2005). This change in behavior brought on by operant conditioning elicited changes in pain-related somatosensory evoked brain potentials and are thought to involve the anterior cingulate cortex and the primary and secondary somatosensory cortices (Flor et al., 2002), but the underlying mechanisms remain largely elusive.

In laboratory animals, it is difficult to isolate and recapitulate operant conditioning as behavioral assays often involve both operant and classical (Pavlovian) conditioning (Li, 2013). However, efforts have been made to develop operant measures to analyze the affective component of pain in animals (for review see Navratilova and Porreca, 2014). One such measure is conditioned place preference (CPP), which has traditionally been considered to be dependent on a classical conditioning mechanism, but is in part, an operant measure of the affective component of pain (Huston et al., 2013). CPP was used to demonstrate in male rats with an incisional paw injury that local analgesia increases a rat's preference toward a chamber associated with pain relief (Navratilova et al., 2012). Physiologically, this motivational behavior was driven by activation of ventral tegmental dopaminergic cells and increased release of dopamine in the nucleus accumbens, which supports the hypothesis that analgesia induces negative reinforcement—elicited by relief of an aversive state—via the mesolimbic reward pathway (Navratilova et al., 2012).

Accumulating evidence suggests that pain perception is shaped by our prior experience with pain and its relief, occurring through the creation or erasure of memory traces in peripheral neurons, the spinal cord and the brain (Ji et al., 2003; Sandkuhler and Lee 2013; Price and Inyang 2015). The term "pain memory" was coined by Dennis and Melzack (Dennis and Melzack 1979) following the observation that exposure to painful irritation of the forepaw in male rats before denervation of dorsal roots in the spinal cord (rhizotomy) led to acceleration and exacerbation of the neuropathic pain. They postulated that the pre-injury irritation created a pain memory that became disinhibited once the descending inhibitory control system was disconnected due to rhizotomy. The findings here can be translated to patients suffering from phantom limb pain who retain somatosensory memories about their pain before the amputation and still experienced the pain sensation following the amputation (denervation) (Katz and Melzack 1990; Flor et al., 2006). With somatosensory memory, many amputees retain a phantom limb's sensation being immobile and in the same position as before its amputation (Katz and Melzack 1990).

Therefore, in part, phantom limb pain may derive from a proprioceptive memory mechanism where patients retain memory engrams of the limb before amputation (Gentili et al., 2002).

Physiologically, these long-lasting pain memories manifest as alterations of both the peripheral and central nervous systems (Flor et al., 2006). Peripherally, increased C-fiber afferent activity has been linked with pain sensation in amputees (Nyström and Hagbarth 1981). This finding was complemented in animals, where blocking C-fiber afferents in male rats with nerve injury attenuated hyperalgesia (Coderre and Melzack 1987). Local nerve block had a similar effect in patients with phantom limb pain where blocking the affected dorsal root ganglion led to immediate pain relief (Vaso et al., 2014). Centrally, among other changes, somatosensory pain memory elicits topographically reorganization of the primary somatosensory cortex (SI) (Ramachandran et al., 1992; Elbert et al., 1994; Halligan et al., 1994; Flor et al., 1995; Doetsch 1998). In animals, the amputation of digits in monkeys led to a reorganization in SI, where the representation of deafferented fingers was taken over by adjacent areas (Merzenich et al., 1984). In dorsal rhizotomy, the extent of SI reorganization was found to be even greater than the amputation of digits (Pons et al., 1991). Stimulation of the SI cortex has been found to induce phantom limb pain, and removal of this region ameliorated phantom limb pain (Head and Holmes, 1911; Appenzeller and Bicknell 1969), demonstrating the importance of somatosensory pain memory in phantom limb pain. Further research is still needed regarding the molecular determinants of pain memory in the context of phantom limb pain and longitudinal studies examining the relationship between pain experience and phantom limb pain.

When it comes to pain memory, children are highly vulnerable to behavioral and perceptual alterations induced by pain experience. Given the malleability of pain memories, childhood presents an opportunistic window to target pain management interventions to mitigate long-term consequences (Noel et al., 2017). Several studies have found that children who remember the nociceptive stimulus as worse than their initial experience rated the pain as greater when re-exposed to the same nociceptive stimulus (Chen et al., 2000; Noel et al., 2012; Noel et al., 2017). Interestingly, in children, pain memory served as a strong predictor of subsequent pain ratings (Noel et al., 2012; Noel et al., 2017). These findings in children are, in part, complemented by animal studies where neonatal injuries were associated with changes in nociceptive sensitivity. In rat neonates, a skin-deep injury to the hind paw led to increased sensitivity to mechanical stimuli three weeks following injury, indicative of long-term hypersensitivity (Reynolds and Fitzgerald 1995). In another study, rat neonates administered an inflammatory stimulus (i.e., complete Freund's adjuvant) showed long-term alterations in pain response during adulthood facilitated by increased spinal circuit input and sprouting nociceptive primary afferent axons (Ruda et al., 2000). Furthermore, rat neonates exposed to colorectal distension led to colon hypersensitivity in adulthood (Al-Chaer et al., 2000). Neonatal injuries in rats also led to hypoalgesia, or reduced nociceptive sensitivity, when exposed to repeated formalin injections and tested for thermal sensitivity in adulthood (Bhutta et al., 2001). Still, in its infancy, similar findings in children and neonates encourage expanding the study of pain memory during development into various species and verifying their validity for translational purposes.

We have recently developed a novel assay to assess pain memory more directly in mice (**Figure 1**) (Martin et al., 2019). This behavioral model exploits prior pain experience to alter nociceptive sensitivity and examine the influence of environmental variables. In brief, we exposed mice to a specific context and administered an acute visceral nociceptive stimulus (i.e., acetic acid) that lasted for approximately 30 min (**Figure 1A**). When mice were re-exposed to the same context 24 h later, mice displayed hypersensitivity to a thermal stimulus. The working model is that exposure to nociceptive stimuli act as unconditioned stimuli leading mice to develop a negative association with their environment. In turn, this increased their nociceptive sensitivity (when returned to that environment), a phenomenon that we refer to as *conditioned pain hypersensitivity*. Interestingly, conditioned hypersensitivity was observed exclusively in male mice, and was context specific. This model does not seem to reflect human-like nocebo phenomena because proglumide (1 and 10 mg/kg), a cholecystokinin receptor antagonist that blocks nocebo responses in people failed to block conditioned pain hypersensitivity in mice (*unpublished observations*). It also does not represent general priming of the pain system because the context where the pain occurred is critical, indicating that memory of the environment is necessary for the observed hypersensitivity. Physiologically in males, testosterone was important as castration, and blocking testosterone abolished the conditioned pain hypersensitivity. Furthermore, atypical protein kinase C (aPKC), which includes the protein kinase Mζ isoform (PKMζ), was found to be critical at both spinal and brain level as inhibition prevented conditioned hypersensitivity. Given PKMζ's postulated role in synaptic long-term potentiation and maintenance of long-term memory (Sacktor and Hell 2017), this finding adds to the accumulating evidence for the existence of pain memory and its effects on pain sensitivity.

The translational potential of our conditioned pain hypersensitivity model is exceptionally high because of contemporaneous experiments we conducted in humans that showed an excellent congruence with the mouse data (**Figure 1B**). Similar to the mouse experiments, healthy participants were exposed to the ischemic tourniquet procedure (i.e., unconditioned nociceptive stimulus) in a specified context. The next day participants returned for a second experimentation day. They were either tested by the same experimenter in the same room (i.e., same context) or a different experimenter in a different building (i.e., different context). Males tested in the same context on the second day reported increased ratings of pain intensity. This pain hypersensitivity was associated with increased stress ratings in males tested for pain sensitivity in the same context, and similar to mice was absent in female participants. These results showed a surprisingly direct translation of context-dependent conditioned

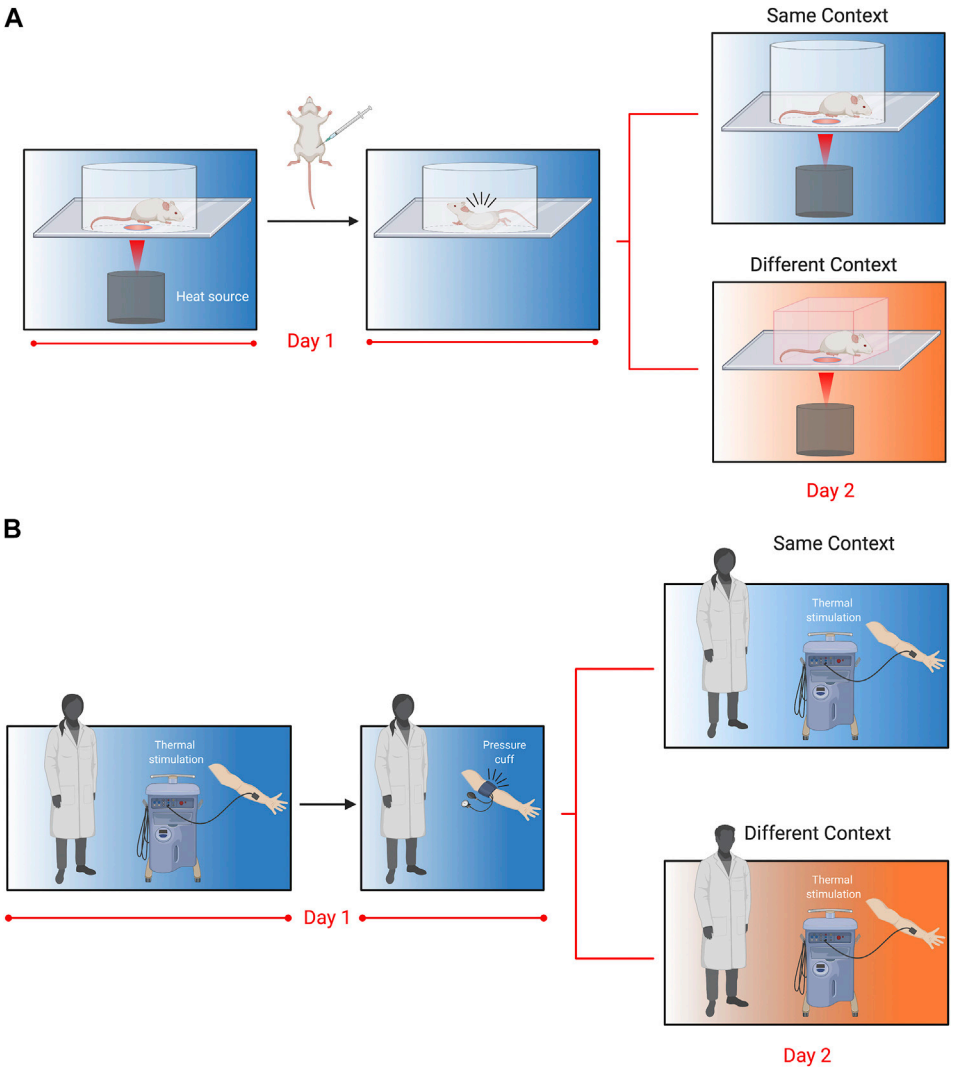

FIGURE 1 | Translational behavioral models to test pain memory in mice and humans as originally reported in Martin et al., 2019. **(A)**. Mice are placed in Plexiglas cylinders and paw withdrawal thresholds evoked by thermal stimuli are measured every 5 min for 30 min. Following baseline measurements, mice are injected with acetic acid (i.p.,; 0.9%), which causes abdominal cramps and nociceptive writhing behavior that lasts for approximately 30 min. Twenty-four hours later, mice are placed either back in the same cylinder in the same room, or in a novel cubicle in a different room, and again tested for thermal withdrawal latencies. Nociceptive sensitivity was increased in mice returned to the same context 24 h following acetic acid injection, but this was only observed in male mice. Follow-up experiments revealed that enhanced nociceptive sensitivity in males was dependent on testosterone, the stress response and atypical PKCs. **(B)**. In the human model, participants were tested for their thermal sensitivity to a heat probe placed on the volar aspect of the forearm. Volunteers were then subjected to a submaximal effort ischemic tourniquet test for 20 min. Twenty-four hours later, participants were tested for thermal sensitivity in the same room by the same experimenter, or a different room (in a different building) and a different experimenter. Participants returned to the same room rated the thermal pain as higher, but this was only observed in men and associated with their pre-test stress response.

pain hypersensitivity between the two species (Martin et al., 2019).

