ABSTRACT

MINNEMA, LAURA MARIE. The Role of GFRα3/Artemin in Chronic Osteoarthritis-Associated Pain. (Under the direction of Dr. B.D.X. Lascelles and Dr. Santosh Mishra).

**Introduction:** Chronic pain is a major health problem posing an enormous economic burden to our society. The lack of therapeutic options, particularly for chronic pain, is driving the current opioid epidemic in humans. One of the limitations in developing potential therapeutics is a lack of understanding of the precise mechanisms in the target disease condition. To overcome this problem, we identified a pharmacological target, GFRα3/artemin, in naturally occurring osteoarthritis in pet dogs and further investigated GFRα3/artemin in a mouse model of OA. Here, GFRα3/artemin was identified as a potential target, the MIA model of OA in mice was phenotyped for chronic pain, and the role of GFRα3/artemin in this chronic pain model was evaluated through behavioral assays and molecular techniques.

**Methods:** Dorsal root ganglia (DRG) from dogs with naturally occurring OA were screened for potential therapeutic targets. Serum and synovial fluid from well-phenotyped dogs were analyzed for artemin concentrations using an ELISA. In mice, the MIA model of OA was phenotype for hypersensitivity and pain behavior in wild-type and TRP channel knock-out mice. Mice were injected intra-articularly with either MIA or a saline sham. Mice were tested for limb use and cold, heat, and mechanical hypersensitivity for 28 days and then DRG were collected for IHC staining to determine whether these mice had de novo expression of GFRα3. Joint capsules were collected for qPCR analysis and IHC staining of artemin.

To determine the role of artemin in hypersensitivity, naïve mice received intraplantar hindpaw injections of artemin (200 ng) and were tested for cold, heat, and mechanical hypersensitivity for 24 hours. To determine whether blocking GFRα3/artemin had potential as an analgesic, calcium imaging was used to validate that an anti-GFRα3 antibody could block
artemin signaling and this same antibody was intra-articularly injected into MIA-injected mice and limb use measured. An anti-artemin antibody was delivered systemically and tested for its efficacy in relieving MIA-induced hypersensitivity.

**Results:** Dogs with naturally occurring OA were found to have increased GFRα3 expression in their DRG, increased serum artemin concentrations, and synovial fluid artemin concentrations that correlated to measures of pain. The MIA model of OA was successfully phenotyped and found to induce decreased limb use and hypersensitivity for hot, cold, and mechanical stimuli out to 28 days. TRP channel knock-out mice also experienced hypersensitivity in this model, as neither TRPV1 or TRPA1 knock-out mice were fully protected from MIA-induced hypersensitivity. In the MIA model of OA, the GFRα3-receptor significantly increased in DRG serving MIA-injected joints compared to normal. Intraplantar injection of artemin resulted in hypersensitivity to hot, cold, and mechanical stimuli, which peaked at 4 hours post-injection. Calcium imaging data indicated that an anti-GFRα3 antibody was able to block neural activation by artemin *in vitro*. Limb use was not improved in mice administered the anti-GFRα3 antibody *in vivo* via an intra-articular injection. Systemic delivery of an anti-artemin antibody was effective for alleviate hypersensitivity to heat, cold, and mechanical stimuli in the MIA model of OA.

**Conclusions:** Our results strongly implicate a role for GFRα3 and its ligand artemin in OA pain in both naturally occurring disease and the MIA model of OA and show that the MIA model in mice is a robust system for further investigation into this system i.e. the development and testing of therapeutics blocking GFRα3/artemin.
The Role of GFRα3/Artemin in Chronic Osteoarthritis-Associated Pain

by
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DEDICATION

This thesis is dedicated to my family, friends, and all who supported me through this process and, of course, to my dog, Izzy, who wagged her tail through it all.
BIOGRAPHY

Laura Minnema is originally from the DC metro area where her interest in research was originally piqued at Thomas Jefferson High School for Science and Technology. She then attended college at the College of William and Mary where she majored in neuroscience and minored in Chemistry. During summers she worked at Syngenta Crop Protection as an analytical chemist interesting her in pharmacology.