The studies outlined here highlight the widespread nature of pain memory and add a layer of complexity to studying pain modulation and mechanisms. On the other hand, this presents us with a unique challenge where novel strategies may be developed to improve pain outcomes by focusing on pain memory to yield positive symptom relief through learning. This is highlighted by placebo analgesia, a phenomenon where an inert treatment produces an improvement in symptoms (Levine et al., 1978). Cognitively speaking, two principal theories were developed as the basis for the activation of placebo analgesia: the expectation of pain relief and learning symptom relief by the repeated association between an active analgesic and therapeutic context (i.e., classical conditioning) (Amanzio and Benedetti 1999). One of the first studies to demonstrate placebo analgesia through conditioning was by Voudouris et al., 1989. In this study, participants were conditioned by repetitive pairings of a neutral non-anesthetic cream with electrical stimulation below their noxious threshold. Following conditioning,

participants formed an associative memory between the placebo cream and the reduced electrical stimulation, which resulted in placebo analgesia to a higher intensity of electrical stimulation. Since then, numerous laboratories have successfully demonstrated placebo analgesia using classical conditioning (Amanzio and Benedetti, 1999; Price et al., 1999; Colloca and Benedetti, 2006; Klinger et al., 2007; Jensen et al., 2012). It should also be noted that conditioning produced a more substantial placebo effect than those induced with expectation (i.e., verbal cues and suggestion) (Colloca et al., 2008). Mechanistically, expectation-induced placebo analgesia is mediated mainly by the endogenous opioid system and is reversible by administering the opioid antagonist naloxone (Amanzio and Benedetti, 1999). However, conditioning-induced placebo analgesia can activate different subsystems depending on the unconditioned stimulus. For example, pharmacological conditioning using morphine engages the opioidergic system, while conditioning using ketorolac, a non-steroidal anti-inflammatory drug (NSAID), activates a non-opioid-based mechanism. While the former is blocked by naloxone, the latter is dependent on the endogenous cannabinoid system and blocked by rimonabant, a cannabinoid receptor type 1 blocker (Amanzio and Benedetti, 1999; Benedetti et al., 2011). These findings are complemented by brain imaging studies that have demonstrated that placebo analgesia is mediated by functional changes in cortical, subcortical and brainstem structures (Petrovic et al., 2002; Wager et al., 2004; Zubieta et al., 2005; Bingel et al., 2006; Eippert et al., 2009a). These include pain and affect processing regions such as the anterior cingulate (ACC), insula, thalamus, hypothalamus, periaqueductal gray (PAG) and rostral ventromedial medulla (RVM) (Wager et al., 2004; Eippert et al., 2009a; Atlas and Wager, 2014) and other higher-order regions that include dorsolateral prefrontal cortex (DLPFC) as well as the nucleus accumbens, which mediates reward behavior (Zubieta et al., 2005). Reduced activity in the spinal cord has also been observed (Eippert et al., 2009b). Furthermore, functional brain imaging studies have identified changes within brain networks in response to placebo analgesia. For example, the activity of rostral ACC shows coupling with bilateral amygdalae and the PAG (Bingel et al., 2006; Eippert et al., 2009a), while a decrease in coupling was found between DLPFC and PAG (Sevel et al., 2015). Collectively, these findings suggest the involvement of the descending pain modulatory pathway, but the precise neural network and molecular mechanisms remain elusive. While the aforementioned studies used classical conditioning to induce placebo analgesia, recent research suggests that operant conditioning can also elicit the placebo effect (Janssens et al., 2019).

Several laboratories have attempted to generate an animal model for placebo analgesia, mainly using rodents via pharmacological conditioning, albeit with mixed results. Using morphine and other opioid agonists as unconditioned stimululi, both mice and rats have been shown to display responses resembling placebo analgesia (Miller et al., 1990; Valone et al., 1998; Bryant et al., 2009; Guo et al., 2010; Guo et al., 2011), while other studies have not (Nolan et al., 2012; McNabb et al., 2014; Akintola et al., 2019). In line with findings in humans, placebo analgesia in animals conditioned with opioids was reversed by opioid antagonists (Guo et al., 2010; Zhang et al., 2013). Further, in rats, placebo-like analgesia was inhibited by an antagonist specific to the μ-opioid receptor subtype in the rostral anterior cingulate cortex–a region implicated in human placebo responding (Petrovic et al., 2002; Wager et al., 2004; Zhang et al., 2013). Guo et al. (2010) also induced placebo-like responses in mice using a non-opioid NSAID, which was not blocked by naloxone. Overall, these animal studies demonstrate that certain aspects of placebo analgesia can be modeled in laboratory animals using classical conditioning and offer potential avenues to decipher pain memory mechanisms and the endogenous modulation of pain.

SOCIAL CONSIDERATIONS

Sigmund Freud first observed that patients with pain problems tended to have family members with pain problems (Breuer and Freud, 1893). While this observation could be attributed to genetic relatedness, similar observations have been confirmed in genetically unrelated individuals living in the same household (Merskey and Spear, 1967; Turk et al., 1987). For instance, spouses of chronic pain patients suffer pain-related symptoms at a higher percentage than the spouses of people with diabetes (Flor et al., 1987b). Indeed, observer reinforcement of pain behavior has been assumed to play a significant role in shaping the severity and duration of chronic pain in the pain patient (Flor et al., 1987a). With this in mind, it is essential to recognize that pain occurs in a social sphere and is commonly communicated to and observed by others. This may start at a young age through children observing parents and other significant persons who teach them different attitudes about pain perception and responding to physical ailments (Baranowski and Nader, 1985). Viewed in this light, immediate family members with chronic pain may act as pain models to shape a child's future pain behavior and experience.

One's pain may be considered a personal experience, but it is rarely private and behavioral responses to pain function to communicate information to others within our social environment. The outward expression of pain universally indicates distress, which may elicit emotional reactions and caregiving actions from those around us. Humans communicate their pain experience through several different behaviors, including touching an injured body part, expressing discomfort through facial grimaces, and using vocal interjections such as "ouch." Laboratory studies largely pioneered by Ken Craig and his colleagues have comprehensively documented the effect of social modeling and observation on psychophysical and psychophysiological responses to pain, finding that exposure of subjects to models exhibiting "tolerance" or "intolerance" to pain dramatically match the perceived level of tolerance (Craig and Weiss, 1971; Craig et al., 1975; Craig and Prkachin, 1978; Craig and Patrick, 1985; Goodman and McGrath, 2003). This is similar to results observed in rodents, where subjects match pain behavior and emotions when tested within a social environment.

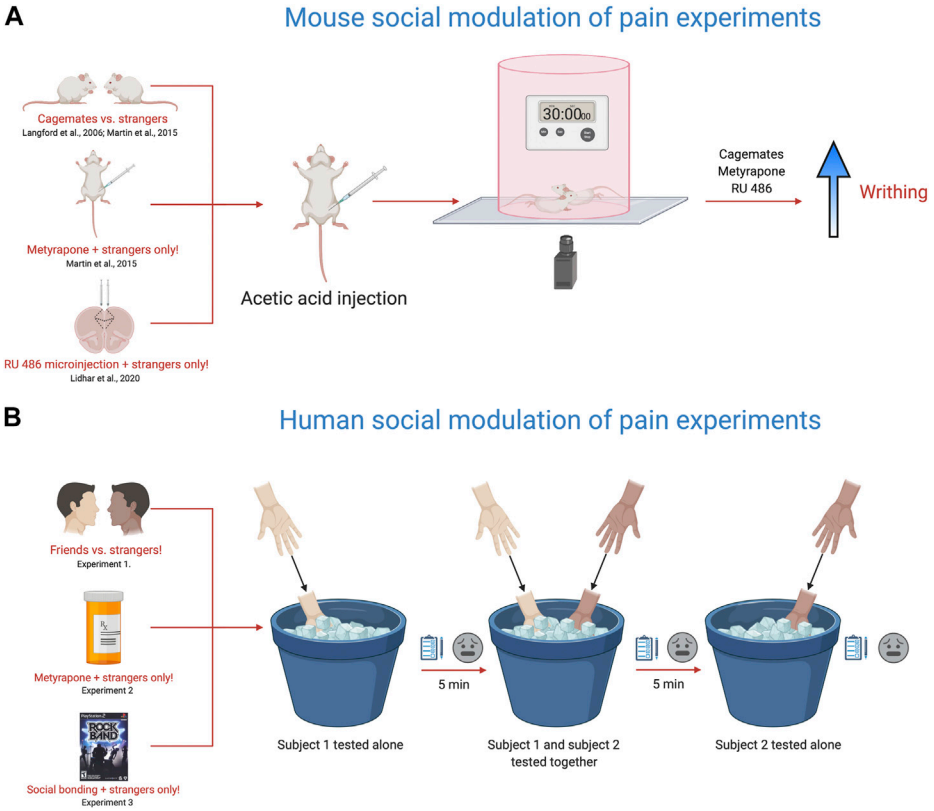

FIGURE 2 | Translational behavioral models to test the influence of social context on pain responses. **(A)**. Using the acetic acid (0.9%) writhing test and manipulating social partner, drug pre-treatment or targeting specific brain areas, the nociceptive responses of mice are altered within the social environment. Placing two mice in a cylinder and injecting both mice with acetic acid (0.9%) enhances the nociceptive response of cagemates, but not strangers as originally reported in Langford et al., 2006. Further work with this model, showed that metyrapone, a glucocorticoid synthesis inhibitor recapitulated the cagemate effect in strangers, with metyrapone-injected stranger mice showing increased nociception (Martin et al., 2015). In addition, targeted injections of RU-486, a glucocorticoid receptor blocker also facilitated the nociceptive response of stranger mice (Lidhar et al., 2020). **(B)**. The cold pressor task was used to measure pain ratings in friends vs. strangers, strangers pre-treated with metyrapone and in strangers after engaging in a shared social experience (Martin et al., 2015). The experiment consists of three testing trials using the cold-pressor task. In the first test trial, subject 1 is tested alone by placing their non-dominant hand in the cold pressor for 30 s and then rating the pain intensity and unpleasantness using a visual analog scale. In the second trial, the second subject is brought into the room and both subjects are tested together by placing their non-dominant hand in the cold pressor for 30 s and then rating the pain intensity and unpleasantness using a visual analog scale. For the third trial, the second subject is tested alone by placing their non-dominant hand in the cold pressor for 30 s and then rating the pain intensity and unpleasantness using a visual analog scale. During trials where the participant is tested alone, the other subject is not present in the room. In calculating the overall pain ratings, the mean difference between a subject's trial when tested alone was subtracted from their trial when tested with another participant. Friends tested together rated their pain higher than strangers, while metyrapone pre-treatment and playing the video game Rock Band (shared social experienced) enhanced the pain ratings in strangers. Overall, these models provide a new framework for studying the influence of social context on pain and offer insight into the fundamental mechanisms that engage the neural circuits responsible for pain modulation via social context. Given the complex nature of social context and social interactions on pain sensitivity in animals and people, dissecting their integral role in mediating pain outcomes is of critical importance.

The social modeling of laboratory pain in humans is strikingly similar to experiments performed in rodents, particularly mice (**Figure 2A**). When mice are tested in the presence of a familiar mouse also given a nociceptive stimulus, the nociceptive behavior of both mice is increased. This was first reported in 2006 and provided extensive evidence that nociceptive behaviors in mice are changed through social interactions and observation (Langford et al., 2006). Mice given a weak acetic acid injection into the stomach display twisting of the abdomen or writhing behaviors. Placing mice in an arena in dyadic pairings of cagemates enhanced the number of writhing episodes evoked by the acetic acid when compared with stranger dyads or mice tested alone. However, this enhancement was blocked by placing an opaque barrier between the cagemate pair of mice, suggesting that social observation was necessary for increased pain responses in cagemates (Langford et al., 2006). In the same study, when the target mouse was placed together with a mouse given a higher concentration of the inflammatory agent formalin, the target mouse licked the paw more compared to when paired with a mouse given the same concentration. In the reverse direction, when a target mouse was placed with a mouse given a weaker formalin concentration, the target mouse licked the paw less. This study showed a bidirectional modulation of nociceptive behavior, reminiscent of "tolerance matching" in the human social modeling experiments (Craig and Prkachin, 1978). While these findings may be explained by vicarious arousal or social

modeling, they also may subserve processes related to empathy, as we've previously suggested (Martin et al., 2014; Sivaselvachandran et al., 2018a; Sivaselvachandran et al., 2018b).

It is conceivable that "pain communication" and "empathy" are synonymous concepts. Accordingly, our laboratory has been studying pain empathy in mice as a means to understand social-communicative pain behaviors (Martin et al., 2014; Sivaselvachandran et al., 2018a). The evidence for empathy in rodents shows that mice and rats consistently imitate arousal states and behaviors of one another; they will even sacrifice personal gain to relieve the distress of a fellow rodent (Langford et al., 2006, Jeon et al., 2010, Ben-Ami Bartal et al., 2011). In one line of research, we have been examining why unfamiliar (or stranger) mice do not show enhanced nociceptive responses when tested in each other's presence. We came up with a relatively simple hypothesis–the social interaction of strangers is stressful, and this blocks the social facilitation of nociceptive responses, as observed in cagemates. To interrogate this hypothesis, we used a drug called metyrapone, which blocks the synthesis of cortisol and is a standard treatment for Cushing's syndrome. To start, we pretreated all mice with metyrapone and tested mice in various social contexts (alone, cagemates, strangers) and dyadic pain status (i.e., only one mouse in pain or both mice in pain). In line with our hypothesis, stranger mice pretreated with metyrapone showed pain facilitation *on par* with cagemates (**Figure 2A**). Therefore, preventing glucocorticoid synthesis seemed to suppress the stress response induced by a stranger's presence and ultimately promoted the display of empathic behavior in mice (Martin et al., 2015). Further, we have recently identified the prelimbic subdivision of the medial prefrontal cortex as an important node in controlling this behavior. Targeted microinjections of RU 486, a glucocorticoid receptor blocker into the prelimbic cortex enhanced nociceptive behavior in stranger dyads, but did not alter nociceptive responses of mice tested alone (Lidhar et al., 2020).

In support of our translational efforts, we designed a human experiment that paralleled our mouse experiments (**Figure 2B**). We recruited healthy participants to determine whether pain ratings were altered when subjects were tested in the presence of a friend or a stranger, similar to our mouse cagemate/stranger experiments. In line with our mouse findings, friends tested together–on the cold pressor task–reported greater intensity and unpleasantness than strangers (Martin et al., 2015). We then randomly divided a separate group of strangers into two groups and administered either 750 mg oral metyrapone or placebo, 60 min before cold pressor testing. Participants receiving metyrapone reported significantly increased pain intensity compared to those pretreated with placebo. These findings were congruent with our mouse experiments suggesting that blocking glucocorticoid synthesis increased pain sensitivity in strangers. Metyrapone also increased other non-verbal pain behaviors such as facial grimacing, handholding and rubbing when strangers were tested together. Finally, we conducted a third study and allowed participants–all strangers–to engage in a brief social gaming experience, where they bonded over the videogame RockBand. We found that stranger dyads who played RockBand together demonstrated increased stimulus intensity ratings, naturally alleviating the social stress induced by the mere presence of a stranger.

In Martin et al. (2015), we attempted an experiment where we administered intranasal oxytocin to participants, but pain responses were not enhanced when tested with a stranger. With regard to empathy and social communication, the neuropeptide oxytocin has received the most attention. It has been widely referred to as the *"love hormone"* because it modulates feelings of social attachment, trust, intimacy and empathy (Bartz et al., 2010; Hurlemann et al., 2010). Ultimately, our oxytocin experiment in humans did not work. We attempted similar–*unpublished*–experiments in mice using oxytocin antagonists to block the social facilitation of pain in cagemates, which also did not work. However, the pain modality and testing context may significantly influence oxytocin pain experiments and ratings. For instance, intranasal oxytocin increased the perceived pain intensity when subjects were asked to imagine that photographs of hands and feet in painful or nonpainful scenarios belonged to someone else (Abu-Akel et al., 2015). Interestingly, this same study showed that when participants were asked to imagine that the appendages were their own, oxytocin did not influence perceived pain intensity. There are also similar rodent experiments where oxytocin administration has been shown to enhance observational learning when a pain stimulus is delivered to another mouse (Pisansky et al., 2017). The observer mouse was administered intranasal oxytocin 30 min before watching another mouse receive a 0.8 mA of electric shock; this caused the observer mouse to exhibit profound freezing behavior, a common sign of fear in rodents. The effects of neuropeptides such as oxytocin are highly complex and as such we have not gone into great detail in this section.

We have previously reviewed the translational aspects of empathy in humans and mice including oxytocin (Sivaselvachandran et al., 2018b) and the studies mentioned here provide evidence for similarities between mouse and human subjects.

CONCLUSION

The primary objective of the current review was to provide an overview of the translational approaches used in pain research, not necessarily clinical (pathological) pain. Of course, clinical pain is the problem, but understanding pain modulation and the relevant models of pain processing in healthy individuals is equally as important. For instance, one of the greatest risk factors for the development of chronic pain may be concurrent or past pain (Puntillo et al., 2001; Katz and Seltzer 2009), with the anxiety of impending pain crucial in determining whether pain sensitivity is enhanced (Ploghaus et al., 2001). Thus, studying pain-related behavior in healthy individuals in

response to prior pain, trauma or social reinforcers are of physiological, and potential therapeutic value. Creatively developing animal models or testing similar phenomena in humans may also increase the validity of animal studies. These types of translational studies will enhance our understanding of pain perception and ultimately lead to improved treatment methods.

Rarely have behavioral pain experiments compared pain modulation using animals and humans in a single paper. In the translational studies that we have conducted, all experiments were performed in-house or in close collaboration with other labs, where the primary author designed and directed all research. Collaboration was especially important because there are considerations for human research that must be taken into account that are not so obvious for rodent researchers and vice versa. While we recognize that it may not always be possible to directly compare multiple species for every paper, a more concerted effort should be made within this domain. Conducting behavioral experiments within a single lab (or with the same personnel) allows for the most optimal experiments to be designed and allows for efficient troubleshooting of procedural problems so that researchers can assess whether translation does or does not exist, or a methodological problem persists. Once translation is suspected to be possible (or not), initial findings can then be replicated and extended by other labs. It is prudent to point out that many investigators may refrain from implementing such an approach in their laboratories because these experiments are laborious, high focus, and require significant numbers of staff to perform. However, where possible we believe that the development of novel translational models to assess pain behavior should be prioritized as they are not technically challenging and may be performed by adequately trained undergraduate researchers. From a behavioral perspective, having mouse and human experiments conducted in parallel is an exciting approach for the initial assessment of model relevancy and species translation.

There exists a diversity of available animal models of human chronic pain but understanding what animal models are relevant to human pain modulation is essential to provide more information about the development of chronic pain. To fully understand pain phenotypes and modulation, a full battery of assays, including multiple modalities and injury types, should be considered (Mogil et al., 2006), and because various artifacts can confound some but not other assays (Callahan et al., 2008). Newer approaches, such as operant techniques (Murphy et al., 2014) or measuring facial expressions of pain (Langford et al., 2010), might be beneficial adjuncts to the standard current batteries. However, most of the papers that use these assays do not conduct conceptually similar experiments in humans. Assessment of facial pain expressions may be a highly valuable translational tool, as pain faces are evolutionarily conserved and would allow for similar modalities of assessment using analogous metrics in animals and humans (Chambers and Mogil 2015).

The reliance on current mouse models, especially inbred mice may not be appropriate for modeling complex human conditions with the explicit purpose of clinical translation. The individual differences commonly observed with human pain conditions are difficult to reproduce with the inbred homogeneous animal populations, particularly genetic and environmental variability (and their interaction). While inbred mice have been preferentially selected (and even necessary) for molecular genetic studies, the strong preference for inbred subjects derives from inertia and the assumption that outbred stocks would amount to increased phenotypic variation and necessitate the use of higher sample sizes. This is certainly a reasonable assumption; however, it is not supported by the current evidence since the coefficient of variability comparing phenotypic outcomes between inbred and outbred populations are similar (Jensen et al., 2016; Tuttle et al., 2018). While mechanistic studies are hugely important for the development of novel therapies and offer a complete understanding for the basis of disease, they may be of little use if those therapies or mechanisms do not translate across species. In this regard, variability in laboratory (i.e., environmental) conditions may improve phenotypic heterogeneity to better capture the variability observed by human disease populations. These include differences in food type, bedding material, cage size, humidity, temperature, light cycles and identification method (Richter et al., 2011). Thus, we are suggesting that the use of heterogeneous animal—especially mouse—populations may provide a better way forward in assessing the generalizability of results across experimental conditions where treatments—especially pharmacological—are the goal.

Aside from rodent research, using populations of domestic animals that display naturally occurring diseases may also be a potential future direction of pain studies. Many domesticated animals display pathophysiological similarities to human pain conditions including bone cancer-related pain and degenerative joint disease (Kol et al., 2015; Klinck et al., 2017). Examining these naturally occurring conditions in various animal species may be part of the solution for conducting effective translational research. Domesticated animals are genetically diverse and are subject to varying environmental conditions. Many domesticated animals are treated like family and are exposed to similar environmental conditions as humans. Watkins et al., 2020 recently showed that a novel DNA-based therapy was well-tolerated, safe and effective for the treatment of advanced osteoarthritis in companion dogs. Limiting the use of companion animals to the sphere of randomized control trials would offer a distinct advantage for the discovery and validation of novel therapies for the treatment of naturally occurring pain conditions such as arthritis and cancer-bone pain (Kol et al., 2015); this would offer a giant step forward. Of course, the widespread use of companion animals would be met with heightened sensitivity and ethical issues, but their inclusion has the added benefit of potentially finding therapies for our most beloved pets as well as chronic pain patients. The inclusion of domesticated animals may increase the validity of findings to offer a higher degree of translational success.

In chronic pain management, our goal is to treat the individual as best we can; however, factors such as experience with pain, psychological profile, and the social environment cannot be accounted for in the same manner as biological targets. These factors drastically alter pain responses and the way we treat individual pain patients, especially when they override or interact with drug treatments. Conclusions drawn from various translational studies have successfully shown the influences of empathy, memory, and individual differences on subjective pain experiences. Animal studies are often criticized for the lack of biological validity, but we have had the most success in developing cognitive and social modulation of pain experiments that translate between species. By focusing a portion of future pain research into developing highly translational pain models, we may drive the enhancement of pain therapeutics and conclusions drawn from animals to humans.

AUTHOR CONTRIBUTIONS

LM conceived of the manuscript. CC wrote the psychological considerations section. HD wrote the biological considerations section. LM wrote the abstract, introduction, social considerations and conclusion sections. LM designed and created the figures using biorender.com. All authors edited and commented on the final version of the manuscript.

FUNDING

This research was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC; RGPIN-2016–06284 to LM) and Canadian Institutes of Health Research (CIHR; PJT-166171 to LM), and the Canada Research Chairs program (950-233114 to LM).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

REVIEW
published: 05 August 2021
doi: 10.3389/fphar.2021.705743

Pain Treatment in the Companion Canine Model to Validate Rodent Results and Incentivize the Transition to Human Clinical Trials

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One of the biggest challenges for analgesic drug development is how to decide if a potential analgesic candidate will work in humans. What preclinical data are the most convincing, incentivizing and most predictive of success? Such a predicament is not unique to analgesics, and the pain field has certain advantages over drug development efforts in areas like neuropsychiatry where the etiological origins are either unknown or difficult to ascertain. For pain, the origin of the problem frequently is known, and the causative peripheral tissue insult might be observable. The main conundrum centers around evaluation of translational cell- and rodent-based results. While cell and rodent models are undeniably important first steps for screening, probing mechanism of action, and understanding factors of adsorption, distribution metabolism and excretion, two questions arise from such studies. First, are they reliable indicators of analgesic performance of a candidate drug in human acute and chronic pain? Second, what additional model systems might be capable of increasing translational confidence? We address this second question by assessing, primarily, the companion canine model, which can provide particularly strong predictive information for candidate analgesic agents in humans. This statement is mainly derived from our studies with resiniferatoxin (RTX) a potent TRPV1 agonist but also from protein therapeutics using a conjugate of Substance P and saporin. Our experience, to date, is that rodent models might be very well suited for acute pain translation, but companion canine models, and other large animal studies, can augment initial discovery research using rodent models for neuropathic or chronic pain. The larger animal models also provide strong translational predictive capacity for analgesic performance in humans, better predict dosing parameters for human trials and provide insight into behavior changes (bladder, bowel, mood, etc.) that are not readily assessed in laboratory animals. They are, however, not without problems that can be encountered with any experimental drug treatment or clinical trial. It also is important to recognize that pain treatment is a major veterinary concern and is an intrinsically worthwhile endeavor for animals as well as humans.

Keywords: cancer pain, osteoarthritis pain, resiniferatoxin (RTX), TRPV1, transient receptor potential vanilloid type 1 receptor, opioid, palliative analgesia, activity monitoring

OPEN ACCESS

Edited by:
E. Alfonso Romero-Sandoval, Wake Forest School of Medicine, United States

Reviewed by:
Abdallah El-Sayed Allam, Tanta University, Egypt
Anthony H. Dickenson, Independent researcher, London, United Kingdom

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Specialty section:
This article was submitted to Translational Pharmacology, a section of the journal Frontiers in Pharmacology

Received: *06 May 2021*
Accepted: *14 July 2021*
Published: *05 August 2021*

Citation:
Iadarola MJ, Brown DC, Nahama A, Sapio MR and Mannes AJ (2021) Pain Treatment in the Companion Canine Model to Validate Rodent Results and Incentivize the Transition to Human Clinical Trials. Front. Pharmacol. 12:705743. doi: 10.3389/fphar.2021.705743

INTRODUCTION

Background and Scope

The companion canine model in pain research is a relatively new addition to the field of analgesia research. In this report we examine the foundational studies that led us to develop the companion canine model, the evolution of this model, provide examples of its use in translation of novel analgesics to phase one trials, outline various other aspect of the model in terms of the pain research ecosystem, and briefly discuss other aspects and other large animal models that may provide potential insights into new indications for analgesic use.

Development of the companion canine model Karai et al. (2004), Brown et al. (2005) began subsequent to our discovery of the calcium overload mechanism for selective inactivation of TRPV1-expressing neurons, axons, or nerve terminals by RTX (Olah et al., 2001). TRPV1 is a heat and inflammation sensitive ion channel Caterina et al. (1997) that is highly expressed by nociceptive primary afferents Cavanaugh et al. (2011a), Cavanaugh et al. (2011b), particularly Aδ and C-fiber nociceptive neurons in the dorsal root and trigeminal ganglion (Mitchell et al., 2014). The companion dog model was developed to test this novel mechanism in a species and a pain condition more closely aligned clinical pain problems in humans, rather than transition from analgesic rodent results directly to human trials. After evaluation of RTX, use of the companion dog model was extended to analgesic assessments of a protein therapeutic agent Substance P-Saporin (Mantyh et al., 1997; Allen et al., 2006; Brown and Agnello, 2013). Saporin is a plant-derived toxin that inhibits protein synthesis. Attachment of the neuropeptide Substance P directs the conjugate to substance P receptor-expressing neurons. When injected into the CSF around the spinal cord the toxin-peptide conjugate is internalize by agonist-mediated receptor endocytosis and the toxin is eventually delivered to the cytoplasm where it stops protein synthesis (Yaksh et al., 2017). This mechanism is used to delete second order spinal cord neurons that are critical in transmitting nociceptive signals to the brain. Again, a novel approach to pain control that could benefit from evaluation in a veterinary canine clinical pain model. The ultimate translation to humans of these two agents led to two divergent results: for the small molecule ion channel activator translation has progressed to multiple clinical trials (NCT00804154; NCT02522611; NCT03542838) whereas the protein therapeutic underwent an abbreviated trial in human cancer pain patients but ended without positive findings (NCT02036281). The crux of the matter partially resides in the *details of drug administration* and tissue distribution and localization of the target receptor. Both agents are interventional and require precise injections at, or near, the site of the intended target. The elements of successful administration and mechanisms of action will be examined in detail.