Laura graduated from William and Mary in 2015 and entered the Comparative Biomedical Sciences program at the NC State University College of Veterinary to pursue a Ph.D. in the concentration of pharmacology. At NC State she has undertaken many leadership roles, particularly in association with the Graduate Student Association. Under the mentorship of Dr. B. Duncan X. Lascelles and Dr. Santosh Mishra, the research focus has been on OA-associated pain and the mechanisms thereof. Upon completion of her Ph.D., Laura plans to pursue a career in the pharmaceutical industry.
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CHAPTER 1

Introduction

Prevalence and risk factors associated with chronic osteoarthritis pain

An estimated 100 million adults in the US suffer from chronic pain (Daubresse, 2013). Chronic pain, a major health problem posing an enormous economic burden to our society (approximately $635 billion a year), lacks therapeutic options that are effective and non-addictive (Gatchel, 2014). This problem was recognized decades ago, but we still lack effective analgesics partly due to a lack of translation of basic science findings into new therapeutics (Kissin, 2010). The lack of effective analgesic options in the 1990s led doctors to turn to oxycodone hydrochloride (OxyContin) to treat chronic pain, and this has contributed to the United States’ opioid epidemic (Hughes, 2019; Scholl, 2018). Yet, even with the opioid crisis ongoing, opioids such as tramadol or oxycotin are being prescribed more often than they should be for chronic pain (Feldman, 2019) despite the CDC guidelines around treating chronic pain, which indicate that “opioids are not first line or routine therapy for chronic pain” (CDC Guidelines).

The need for effective therapeutics for chronic pain becomes more urgent when one considers that chronic pain is becoming more prevalent as the general US population starts to skew older and the baby boomer generation reaches retirement age (Blyth, 2017). The majority of chronic pain cases in the US are due to musculoskeletal pain. Epidemiological studies around the world have found that 13.5% to 47% of the general population have musculoskeletal pain. Musculoskeletal conditions are the main cause of disability in older age groups and are the most common medical reason for long leaves of absence (Cimmino, 2011). Chronically painful
musculoskeletal conditions can lead to low physical activity level, poor mobility, frailty, depression, cognitive impairment, falls, and poor sleep quality (Blyth, 2017).

Osteoarthritis (OA) is a degenerative joint disease and a major component of chronic musculoskeletal pain in the US. Osteoarthritis frequently affects the knees, hips, hands, feet, and spine (Miller, 2017). In individuals over age 60, 10% (males) and 13% (females) of people have knee osteoarthritis (Zhang, 2010) which, in part, is due to the relatively high rate of obesity [39.8% of American adults are classified as obese (Hales, 2017), while 71.6% are either obese or overweight (CDC FastStats: Obesity and Overweight, 2016)]. Obesity is a huge risk factor for osteoarthritis (Guilak, 2011), especially in weight bearing joints such as the knee. The incidence of OA in particular is expected to increase as this increasingly obese and overweight US population becomes older (Hales, 2017).

Obesity can contribute to OA progression both mechanically and metabolically. Obese and over-weight individuals put more weight on their joints, particularly the knee joint (Zhang, 2010), increasing the incidence of OA in this population. Obese and overweight individuals also have an increased incidence of OA in non-weight bearing joints, which indicates that there are metabolic differences contributing to the development and progression of OA in this population (Berenbaum 2016). Inflammatory cytokines, TNF-α, and other adipokine signaling molecules are produced by adipose (fat) cells and an overabundance of fat in the body can lead to dysregulation of these signaling molecules with downstream effects such as inflammation, cartilage destruction, and changes in the synovial fluid (Pottie 2006, Livshits 2010).

Type 2 diabetes is also a risk factor for OA and is very common in overweight and obese individuals, which in the United States is 71.6% of adults (CDC FastStats: Obesity and Overweight, 2016). About 1 in 10 Americans have diabetes, with 90-95% being type 2 (CDC:
Type 2 diabetes). Diabetes has been identified as an independent risk factor for OA (Schett, 2012). A study investigating the association between diabetes and both hand and knee OA found that individuals with diabetes were twice as likely to have either hand or knee OA, indicating that differences in metabolism and not mechanical loading in diabetic patients is contributing to the development of OA (Nieves-Plaza, 2014).