This review mainly focuses on these two interventional agents and their use in canine disorders that exhibit a prominent pain component. We will not examine to any great extent studies performed on cats, and the reader is referred to other reviews to access the feline literature (Klinck et al., 2017; Birder and Andersson, 2018; Klinck et al., 2018; Lascelles et al., 2018). However, we briefly consider work done in swine, goats and horses. We also acknowledge that, in the course of drug development efforts, many studies of analgesic agents are conducted with purpose-bred dogs, especially to fulfill the toxicology requirements of an Investigational New Drug (IND) application to the Food and Drug Administration (FDA). However, the results of such evaluations, while undeniably important, frequently do not include investigation of efficacy in a companion canine pain disorder, are generally proprietary, and are often unpublished. While some published toxicological studies are referred to throughout this review, improved access to such data may facilitate future analgesic drug development efforts.

Where Do Companion Models Fit in Drug Development Schemes? One of the main questions translational pain scientists might have about the topic of large animal models is why and in what ways are canine models superior to current approaches? The answer is that these models are not necessarily superior, rather they play a different role in the development process than rodent models. One place for the companion canine model in translational drug development efforts is shown in **Figure 1**. When interposed between the early developmental stages and a potential phase one clinical trial, positive canine results provide strong validation of preceding cellular and rodent model data. This is especially true for the interventional toxin approaches outlined here. As noted, the idea of stepping from rodent chemo-axotomy or neuronal deletion an equivalent trial in human spinal cord or ganglion is a large leap despite obtaining strong efficacy and safety in rodents and healthy laboratory canines. The use of companion canine models does not need to be a sequential step in the development process. Many questions can be answered to improve human phase two and phase three clinical trial designs by using companion animals in parallel with the conduct of an ongoing phase one or two trial. Examples include evaluations of drug/drug interactions, dosage protocols (frequency, escalation paradigms), and side effect monitoring; all can inform and accelerate human drug development if deliberately thought out and implemented early on. Our experience the more novel interventional agents was that evaluating performance of these approaches in a transitional model closer to the human greatly facilitated our understanding of administration procedures, dosing parameters and safety well in advance of both the IND campaign and first-in-man human clinical trials in the intrathecal compartment. The companion canine model filled this critical gap by treating naturally occurring disease in (often elderly) animals with pre-existing conditions. Beyond this, the anatomy of a dog or pig is much more comparable to human, and better suited to examining interventional agents. There are also therapeutic gaps for effective treatments for animals with painful conditions including palliative care for cancer and control of osteoarthritis pain that need to be filled. Other than opioids or NSAIDS, no other effective treatment for pain from advanced cancer is available, and less than satisfactory pain control is available for arthritis. This situation is identical to that in humans and the use of companion canine models allows the unmet needs of *both* species

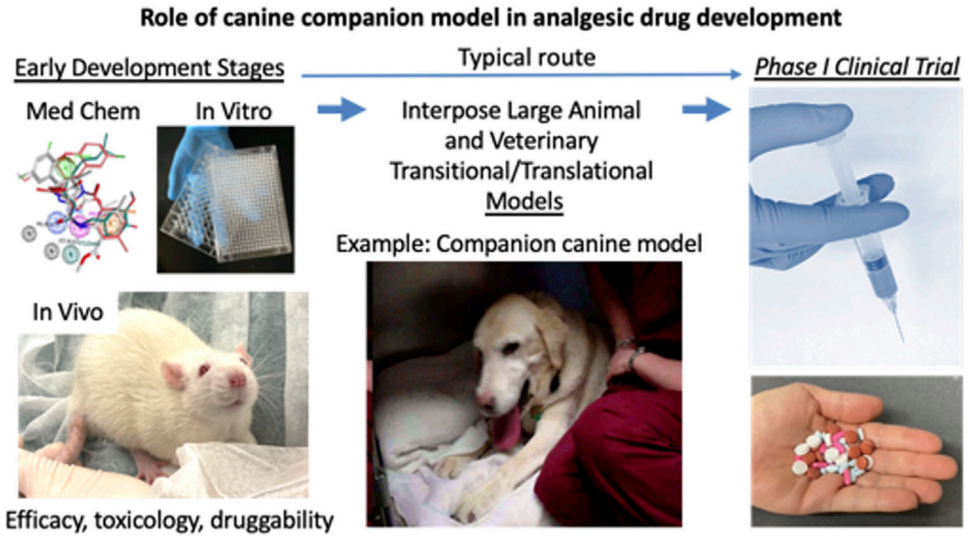

FIGURE 1 | Role of companion canine model in analgesic drug development. The drug development process progresses from left to right. The early development steps include, discovery, medicinal chemistry, *in vitro* screening, *in vivo* testing in rodent models, and optimization for target engagement, adsorption, distribution, metabolism, excretion. Usually, after manufacturing processes and toxicology are conducted, the candidate molecule goes into phase I testing. What is often lacking, despite positive result from mouse or rat studies is a clear indication of whether the compound produces analgesia in clinical pain states. These would be indications arising from natural diseases. To address this gap the veterinary companion canine model is interposed between the two major milestones in drug development. The two main clinical conditions that have been used for analgesic drug evaluation are pain from osteosarcoma and osteoarthritis. These conditions both impact motor activity and performance which provide obvious endpoints for evaluation. Since the pain from the conditions affects activities of daily living, the owners have multiple parameters from which to judge improvement in their animal's behavior. Formal, validated scales have been developed for evaluation of both pain severity and pain interference with activity (Brown et al., 2007; Brown et al., 2008; Brown et al., 2009; Brown et al., 2010; Brown et al., 2013a; Brown et al., 2013b; Brown, 2014). Other parameters available include objective measures such as force plate determinations of weigh bearing and analysis of video recordings. Because a weight bearing limb (or limbs) is involved in both disorders and either a fore or hind limb can be affected, gait and force plate measurements sometimes can be difficult to obtain in a reproducible or reliable manner (Iadarola et al., 2018). However, the range of potential available endpoints is broad enough to be adapted to a variety of conditions.

to be addressed. Some of the advantages of the companion canine model and comparison to rodent models are listed in **Table 1**.

RESINIFERATOXIN FOR INTERVENTIONAL, PERSONALIZED PAIN MANAGEMENT

Foundational Studies Defining the RTX Mechanism of Action

The eventual clinical use of RTX had its beginning in our basic research into the question: How does pain start? It was well appreciated that nerve endings and axons in the skin and deep tissues are the physical, cellular-level structures that receive and transmit painful stimuli originating from frank tissue damage or external sources (e.g., heat). However, the range of effector molecules in the nerve endings responsible for transducing noxious stimuli into electrical impulses remained to be defined at the molecular level. In 1997, the TRPV1 receptor was cloned by Caterina et al. (1997), and starting from this sequence, a full-length cDNA was isolated from rat dorsal root ganglion and we made a fusion protein with the mammalian codon-optimized "enhanced" Green Fluorescent Protein (TRPV1-eGFP) (Olah et al., 2001). We used the fusion protein to make a stably expressing cell line for live cell imaging. At that time, our experiments were more curiosity-driven rather than consciously translational. TRPV1 conducts calcium ions and, as an aside, making the cell line required use of a weak promoter to prevent overexpression of TRPV1. We found that overexpression with the cytomegalovirus (CMV) promoter caused the cells to become leaky to calcium and undergo apoptosis. Thus, in the first set of cultures using the CMV plasmid, cells that retained the TRPV1 insert were negatively selected and those that recombined the cassette to remove the TRPV1 portion of the insert but retain the antibiotic resistance gene were positively selected. Substitution of a minimal metallothionein promoter for the much stronger CMV promoter generated a viable cell line that expressed visible amounts of the TRPV1-eGFP fusion protein that was functional and produced a robust calcium influx upon activation (Olah et al., 2001).

Based on observations made by vital imaging of the stable TRPV1-eGFP cell line and primary cultures of DRG neurons (many of which express TRPV1) it became apparent that binding of RTX to TRPV1 opened the ion channel and induced a substantial calcium overload. Exposure of the culture to RTX could kill the cells in a matter of minutes Olah et al. (2001) and RTX was much more potent compared to capsaicin, ~500 times in this assay (Kárai et al., 2004). The idea of using RTX to effectively manipulate, in fact, literally kill TRPV1 neurons or their processes for pain control became apparent from watching RTX-induced calcium cytotoxicity on the stage of a microscope

TABLE 1 | Advantages of companion animal models.

Canine model	Attributes and elements	Rodent models
Owner is familiar with animal's activities of daily living (ADL)	Activity levels eating activities and food preferences sleeping, gait and movement	Monitoring of activity is possible in the home cage, open field or by other devices
Owner is familiar with animal's "personality"	Emergence of neurological or "psychiatric" problems may be evident	Must actively assess presence of anxiety or depression
Naturally occurring diseases (actually not models)	Pathologically similar to human disorders and occur over a long time period. No need for artificial inducing agents	Needs inducing agents, e.g., carrageenan inflammation or iodoacetate or kaolin for joint osteoarthritis models
Exhibit complex behaviors spontaneously	Pets will seek out the owner and respond to family or visitors, validated outcome measures are available	Exhibit complex behaviors, need experimental settings or apparatus for measurement
Many breeds, genetic diversity	Higher prevalence of disorders in some breeds can be used to enrich for recruitment	Outbred strains or inbred strains can be used to explore specific traits
Tissues more similar to human	At the histological level, dog cells are more similar to human than are rat cells	Tissues less similar to human. There are differences in transcriptome between the 3 species iadarola et al., (2018)
Efficacy measures can be judged by multiple observers in the home or clinic	Nearly continuous monitoring is possible. Can be correlated with activity monitor readouts	Efficacy generally measured by evoked responses to acute stimuli, inflammation and incision models for hyperalgesia, models of joint pain, chemotherapy induced peripheral neuropathy etc. Brown et al., (2008), Brown et al., (2013a), Brown et al., (2013b)
Allows better estimation of starting dose in human phase I trial	The doses used in the companion animal model were almost identical to those use in human trials for cancer pain and osteoarthritis	Provide initial dose-response of candidate drugs that may approximate starting dose in humans
Positive results provide strong incentive to proceed to human clinical trials or provide an indication for a "no go" signal	This is an important advantage. Clear-cut analgesia in the canine companion model is a strong predictor of positive translation in human trials	Positive results begin the process of translation

using live cell imaging of the TPRV1-eGFP cell line (Olah et al., 2001). This result launched a series of *in vivo* studies in rats in which the effects of RTX on peripheral nerve endings in hind paw Pecze et al. (2009), Cruz et al. (2014), Raithel et al. (2017), TRPV1 neurons in trigeminal and lumbar dorsal root ganglia Karai et al. (2004), and perineural routes of administration Neubert et al. (2008) were explored. In all of these cases RTX produced significant and substantial analgesic activity Iadarola and Mannes (2011), Iadarola and Gonnella (2013) and these results formed the underlying impetus for development of the companion canine model and for a human clinical trial in cancer pain.

Preclinical Animal Models for Translation of RTX

Once the mode of RTX action was clarified and preclinical rodent studies showed efficacy, the idea of conducting a human clinical trial by injecting RTX somewhere along the route of the nociceptive primary afferent neuron gained momentum. As noted, there were reservations about killing cells in the DRG centering around the possibility of generating a denervation hyperesthesia or a synaptic rearrangement in the spinal cord dorsal horn that would be deleterious in some way. We had not seen this in the rat studies. Even when we unilaterally injected RTX directly into the trigeminal ganglion Karai et al. (2004) we did not observe abnormal scratching behavior directed towards the facial dermatomes nor did we see a unilateral neglect syndrome. The analgesia also was durable and did not diminish over time. Using the capsaicin eye wipe test, no loss of analgesic efficacy was detected in a group of trigeminally injected rats observed for a full year Karai et al. (2004) and no impairment of the blink reflex was seen Karai et al. (2004), Tender et al. (2005), Bates et al. (2010), indicating fiber-type selectivity of RTX treatment. Long duration results also were obtained with lumbar intrathecal injections and inhibition of hind paw thermal nociception as the endpoint. In the paw, thermal analgesia occurred without affecting mechanical withdrawal in the von Frey hair test. These rodent studies formed the basis for additional transitional studies in monkey and dog.

RTX injections made directly into the trigeminal ganglion of rhesus macaques further impelled the therapeutic development of RTX (Tender et al., 2005). Here, target engagement in the trigeminal system was assessed by the capsaicin eye wipe test. As in the rat, unilateral trigeminal injection produced a unilateral loss of capsaicin-induced eye wipe. The analgesic effect was durable and lasted at least 3 months. There was also a loss of peripheral plasma extravasation indicating that TRPV1 neuronal cell bodies had been exposed to RTX and were deleted by the intraganglionic injection. This was confirmed upon necropsy and immunocytochemical staining of the ganglion. A significant loss of TRPV1-immunoreactive neuronal perikarya was observed. The analgesia and TRPV1 neuronal deletion occurred without affecting healing of the scalp incision, other facial functions, or swallowing or feeding. Again, these results, while strongly encouraging, were still in the arena of experimental nociception rather than a test in a spontaneously occurring disease entity.

This knowledge gap was rectified in the first canine study which tested the efficacy of intrathecal RTX in eight companion dogs with osteosarcoma, debilitating osteoarthritis, or both (**Figure 2**). The owners had brought their animals to the veterinary clinic because pain was not well controlled after exhausting standard of care

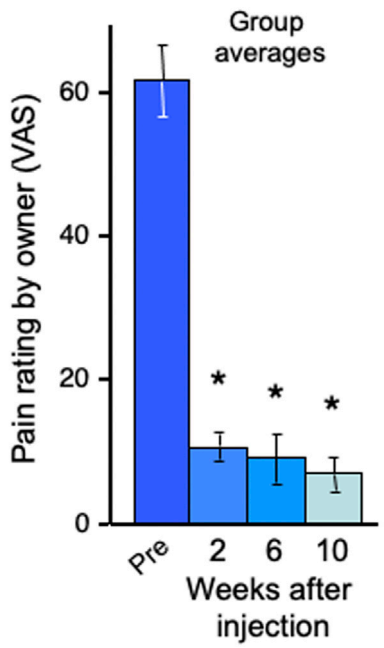

FIGURE 2 | Efficacy and long duration analgesic action of RTX in companion canine model. RTX analgesia was tested in naturally occurring neoplasms and/or osteoarthritis in the dog after a single intrathecal dose of a single intrathecal dose of RTX (1 μg/kg) administered under general anesthesia via cisternal puncture. Reports by the owners using Visual Analog Scale rating demonstrated a sustained reduction in pain in the eight dogs. The bars represent the average rating ±SEM (*ANOVA with Scheffe's post hoc test; $p < 0.05$). The animals initially presented with limb guarding as they walked, but this improved over time and daily activity increased, as was evident from video recordings. In fact, the entire demeanor of the dogs appeared improved following intrathecal RTX treatment. The efficacy of RTX action was further demonstrated by discontinuation or reduction in the use of supplementary analgesics (opioids and NSAIDs in all eight dogs). As the time points on the *X* axis show, the RTX-induced analgesia did not diminish despite neoplastic advancement. These data formed a bridge between the initial rodent data Karai et al. (2004) and the human clinical trials in cancer pain as depicted schematically in **Figure 1** and with preliminary human result in **Figure 3**.

options. For these animals a single 1 μg/kg dose of RTX was infused over 10 min into the cisterna magna. Pain intensity was rated by the owners on a 0 to 10 visual analog scale before, and at 2, 6, and 10-weeks post injection. A significant analgesic action was observed at each post-injection time point and amounted to an ~85% decrease compared to the pre-injection pain rating (**Figure 2**, from Karai et al., 2004). These data show that the analgesic action of a single injection was durable and lasted for the remainder of the animal's lifetime. In that same study, the actions of RTX were also examined at the cellular level *in vitro* in primary cultures of human DRG neurons. RTX produced a marked and sustained increase in calcium influx in a subpopulation of DRG neurons but nearby, nonresponding, non-TRPV1-expressing neurons maintained normal calcium levels. This further added to the idea that RTX could be used as an interventional analgesic in humans. These data represented a progressive series of steps from cell-based assays, to rodent models, to clinical pain in companion canines, to human DRG neurons but a key element in the decision process to proceed to human clinical trials (NCT00804154) were our observations of reproducible and sustained analgesic efficacy, without development of behaviors indicative of denervation hyperesthesia in the companion canine model of osteosarcoma pain (Karai et al., 2004; Brown et al., 2005; Brown D. C. et al., 2015).

Delineating the Site of Action of RTX

At the time of these earlier experiments, the intrathecal actions of RTX were attributed to deletion of TRPV1-expressing DRG neurons. However, further studies in rat, dog, and human revealed that when RTX is administered into the spinal CSF the most vulnerable neural structures are TRPV1-containing axons of Aδ- and C-fiber in the dorsal roots that are bathed in spinal CSF. In fact, the neuronal cell bodies and their axonal connection to the periphery are largely spared in comparison to the centrally projecting axons in the cauda equina. These issues are examined in detail in Sapio et al. (2018) and the mechanism of axonal susceptibility now governs the mechanics of intrathecal RTX administration (Sapio et al., 2018). These mechanistic insights point towards better results being obtained with small volumes of concentrated RTX, which in these studies can be optimized further by a slow infusion rate to keep the drug confined to the cauda equina and not distributed widely in the spinal CSF space (Shafer et al., 1998; Eisenach et al., 2003). The exact parameters continue to be evaluated in humans, but we anticipate that, by limiting dispersion of the drug solution, stronger analgesia will be obtained with less thermal denervation outside the area of interest.

The Value of a Transitional Model: Lessons from Resiniferatoxin

We have mainly described the mechanistic and preclinical rodent studies that led to the initial work with RTX. However, this work was done in parallel with canine studies which gave invaluable insight into the utility and usage of the drug. In the next sections, we describe the contributions of these transitional studies. The majority were conducted in companion canines with naturally occurring disease, but we will also address the advances that came from pig and other non-rodent animal models.

Development of the Canine Brief Pain Inventory and Other Scales

One of the key elements in preclinical animal studies is the selection of a valid and informative analgesic endpoints. In the canine, pain evaluation tools have been developed and validated to allow usage by the research community. It is important to note that many elements relevant to the impact of pain on "life experiences" are not captured by pain rating scales alone. This includes the ability of pain to interfere with the activities of daily living and estimates of overall improvement in the well-being and quality of life of the companion animal. In humans these elements are measured as part of the human Brief Pain Inventory (hBPI).

To capture these elements in an evaluation methodology, a canine Brief Pain Inventory (cBPI) was developed and validated by Brown and others in several reports (Brown et al., 2008; Brown et al., 2013a; Giuffrida et al., 2017). Additional questionnaire instruments were also developed including the Canine Owner-Reported Quality of Life assessment (CORQ) (Giuffrida et al., 2018). Development of these instruments was critical to the methodological standardization of the companion canine model in pain research. The cBPI was validated to evaluate chronic pain in two disease states osteoarthritis Brown et al. (2008) and bone cancer Brown et al. (2009). These were followed by cBPI measurements of reduction in pain intensity and interference with activity in a series of cancer pain studies evaluating RTX and substance P-saporin Karai et al. (2004), Brown et al. (2005), Wiese et al. (2013), Brown D. C. et al. (2015), Sapio et al. (2018) and for evaluation of carprofen for osteoarthritis (Brown et al., 2008; Brown et al., 2010; Brown et al., 2013a; Brown et al., 2013b; Brown, 2014). The cBPI was ultimately used as the primary outcome for the pivotal studies that lead to the approval of Galliprant® (a small molecule EP4 antagonist) and Librela ® (an anti-NGF monoclonal antibody) for the treatment of pain in dogs with osteoarthritis. For analgesia studies, the cBPI scales were instrumental in quantitating the amount of pain relief in an open label study of RTX administered intraarticularly for canine osteoarthritis Iadarola et al. (2018) and by the intrathecal route in a double-blind clinical trial of bone cancer pain (Brown D. C. et al., 2015). The translational aspects of these two research efforts and problems encountered are treated in more detail in the next sections. It is important to recognize that in both cases RTX successfully transitioned to the IND and clinical trial stage and beyond. Nonetheless, many literature reviews of how to improve translational pain research often mention use of larger animal models but rarely include these examples. Canine companion models are still underutilized given their predictive utility and ability to enable clinical research. While preclinical rodent models are not replaceable, incorporation of a companion canine study can facilitate translational advancement in a more tangible way. The advantages of the companion canine model will be outlined in the following sections (see **Table 1**), as well as barriers that prevent the wider usage of these models. These barriers include the need for personnel to recruit and screen candidate patients, the requisite expertise and veterinary care facilities required to execute treatment and evaluation successfully, and the costs of canine studies. Nevertheless, a compelling case exists for how these studies can generate more predictive preclinical data that facilitates more rapid and reliable translation to new therapeutics (see **Figure 1**).

Activity Monitoring as a surrogate endpoint for pain and evaluation of oral NSAID analgesia

Importantly, the subjective measures can be augmented with more objective methodology such as activity monitoring Dow et al. (2009), Michel and Brown (2011) and force plate measurement for weight bearing (Iadarola et al., 2018). For the endpoint of activity, use of a wearable monitor was evaluated in a randomized placebo-controlled analgesia study conducted in 70 arthritic dogs. Activity was acquired at one-minute intervals continuously for 21 days: 7 days prior to and 14 days during treatment with the NSAID carprofen (Brown et al., 2010). The activity monitor detected significantly more activity during the carprofen treatment phase than at baseline. The activity monitor was able detect improvements in activity with respect to baseline in the carprofen group and the difference in improvement between the placebo group and the carprofen group. There are several notable elements related to this study. First, the ability of the companion model to differentiate placebo from active drug indicates detection of a true analgesic action. Second the companion model was useful in detecting analgesia using an orally dosed drug, rather than the single administration RTX type of interventional agent. Thus, the model appears suitable for assessment of more conventional orally bioavailable drugs as well as novel treatments Lascelles et al. (2018) and two additional successful development programs were noted in the preceding section. The use of activity monitors or other wearable monitors is an evolving prospect for analgesia studies. Important factors to consider are the proper data to collect from activity monitors, which algorithms to use, how to interpret changes, whether to monitor during daytime or nighttime or during sleep, and with or without a companion dog, for example, are all important parameters for these technological innovations. They represent important techniques that can augment typical questionnaire-based evaluations of analgesics with objective endpoint whether applied to humans or animals.

Treatment of Naturally Occurring Bone Cancer Pain by Intrathecal RTX in the Canine Companion Model

Osteosarcoma is a fairly common cancer in large breed dogs and generally localized to the appendicular bones (Morello et al., 2011). Thus, two routes were chosen: intracisternal for fore limb tumors and lumbar cistern for hind limb tumors (Brown D. C. et al., 2015; Sapio et al., 2018). While is it possible to thread a catheter from the cisterna magna to the desired level of the cord Marsala et al. (1995), preliminary studies showed that needle access to the intrathecal CSF space at both sites was straightforward and could be reliably performed percutaneously without touching the spinal cord itself. A series of three cancer pain studies were performed in companion canines (Karai et al., 2004; Brown et al., 2005; Brown D. C. et al., 2015; Sapio et al., 2018). The first in 2004 (see **Figure 2**), a larger follow up in 2005, and a randomized single-blind study in 2015 of 72 animals. Two additional studies examined target engagement, site of action and histopathology (Hockman et al., 2018; Sapio et al., 2018). In the two early studies Karai et al. (2004), Brown et al. (2005), significant analgesia was observed that was durable despite progression of the bone tumor with some dogs being evaluated out to 3.5 months. In addition, after RTX treatment many animals (12 out of 18, 66%) were able to have reduced or completely eliminated their

concurrent analgesic medications which consisted of non-steroidal anti-inflammatory drugs, steroids and opioids. The target engagement studies showed overall consistent results which included 1) loss of molecular markers in spinal cord reflecting loss of TRPV1+ nerve terminals in dorsal horn (e.g., substance P, CGRP or TRPV1 staining), 2) analgesia to thermal stimuli or decrease in cancer pain, 3) minimal drug-related adverse events, and 4) minimal toxicity to non-TRPV1+ neuronal cell bodies in the DRG (Hockman et al., 2018; Sapio et al., 2018). There was some evidence for neuronal loss, which was anticipated, but it was less than expected, which is consistent with retention of the majority of TRPV1+ neurons in the DRG. These observations are the basis for identifying TRPV1-containing axons in the dorsal roots as the most vulnerable neural structure, and that selective chemoaxotomy in the CSF space is the basis for RTX analgesia (Sapio et al., 2018).

The double-blind study used a time-to-event analysis as the primary endpoint, evaluating how long it took for dogs to require additional standard-of-care intervention following IT injection of RTX or placebo. In this study certain procedural effects occurred, and a detailed examination of them provides insight into experimental complications that can be encountered with the companion canine model and how to anticipate and avoid unexpected events. Apparent spinal headache symptoms in some dogs, which did not improve until the second week post injection confounded the short term cBPI assessment. The apparent post-dural puncture headache was seen in 17 out of 18 animals treated by the intracisternal route and may have influenced owner evaluations of pain severity or interference related to osteosarcoma pain in the first 2 weeks. As a consequence, early treatment-related improvements were not significant. Despite this impediment to cBPI measurements, significant differences at later time points were detected for analgesic endpoints such as 1) improvement in lameness (rated by a veterinarian blind to the treatment rather than the owner) and 2) time to unblinding in order to obtain additional analgesic treatment (significantly shorter for the vehicle animals). The important point is to recognize that RTX is an interventional agent, and any procedural problems can affect drug performance and impact pain evaluations especially at early post-injection time points.