The United States and other developed countries face a situation where an increasingly obese, diabetic, and aging population will be in even greater need of effective, non-addictive analgesics for chronic pain.

**Current pharmacological approaches to managing OA pain**

Osteoarthritis is defined as a degenerative joint disease involving all components of the joint. It is characterized by cartilage damage and loss, the formation of osteophytes, changes in subchondral bone, inflammation of the synovium, and thickening of the joint capsule (Dieppe, 2005). These changes can be caused by general wear and tear or initiated by joint injury or destabilization. One hallmark of OA is the associated joint pain; however, OA-associated pain does not correlate well with many clinical landmarks of the disease, such as radiographic evidence of joint damage (Finan, 2013). The thickening of the synovium caused by inflammation (synovitis) was found to strongly predict the presence of joint pain in the knee (Baker, 2010). Unrelieved pain can result in progressive sensitization in the peripheral nervous system and central nervous system, resulting in greater pain. All of these factors make the treatment of OA-associated pain difficult.

There are no known disease modifying treatments yet available, so all treatments for OA focus on addressing the associated pain and reducing inflammation – they are symptom modifying. The primary treatment options for OA-associated pain are non-steroidal anti-
inflammatory drugs (NSAIDs) (Feldman, 2019). NSAIDs provide relief for many patients, however, for others the pain-relief is insufficient or the side-effects of NSAIDs are intolerable as pain management for OA requires chronic, long-term use. NSAIDs can cause stomach ulcers, liver or kidney problems, and high blood pressure. These side-effects can preclude NSAIDs as a treatment option for patients with liver or kidney problems, heart disease, or gastrointestinal disease (Miller, 2017).

Recently the selective serotonin reuptake inhibitor (SSRI) duloxetine was approved for the treatment of OA-associated pain (Miller, 2017; FDA Reference ID: 2860327). It likely works by modulating some of the adverse central changes that occur in the pain processing system due to prolonged unrelieved pain. Duloxetine is efficacious in reducing OA-associated pain compared to placebo, however, the side-effects of SSRIs make them an unattractive option for many patients. In the double-blind placebo-controlled study that determined duloxetine reduced OA-associated pain, about 50% of patients in the treatment group experienced adverse effects, most commonly dry mouth, drowsiness, dizziness, nausea, constipation, decreased appetite, insomnia, abnormal feces, and thirst (Wang, 2017).

There are a few treatments for osteoarthritis that are administered intra-articularly including corticosteroids to lower inflammation and hyaluronic acid to increase the lubrication properties of synovial fluid. Corticosteroids can be effective at relieving joint pain acutely, but due to the need for injections every 1-4 weeks they are mainly used as a short-term solution (Gossec, 2004). Initial reports from two randomized studies for intra-articular injections of hyaluronic acid suggested they provided similar relief as NSAIDs indicating that it could be used as an alternative or supplemental therapy (Ayral, 2001). Hyaluronic acid injections can provide pain-relief for up to a year with most data being from patients with knee OA (Gossec 2004).
The most promising novel treatments for OA-associated pain are the anti-nerve growth factor (NGF) monoclonal antibodies (Bannwarth, 2015; Dimitroulas, 2017), which have been shown to be effective at providing prolonged pain relief in both preclinical pain models as well as in clinical trials in advanced OA patients (Seidel, 2013). While anti-NGF’s efficacy is not in question, concerns have been raised about its safety (Bannwarth, 2015; Dimitroulas, 2017). Adverse effects of anti-NGF include transient paraesthesia and edema, rapidly progressive OA (<1.5%), and, in a small number of patients treated with both anti-NGF and NSAIDs, osteonecrosis. The adverse effects of anti-NGF are dose-dependent, with lower doses being safer, but lower doses are also less effective for pain-relief (Mullard, 2018). Because of safety concerns clinical trials for the anti-NGF antibodies were halted but then restarted in 2012 with risk mitigation strategies put in place. They are now close to FDA approval for OA pain that cannot be managed by other drugs - the FDA accepted the submission of Pfizer’s Biologics License Application for monoclonal antibody tanezumab on March 2\(^{nd}\), 2020. This application includes 39 clinical studies evaluating the safety and efficacy of tanezumab. Approval of this drug could occur before the end of the year (Pfizer and Lilly announce tanezumab BLA accepted). The lack of other treatment options and the efficacy of anti-NGF for the alleviation of OA-associated pain are the main reasons these drugs, specifically tanezumab, are moving forward and will probably be approved for the treatment of OA-associated pain with specific consideration for abnormal joint side effects (Seidel, 2013).