It was, therefore, suggested that trial designs with interventional agents should include extended periods of evaluation as part of the primary outcome and multiple independent endpoints for determination of analgesic actions to account for any variability due to administration parameters. Also, progression of the disease can be an issue if bone tumors or arthritis develops outside the zone of initial drug delivery. If the candidate analgesic is an oral drug, evaluation is less likely to be influenced by procedural parameters. However, other factors such as drug adsorption, metabolism and pharmacogenetics need to be incorporated and consideration given to behavioral and differences among breeds (Ostrander et al., 2017; Bowden et al., 2018). Caregiver placebo effects associated with an owner-evaluator are another factor that needs to be monitored. Lastly, the influence of expectations and pre-conceived bias with respect to breeds and pain sensitivity may need to be taken into consideration (Gruen et al., 2020). These details are enumerated to reinforce the idea that the interplay between trial design, owner evaluations, use of the canine BPI, procedure-related effects, analgesic activity, timing of endpoints, and use of independent trained observers all need deliberate consideration at the start of a proof-of-concept efficacy trial.

Treatment of Human Cancer Pain by Intrathecal RTX

Two studies of RTX to treat intractable pain in advanced cancer have been conducted, one using an intrathecal route and the other an epidural route. Preliminary data on for the intrathecal trial are available in abstract form Heiss et al. (2015), Pomeraniec et al. (2020) and preliminary epidural results can be found at <https://www.cancernetwork.com/view/interim-analysis-phase-ib-study-resiniferatoxin-generates-positive-data>. Additionally, molecular and analgesia data were published from one intrathecal human patient whose DRG and spinal cord were obtained after autopsy (Sapio et al., 2018). This patient's data are presented along with data from a small cohort (N = 5) of companion canine dogs with osteosarcoma analyzed in parallel (**Figure 3**). The dogs exhibited significant decreases in their pain severity and pain interference scores post treatment. Similarly, the human exhibited a 52% decrease in pain severity and similarly pronounced reductions in opioid use. These results exemplify the strong analgesic response that is possible with intrathecal RTX in pain from advanced cancer.

PROTEIN THERAPEUTICS FOR CANCER PAIN

Substance P-Saporin and Substance-P *Pseudomonas* Exotoxin

The companion canine model has also been used to evaluate another type of long-acting pain control agent; a protein therapeutic called substance P-saporin (SP-SAP) (Brown and Agnello, 2013). This compound, and a similar conjugate of Substance P with a truncated, bioengineered *Pseudomonas* exotoxin (SP-PE35) Iadarola et al. (2017), act on second order spinal cord neurons expressing the receptor for substance P, the Neurokinin one receptor (NK1) coded for by the TACR1 gene. Substance P is an 11 amino acid, C-terminally amidated peptide released from primary afferent endings onto NK1-receptor-positive spinal cord neurons. The agonist-bound receptor complex in internalized into the neuronal cytoplasm in clathrin-coated vesicles in a process referred to as agonist-mediated endocytosis (Mantyh et al., 1995). After internalization, the two protein toxins are released from the endosome into the cytoplasm and their enzymatic activity stops protein synthesis. Without protein synthesis the NK1+ neurons die, eventually removing these critical populations of neurons from the nociceptive transmission pathway to the brain. In preclinical rodent studies intrathecal injection of the two

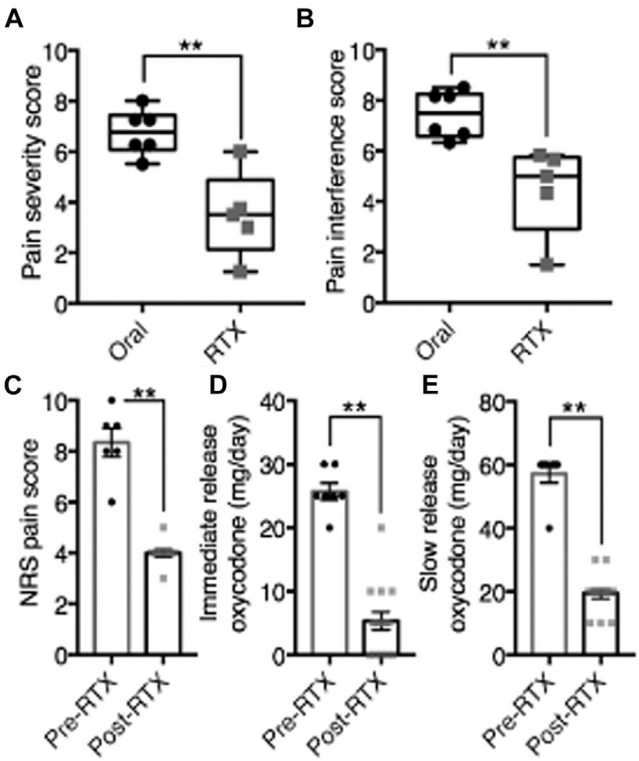

FIGURE 3 | Analgesic action of intrathecal RTX in companion canine osteosarcoma model. compared to cancer pain control in a human patient. Dogs with appendicular osteosarcoma (n = 6), and a human patient with intractable cancer pain (n = 1) were injected intrathecally with RTX (1.2 μg/kg and 13 μg, respectively). Subsequent to IT RTX treatment, dogs were released to their owners and pain assessments were made at monthly intervals. Owners for each dog rated pain severity and interference with function using the Canine Brief Pain Inventory (79). Pain Severity **(A)** and Pain Interference **(B)** scores for companion dogs treated with RTX were significantly decreased relative to dogs kept on traditional standard of care oral pain medications alone. Data from the one human patient were collected over 7 days before RTX and 15 days after RTX injection while an in-patient on the oncology ward. Beginning immediately after RTX treatment, NRS pain scores (0–10) decreased from about eight to about 4 **(C)** Concurrently, self-administration of immediate release oxycodone was decreased **(D)** as was slow-release oxycodone during the same period **(E)** Statistical comparisons were made using two-tailed Mann-Whitney U test (**, $p < 0.01$) (data from Sapio et al., 2018, with permission). The analgesic effect in the human is remarkably similar to that seen in treated dogs in **Figure 2** and reductions in canine analgesic drug treatments are also observed (Brown et al., 2005; Brown D. C. et al., 2015).

agents resulted in analgesia to a variety of experimental stimuli or hyperalgesic states (Mantyh et al., 1997; Wiley, 2008).

Treatment of Naturally Occurring Bone Cancer Pain by Intrathecal Substance P-Saporin in the Canine Companion Model

Three studies have been conducted with this reagent in the dog. Two published reports evaluating the toxicology and basic neurobiology of SP-SAP in dogs provided positive information on dose response and target engagement, such as loss of NK1 receptor expressing neurons in the dorsal spinal cord. The third report examined pain control in companion canine dogs with pain from bone cancer using the intrathecal route of administration (Allen et al., 2006; Wiese et al., 2013). In two of the studies some animals exhibited a motor function deficit (e.g., motor weakness) which prompted euthanasia in several cases. The basis for this needs further study. This is mentioned to reiterate the point that these dog studies can be subject to experimental variables like any other interventional drug assessment. However, in the companion study, despite procedural variables that may have affected owner's early assessment of pain using cBPI instruments, two other endpoints were acquired as the primary outcomes (Brown and Agnello, 2013). These measured the timing of, and the necessity for, unblinding and analgesic dosage adjustment. The two parameters were significantly different between the control group versus SP-SAP treated animals which is consistent with an analgesic action. In this experiment the SP-SAP was administered into the cisterna magna for fore limb tumors

and at the lumbar enlargement of the spinal cord for hind limb tumors, thus the location of drug administration matched the location of the relevant spinal cord NK1+ neurons. SP-PE35 has not been tested in canines yet. These canine studies formed a major translational foundation for a human clinical trial.

Treatment of Human Cancer Pain by Intrathecal Substance P-Saporin Conjugate

A small cohort of cancer pain patients ($n = 3$) was treated with SP-SAP and reported in two abstracts (Frankel et al., 2014; Frankel et al., 2016). This trial commenced at a no observable effect level dose and did not reach doses that had a clinical analgesic effect. The amounts tested were 1, 2, and 4 μg which were not in the range of the effective doses in the dog studies (~20 μg and higher). In addition, the SP-SAP was delivered into the lumbar cistern. While this is the accepted route for a lumbar puncture it is somewhat remote from the location of the relevant NK1+ neurons in the superficial dorsal horn of the lumbo-sacral cord. In fact, the lumbar cistern is ~30 ml s, and while some volume is taken up by the dorsal roots, this site likely caused a dilution of the SP-SAP which also may have contributed to a lack of analgesic action. It seems that with some adjustment of the dosing and delivery parameters to more closely match the canine studies, a resumption of the clinical trial would be worthwhile.

Treating Osteoarthritis Pain

An Example of Translational Progression Using the Canine Companion Model:

The progression of research from rodent to canine to human is exemplified by the development of RTX for the treatment of osteoarthritis pain. It was evident from our initial RTX studies in 2004 that this compound was a very versatile interventional pain control agent suitable for multiple pain indications. In the rat, in addition to analgesic and anti-hyperalgesic actions of RTX on hind paw inflammation Neubert et al. (2003) or hind paw incision Raithel et al. (2017), intraarticular RTX had been shown to inhibit carrageenan-induced knee inflammation (Kissin et al., 2005). While these studies succeeded as "activity detectors" and gave an idea of a starting dose, they did not predict performance in pain from naturally occurring osteoarthritis in either canines or humans. Just prior to intraarticular RTX, an injection of bupivacaine was administered into the index joint to block the acute nociceptive actions of the 10 μg dose of RTX (Iadarola et al., 2018). Using the cBPI scales we detected decreases in pain severity and interference scores across all animals (**Figures 4A, B**). The onset could be seen by 2 days in most dogs and significant decreases were obtained at days 7 and 21. There was also an increase in weight bearing by the treated limb, an objective endpoint (**Figure 4C**). What was remarkable, was the long duration of therapeutic effect obtained from a single injection: suppression of pain, improvement in gait, weight bearing, and improvement in the dog's activities of daily living all lasted 4 months or longer. The median time at which the owners sought re-treatment for their animal was 5 months and one dog displayed pain relief for more than 1 year (**Figure 4D**). Two to 3 years after injection there was no indication that the period of decreased TRPV1 innervation accelerated joint degeneration (Charcot joint) in any of the dogs. These strong positive results in naturally occurring OA were very incentivizing (see video: http://links.lww.com/PAIN/A627) (Iadarola et al., 2018). It must be pointed out that this was an open study and while not placebo controlled, the attached video, and other videos sent by the owners depicting robust spontaneous activity, mitigates attributing all of the improvement to placebo. However, in the human (**Figure 4E**), the placebo effect is both strong and of long duration, even though there was only one treatment session (discussed below).

The compelling efficacy of intraarticular RTX in the companion canine model, without apparent localized side effects, formed the impetus for toxicological studies in purpose bred dogs and prompted a full development program for control of clinical signs of osteoarthritis pain in companion animals, by one of the authors (AN, unpublished), in parallel to human clinical trials. In these studies, while the dogs can display short-term responses to drug administration (feeling hot, panting and salivating) no joint toxicity was observed. Furthermore, a group of aged beagles with light to moderate OA (not requiring NSAID treatment) was treated in the hip joint with no safety signal of concern. These observations in aged and laboratory dogs inform about the safety of the drug locally and form a path for further studies in humans beyond the knee joint. The lack of observable toxicity at the therapeutic dose for the animals or at the cellular level for the joint capsule provided essential information for submission of the investigational new drug (IND) application to the FDA. The question posed for the current topic is: How well did the companion canine results predict efficacy when translated to the human? In the case of RTX, the information gathered through companion animal studies provided much value and helped accelerate the human drug development program compared to traditional expected timeframes.

Treatment of Human Osteoarthritis Pain by Intraarticular RTX:

After the IND milestone was reached, a double-blind placebo-controlled phase I trial was designed and conducted in humans (Leiman et al., 2020). The starting doses (between 3 and 30 μg delivered in five or 10 mls of saline) were justified from the dog studies, although the volume is considerably larger in this human study than in the initial companion work (**Figure 4E**). Efficacy was measured with the Western Ontario and McMaster Universities Arthritis Index (WOMAC scale) and a numerical pain rating scale. Commencing at about day 21 differentiation from the placebo injection began. At the 4-weeks evaluation point, the 12.5 μg dose was significantly different from placebo (green line, **Figure 4E**). The analgesia was sustained out to 12 weeks, and anecdotally, as long as 1 year. No serious adverse events occurred and the adverse events that were observed were minor, transient, and did not correspond to dose escalation. While these data are preliminary, the

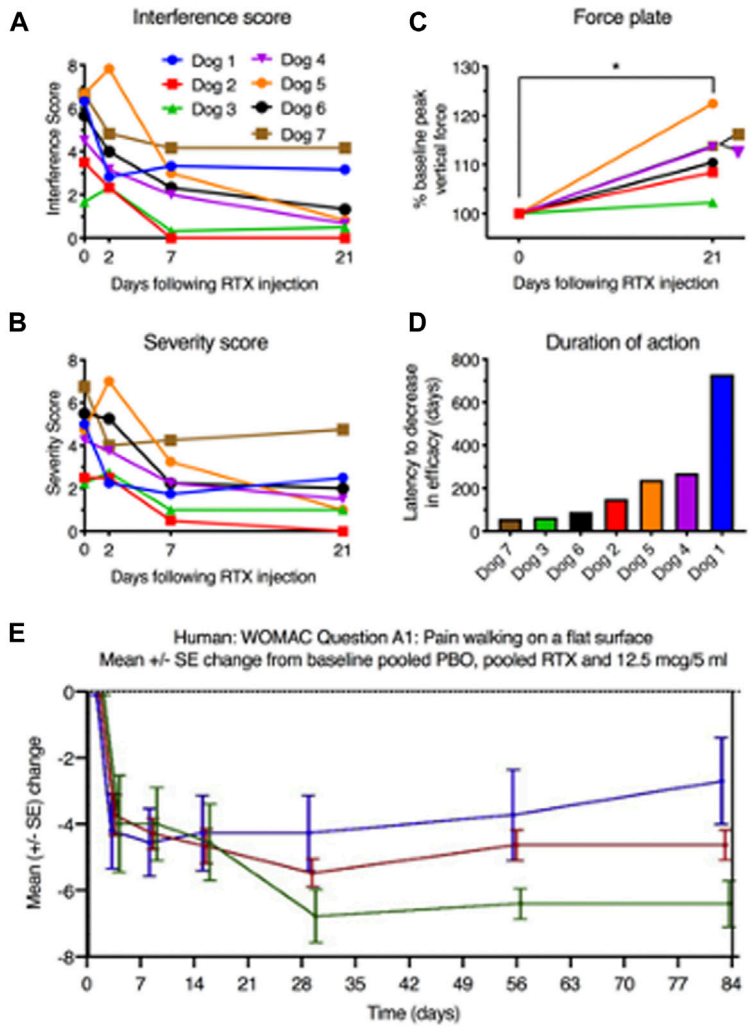

FIGURE 4 | Analgesic action of intraarticular RTX in companion canine osteoarthritis model compared to preliminary data from a human clinical trial. These five panels show the fluid transition from the companion canine model to a human osteoarthritis clinical trial. In both species RTX was delivered intraarticularly (fore or hind limb in the dog and into the knee joint in the human). The canine work is from reference 37, the human clinical data are adapted from a published abstract (Leiman et al., 2020). Note the duration of the 5 ml placebo injection which only begins to wane after 14 days despite the fact that only one injection of either vehicle or RTX was delivered reducing the opportunities for reinforcement such as daily oral medication which are absent with this interventional single injection treatment.

correspondence between the dog and human studies is apparent: Both showed strong efficacy and long duration. Obviously further follow-up is needed in both species but at least preliminarily, effective translational prediction from the companion canine model is substantiated.

One difference between the canine and human osteoarthritis studies was the long duration needed to differentiate the active drug arms from placebo, which was about 3 weeks in humans. This is a substantial difference from the dog study where onset of action was discernable in 2 days. This was an open label study and comparison to vehicle controls was not possible. There may be a myriad number of explanations for this difference. One factor is that the placebo response in human pain studies is known to produce a strong analgesic action regardless of the treatment. However, with tanezumab, an anti-NGF antibody, patients with osteoarthritis pain experience an analgesic response that differentiates from placebo as soon as 1 week after treatment. It is possible that other factors, perhaps related to intraarticular instillation of the vehicle, might have produced short term actions that are additive with the placebo response. Comparison to just a needle insertion into the joint was not made. More studies will help to resolve some of the administration procedural elements that shape the human response. The important point is that effective and long-term analgesia was obtained in the human and

these two therapeutic characteristics were predicted using the translational companion canine model. Additionally, other important information is generated during the course of the canine trials. These include procedural steps such as anesthesia, justification of starting dose, type of interventional administration protocol, general side effect profile, and duration of action, which is a critical factor for determining dosing interval and how long to conduct the study are all robustly anticipated. Thus, the companion canine model provided actionable data on potential clinical efficacy in humans in two different clinical pain conditions, osteoarthritis and advanced cancer, and was a key element in guiding the expenditure of effort to reach the stage of human clinical trials.

Swine Model for Interventional Analgesics

Fine Tuning Periganglionic or Intraganglionic Routes of Administration

Preclinical swine models are preferred in cases when the injection technique and/or anatomical precision is critical to the success of an experimental therapy. Recent advances have led to an increase in needle guidance and precision medicine technologies to deliver local analgesics and lesioning agents (Wood et al., 2007; McKnight et al., 2020). As the capability to deliver injections directly into the DRG grows, RTX stands out as an ideal candidate for injection in or around the ganglion. Several elements such as a strongly lateralized pain problem, dermatomal locations of a tumor or injury above the cauda equina, motivate the more localized approach of drug delivery directly to DRG neurons than afforded by intrathecal or epidural injection routes. Work towards optimizing periganglionic administration has been evaluated in a pig model. Close ganglionic injection of small volumes and amounts of RTX to lumbar DRGs on one side of the pig's body selectively ablated TRPV1+ pain fibers unilaterally (Brown J. D. et al., 2015). Four ganglia were treated with vehicle on the other side. Control ganglia were unaffected neurochemically and behavioral responses to thermonociceptive stimuli were also intact on the vehicle side. This method is ideal for applications where the peripheral generator is driven by an identifiable ganglion or ganglia that can be individually targeted with precision guided injections and could also be appropriate for unilateral pain problems where ablating bilaterally would be unnecessary (e.g., postherpetic neuralgia). One pain indication for which direct ganglion targeting can be used is trigeminal neuralgia, an extremely painful disease the has origins in the trigeminal ganglion. While the exact etiology is not fully understood for trigeminal neuralgia, RTX may be an ideal candidate for an injectable therapeutic agent and may be more beneficial than other types of non-specific, ablative procedures such as radiofrequency lesion. For the patients and the interventionalist, selectively targeting TRPV1 neurons in the trigeminal may provide a permanent therapeutic response, which is important given the technical requirements of the injection (Tender et al., 2005). Here again the data from the large animal model supports the preliminary preclinical results obtained in rodent models and reinforces the confidence for obtaining a positive analgesic response in a human clinical trial.

This idea is bolstered by recent studies supporting the role of the peripheral generator in the etiology of several types of pain, including phantom limb pain, which was previously hypothesized to have a central origin. Recent studies have shown that lidocaine inhibition of the ganglia can prevent pain in the phantom limb, strongly supporting a DRG location for the peripheral generator in this pain condition (Vaso et al., 2014). This has been investigated with lidocaine or bupivacaine injections to "find" the source of the pain problem followed by ablation to extend the duration of the analgesia. These methods have seen success through ablation of an active neuroma (in cases where it could be identified), nerves, and nerve roots proximal to the ganglion depending on the patient (Ramanavarapu and Simopoulos, 2008; Wilkes et al., 2008; West and Wu, 2010; Imani et al., 2012; Zhang et al., 2017). As these patients are rare, most of this work has been done in case reports or in clinical practice, and more studies are needed to refine effective approaches based on evidence. However, RTX is ideally suited to replace radiofrequency or other ablative methods in this and other applications, as it spares normal sensations. These studies also reinforce the role of a peripheral generator in what was previously considered a form of central pain.

Anecdotal "Very" Large Animal Experiences

Goat and Horse

Using models based on other, larger animals is another topic that is raised frequently in reviews of animal models for analgesic drug development. **Table 2** lists a selection of studies that have used the companion canine model. At present a limited amount of experience treating pain with RTX in larger animals, which so far are horses and goats (**Figure 5**). These few cases, albeit anecdotal, are instructive. The horse in **Figure 5** was a working horse and a source of livelihood for the owner. It became incapacitated by pain in the lower forelimb subsequent to an injury. RTX treatment was by intraneural injection. After establishing a dense local anesthetic block proximally, the nerve was surgically exposed and RTX was delivered intraneurally. There were no adverse events and the horse returned to work and could be mounted and ridden. Unfortunately, pain behaviors returned after about 3 months and the animal was euthanized. The goats that were treated came from a herd of Nubian goats located at the Bronx and Queens Zoos in New York. They developed osteoarthritis subsequent to infection with a caprine retrovirus (Crawford et al., 1980; Crawford and Adams, 1981; Blacklaws, 2012; Gomez-Lucia et al., 2018). The animal illustrated had arthritis in the forelimbs and was injected with RTX intracisternally while under isoflurane anesthesia. Recovery was uneventful and the goat returned to his exhibit space. Reports from his keepers indicated that considerable function had been restored: "Yesterday Simon (the goat) was head-butting other goats and people … something he hasn't done for some time. It is the impression of the staff that he is doing fantastically. They are really pleased." "The latest update: Simon has been seen doing things that he has not done for some time–standing with his front legs up on the fence; being up on a stump…. All in all, the Zoo staff is VERY impressed with his response." A second arthritic

TABLE 2 | Examples of canine companion and other large animal models in pain research 2004-present.

Publication	Species, pain indication and technical aspects	Comments
Karai et al. (2004)	Experimental models in rats after intra-trigeminal or intrathecal RTX and in dogs with osteosarcoma, drug formulation, dose-ranging	First *in vivo* demonstration of intrathecal RTX analgesia in canine companion models of osteoarthritis and osteosarcoma pain
Brown et al. (2005)	Companion dog, unilateral osteosarcoma, dose response, hemodynamics and analgesia	Efficacy demonstrated for intrathecal RTX in cancer pain, reduction in use of other opioid and non-opioid analgesics
Tender et al. (2005)	Rhesus monkey, intra-trigeminal RTX as a model for control of facial pain, head and neck cancer pain, and trigeminal neuralgia	Unilateral microinjection into TG. Target engagement shown by ipsilateral loss of capsaicin induced eye wipe and plasma extravasation. No Adverse effects on skin, wound healing, feeding
Brown et al. (2007), Brown et al. (2008), Brown et al. (2009), Brown et al. (2010), Brown et al. (2013a), Brown et al. (2013b), Brown D. C. et al., (2015), Brown J. D. et al., (2015), Wiese et al. (2013), multiple additional publications	Development and validation of multiple metrics for canine pain in osteosarcoma and osteoarthritis	The brief pain inventory and other metrics were adapted and validated to evaluate spontaneous pain states in the canine companion model, objective endpoints such as activity monitoring, and evaluation of analgesic agents
Allen et al. (2006), Yaksh et al. (2017)	Canine toxicology studies of substance P-Saporin conjugate demonstrating target engagement and evaluation of adverse events	Examination of a ligand-directed plant protein that blocks protein synthesis in substance P receptor (NK1) expressing second order spinal cord neurons
Brown and Angello (2013)	Canine companion model, osteosarcoma pain, evaluation of substance P-Saporin delivered intrathecally	Veterinary clinical trial of a ligand-directed bioengineered pseudomonas exotoxin that blocks protein synthesis in NK1 expressing second order spinal cord neurons
Brown D. C. et al., (2015)	Double blind evaluation of RTX in canine bone cancer	A veterinary clinical trial of intracisternal or intrathecal RTX, owner and veterinarian evaluation of spontaneous pain and interference with daily activity
Brown J. D. et al., (2015)	Swine, experimental heat pain, unilateral lumbar peri-ganglionic infusion of RTX	CT image-guided delivery of RTX demonstrates lateralized confinement of injection], analgesia to laser heat pulse and loss of TRPV1 from ganglia
Iadarola et al. (2018)	Canine companion model, osteoarthritis pain, evaluation of RTX delivered intraarticularly	A single injection of RTX produced long-duration analgesia, increased activity with no side effects and without negative side effects
Maus et al. (2019)	Swine, technical evaluation of MRI for peri- or intra-ganglionic infusion of gadolinium contrast agent	MRI and contrast agent for visualization of needle placement and small infusion volumes into DRG. Potential for gene delivery
Guedes et al. (2017)	Horse, laminitis, evaluation of soluble epoxide hydrolase inhibitors as analgesics	Initial studies of efficacy for this prototype enzyme inhibitor

goat was treated, with similar therapeutic response. These were preliminary pilot studies and the results, while demonstrating robust analgesia, remain unpublished but could be extended with a controlled study in settings where more animals need treatment.

The horse has also been used for evaluation of other analgesics. An example being developed by Hammock and others are soluble epoxide hydrolase (sEH) inhibitors (Inceoglu et al., 2006; Wagner et al., 2017; Wagner et al., 2020). This approach centers around inhibition of the degradation of a class of arachidonic acid-derived epoxy fatty acids. The accumulation of these fatty acids is associated with better outcomes in terms of inflammation, primary disease progression, and pain. In addition to rodent models, this class of drugs has been evaluated in transitional models similar to RTX. One such study was done in companion canine animals with naturally occurring arthritis, which was evaluated primarily using the cBPI. This revealed sEH inhibition was effective in blocking arthritis pain in dogs (Wagner et al., 2017). Large animal studies of sEH inhibitors were initiated for treatment of severe refractory pain in horses with equine laminitis, a condition characterized by lameness requiring humane euthanasia (Guedes et al., 2013). The preliminary study was extended to a larger clinical case series, with both showing positive results and reinforcing the utility of the equine approach (Guedes et al., 2017)

Molecular Transcriptome of Canine DRG

Molecularly-Validated Target Selection

We have described in this review that several models, particularly the dog and the pig have distinct advantages over the rodent in predicting efficacy, dosing and usage in humans. It is important to note the different ways the process of using the companion canine model builds on itself. In the canine intraarticular RTX osteoarthritis Iadarola et al. (2018) and intrathecal RTX bone cancer pain investigations Sapio et al. (2018), the molecular transcriptomics of the canine DRG were examined. These were the first studies to define the molecular biology of the DRG neuronal transmission step in the canine pain pathway. In the RTX osteoarthritis report, the complete molecular gene expression profile of canine dorsal root ganglion was determined by next generation RNA-Sequencing (Iadarola et al., 2018). These state-of-the-art results bring the molecular knowledge base for canine pain studies on a par with the human, rat and mouse. These studies allow for a complete molecular framework to be obtained and the "druggable nociceptome," which contains all the

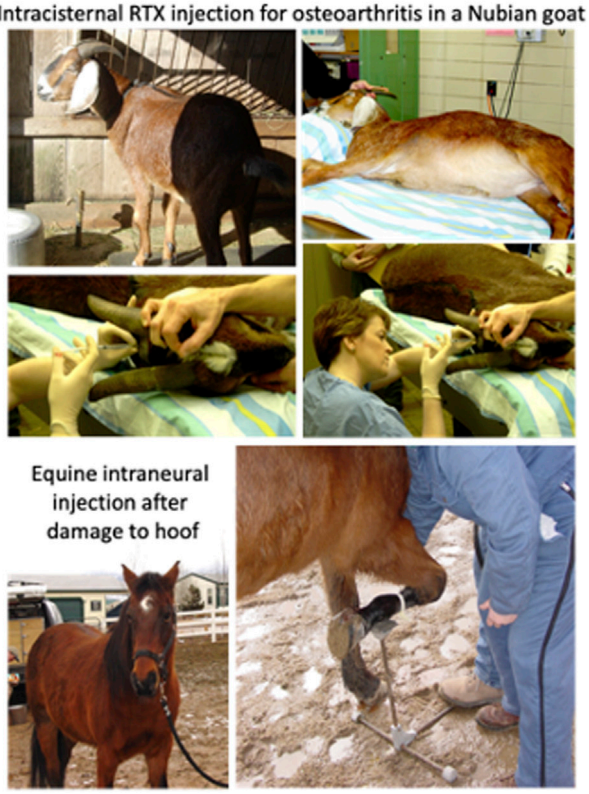

FIGURE 5 | Consideration of goat and horse as models for analgesic drug evaluation. As discussed in the article, painful conditions in the horse and goat represent other opportunities for animal analgesic assessment. The goats were treated intrathecally for painful osteoarthritis by RTX injection into the cisterna magna by one of the authors (DCB). This produced noticeable improvements in activity. There were only two goats that had the arthritic problem, and this highlights the consideration of incidence and consistent access to a population with the appropriate disorder. The horse in the example was treated intraneurally with RTX and is shown just prior to surgical exposure of the nerve. This injection also produced a long duration analgesia (~3 months) but it was not permanent (as expected with a distal peripheral injection). Compared to the goat, many more horses are candidates for such treatment, for example, laminitis is estimated at 10% of the equine population (Pollard et al., 2019). Other species have also been discussed in the literature (12, 13) and present challenges unique to each species.

genes that might code for proteins that transmit or modulate nociceptive transmission, to be identified. This gene can be bioinformatically extracted from the total transcriptomic library and used to design future analgesic drug trials. If contemplating a drug trial, especially a drug designed to act on primary afferent nociceptors, knowing whether the target molecule is expressed in the DRG of the animal model, as well as the human, is an extremely important consideration for selection of the model animal, and ultimately to the success of the trial. For example, clinical trials and animal studies of Kappa opioid receptor agonists may exhibit analgesic and anti-pruritic actions, particularly for visceral modalities (Kaski et al., 2021). However, while published studies have shown anti-pruritic actions of kappa agonism Fishbane et al. (2020), reports of analgesic effects remain largely unpublished as of 2021. More complicated peripheral agonists at delta-kappa opioid heterodimeric receptors have also exhibited analgesia in the rat. However, deep RNA sequencing of *canine* DRG shows that kappa opioid receptors are not expressed in canine DRG, a finding which would eliminate evaluation of peripheral kappa agents in a canine model. Thus, knowing the transcriptomic profile of dog DRG may help guide decision making and whether or not to incorporate the companion canine model into drug development effort.

FURTHER ADOPTION OF THE CANINE COMPANION MODEL

It is disconcerting that, while bemoaning the lack of translational success using rodent models of nociception, neuropathic pain, cancer pain, and affective components of pain, the research community has been slow to adopt alternatives to the traditional approach. There is a recognized need for new animal models such as the companion canine model but, apparently, there is little interest in developing programs that utilize these types of approaches. This may be due in part to difficulties in establishing the research infrastructure, and in part due to the limited applications to which this type of animal research is relevant. For example, the mainstay mechanistic research performed in most university settings is generally not appropriate in larger animals due to costs, logistics, and ethics. The appeal of rodent models, and the fallacy of rodent models, lies in the perception of standardization and repeatability. However, it is debatable whether analgesic efficacy in a given homogenous rodent model is broadly predictive of the human translational value, due to both the heterogeneity of naturally occurring disease, the difference in metabolism and kinetics, and various other factors associated with individual humans and complexities of underlying degenerative disease processes. The canine transitional model provides accelerated assurance that the analgesic efficacy is translational. It can also be accurate if conducted following good clinical practices with control groups. Research on this level is very practical for developing evidence for *transition* from laboratory work into human clinical trials. Rather than discover new mechanisms, analgesia research in larger animals builds on the laboratory based mechanistic insight to ask an important question: "Is the compound an effective analgesic?" Even if using a novel reagent, results from such analgesia studies are frequently judged as deficient since they are perceived as not providing new mechanistic insight. This "insufficient novelty" not offset by the gain in new knowledge that the analgesic agent under consideration actually works. Unfortunately, the "mechanism" viewpoint may extend to grant reviews, as this kind of work may not be prioritized because of the prejudice against the value of a practical line of inquiry. Nevertheless, as we show in this review, the combination of positive results in rodents, with a positive outcome in a companion canine model constitute invaluable translational milestones for a successful outcome in human trials.

TABLE 3 | Elements related to translational success.

Transcriptional Validation
Is the target gene expressed in target tissue? In some cases, the foundational data needs to be verified. Solution: Measurement of gene expression levels for target molecule by RNA-Seq
Neuronal and neural circuit cross-species validation
Are the neuronal populations in the model species the same as in human? Is the relevant neuronal population in PNS or CNS? Centrally, is the circuit "wiring" the same? Are the transmitters and peptides in the neurons the same? With peripheral tissue damage are the plastic changes in spinal cord the same?
Peripheral validity
Are inflammatory mediators similar? Do they perform the same function? Are the local tissue transcriptomic changes the same? Are the infiltrating leukocyte populations the same? Is the timing of inflammation/damage-response similar? Again, some of the outstanding gaps can be filled by conducting RNA-Seq on the model (26,38), anatomical techniques. And methods that combine both such as spatial transcriptomics
Types of preclinical models
Standard rodent inflammation and nerve injury models of various types + more specialized models such as burn, osteoarthritis and osteosarcoma. This is a vast literature. With judicious interpretation they are good initial "activity detectors." As soon as larger animals become subjects the number of studies falls off precipitously. In the dog the primary models are osteoarthritis and osteosarcoma
Interpretation of data from models
Dogs like other animals can be influenced by the testing environment (clinic or outdoors), who is in the vicinity or handling them and the presence of other dogs to interact with. These factor need to be accounted for in the study design and data interpretation and can be added into the testing protocol. Ideally, efficacy should be obvious. If complex statistics are needed to discern an analgesic effect, a similar situation will likely pertain to a human trial.
Measurement Tools
Pain rating methods include the canine brief pain inventory and other scales and subjective evaluations. For pain disorders affecting the limbs locomotion and force plate measurements can be obtained. However, such testing can be painful if the pain from the underlying condition is severe, and this may make obtaining reliable pre-analgesic testing difficult to acquire and assess for comparison. Similar considerations apply to gait analysis
Problems that May Be encountered
Damage to the injection site from the physical injection procedure can occur if the active pharmaceutical is administered by an interventional procedure. Orally administered drugs may cause aversive effects such as nausea that may not be obvious but influence testing. The main requirement is that the treatment produce a robust analgesia. Marginal analgesics may not produce a signal but theoretically could be active to a limited extent in humans

SUMMARY

Translational advantages of the companion canine model

As noted, one of the subjects mentioned frequently as a possible remedy for the lack of translational success of mouse- or rat-based models was the inclusion of large animals into the repertoire of pain research. As we show in this review, evaluating drugs in larger non-rodent species can serve as an excellent "accretive second level of assessment" for efficacy and side effects and provides a strong set of observation for informed decision making about further drug development needs. One advantageous characteristic is that the pain-causing problems frequently arise from naturally occurring diseases or disorders and thereby are not really models at all, but bona fide pain problems that are very similar to the human disorders. We used dog osteosarcoma pain as part of the preclinical basis for our human phase I trial of RTX in cancer pain and for many other disorders. Several aspects that can influence the success of translational pain research are summarized in **Table 3**.

The questions that are pertinent to the companion canine model are: 1) What advantages accrue to the translation to human and/or veterinary patients? 2) How well will the information obtained translate to phase I trials and beyond? 3) How complex of a study design is needed and for how long? and 4) Will the study follow Good Clinical Practices guidelines? Clearly one advantage is pharmacological. The results obtained provide strong data for 1) dose justification 2) duration of action, 3) strength/efficacy of analgesia, 4) potential side effects, 5) potential for evaluation of drug interactions, and 6) other types of behaviors that may emerge while the dogs are in the care of their owners. The companion canine model has additional advantage of continual observation of the treated patient by one and often many observers. Both pain rating, daily interference, owner evaluation of behavioral metadata, and evaluation of spontaneous behaviors, can all be obtained remotely, and more specialize evaluations can be conducted at scheduled visits to the veterinary clinic. Assuming all goes well, the data obtained should be additive with preceding rodent data.

Lastly, evaluating a potential analgesic in the companion canine model has the potential to be a cost saving step in the development life cycle for industry. One example where the companion canine predicted failure in human clinical trials was that of TRPV1 antagonists, which were unsuccessful in the dog, and subsequently failed in clinical trials (Lascelles et al., 2018). This lack of efficacy in the canine model was an early warning sign that these drugs lack efficacy, as the molecular target is conserved in the dog, where it has a very similar function (Iadarola et al., 2018). An example of where the companion canine model could have been informative might be EMA401. This is an angiotensin II receptor two antagonist that showed some analgesic activity in a small double-blind placebo controlled clinical trial in human neuropathic pain. The clinical trial results did differentiate between placebo and active agent, although the difference was not that large. EMA401 was subsequently acquired by Novartis and, during more extensive trials, it failed to show analgesic activity. While it is hard to model human neuropathic pain in animals, if for example, no activity was seen in a canine companion cancer or osteoarthritis trial, it may have modulated the ~400 million dollar investment Novartis made upon acquisition and testing of this early-stage agent. This is aside from transcriptome analyses which show a very low expression

level of the *AGT2R2* gene in human or canine dorsal root ganglia. Another example is UBX0101 an anti-senescence drug. This molecule is an inhibitor of a protein-protein interaction between MDM2 and p53 where MDM2, which codes for a nuclear-localized E3 ubiquitin ligase acts as a negative regulator of the P53 tumor suppressor protein. Two human clinical trials of intraarticular injection of UBX0101 for osteoarthritis pain were conducted. The results were less than satisfactory. Aside from considerations of dose, pharmacokinetics, and schedule of administration as potential problem points, and assuming that the MDM2-P53 molecular interaction is similar enough in dog and human to support UBX0101 binding, a hypothetical case can be made for interposing a companion canine osteoarthritis study prior to the second phase two study (or earlier) as suggested in **Figure 1**. In this case, if the drug had failed to produce a robust analgesic effect or enhancement of daily living activities in canine osteoarthritis, then the project could have been terminated early and saved Unity Biotechnology and its investors approximately 80 million dollars. While these are hypothetical examples, the use of this model, especially for interventional approaches, can provide valuable performance information to make informed go-no-go decisions during a drug development program. This is not to say that problems are not encountered when using the companion canine model itself, as pointed out in both the RTX and SP-saporin sections and the potential for problems has to be recognized and guarded against as in any experiment. Another factor is having a proper alignment between the animal pain conditions and the intended human pain indication. While this may not always be possible, the many aspects of knowledge gained from a canine trial are generally applicable to evaluation of analgesic performance and potential side effect profiles across multiple pain indications.

In conclusion, interposing a large animal transitional model such as the companion canine model between the early stages of analgesic drug identification and the later stage of human analgesic clinical trial can provide many advantages. Obtaining an unbiased, objective assessment of analgesic activity, or lack thereof, is valuable, actionable information and a basis for guiding further development.

ETHICS STATEMENT

Written informed consent was obtained from the relevant individuals for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

MI Wrote the manuscript and generated figures. DB Wrote the manuscript. AN Wrote the manuscript. MS Wrote the manuscript and generated figures AM Wrote the manuscript.

FUNDING

1ZIACL090033-08, Integrative And Molecular Studies Of Pain And Pain Control, Clinical Center, NIH.

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Conflict of Interest: Author DC was employed by Elanco Animal Health company and author AN was employed by Ark Animal Health company. MI is on the Scientific Advisory Board of Ark Animal Health company.

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