**Failure of translational pain research**

Despite the recent success of anti-NGF antibodies, there has been minimal progress in the development of new classes of analgesics. The success rate for analgesics to get from phase 1 to passing phase 3 clinical trials, over the period 1991 to 2000, was only 17% and many of those
“successes” were simply reformulations of already approved compounds (Mogil, 2010; Yekkirala, 2017; Kissin, 2010). Very few new pain drugs have entered the market, with the primary reason for most failures being a lack of efficacy (Mogil, 2010).

Part of the reason that the success rate is so low for new analgesics is a lack of translation from preclinical pain models, typically in mice, to chronic pain in humans. Notable failures of translation include peripheral cannabinoid CB1 receptor agonists, substance P neurokinin-1 (NK-1) receptor antagonists, fatty acid amino hydrolase inhibitors, and TRP channel antagonists. These drugs all showed efficacy in mouse models but failed in the clinic (Borsook, 2014; Yekkirala 2017).

Part of the reason analgesics have failed to translate from bench-top research to the clinic is due to the complexity of the pain. The mechanisms of pain are highly conserved across mammals, but the experience of pain can vary widely and cannot be directly measured (Malfait, 2013). To improve the translation of analgesics there needs to be two main improvements in the discovery stage: 1) relevant measures of pain and 2) relevant models of pain. OA is a complex disease that encompasses many different pain and disease phenotypes. To discover more relevant analgesics for OA-associated pain, we need to understand how OA-associated pain develops and persists, which potentially involves pathways unknown to OA pathogenesis. It is difficult to capture this complexity in a rodent model. Pharmacological targets should be identified in the naturally occurring disease and then evaluated in a relevant rodent model. While there has been little success in translating potential new analgesics from rodents to humans, back-translation, from humans-to-rodents has always been successful (Mogil, 2010). Anti-NGF is one of the only analgesic drug development successes in recent history and part of the reason for this success is that NGF was determined to be elevated in OA-patients (Miller, 2017). NGF has been the
exception to the trend by showing efficacy in the clinic, indicating that analgesics targeting neurotrophic factors should be pursued further in the naturally occurring disease.

**Role of neurotrophic factors in pain**

Neurotrophins are a family of proteins that support neuronal survival, development, plasticity, and function. There are two main classes of receptors that neurotrophins activate: tyrosine kinases (growth factor receptors) and p75 receptors (members of the tumor necrosis factor receptor superfamily) (Huang, 2001).

Neurotrophins are necessary, both in development to signal neuronal survival and in maturity to signal for peripheral pain (Pezet, 2006). In development neurotrophic factors are essential for forming neuronal connections in the central nervous system and target innervation in the periphery. Neurotrophic signaling controls cell division, survival, growth, and differentiation (Huang, 2001). After initial development, these factors persist to play a role in neuronal function in adults as part of neuronal plasticity which is an important feature of chronic pain (Pezet, 2006).

Pain signaling around the joint is meant to indicate damage and discourage limb-use in order to protect the joint from further injury, however, in OA the pain sensing system can function in a maladaptive manner leading to hypersensitivity and allodynia (Costigan, 2009). Neurotrophic factors play an important role in chronic pain. Direct and indirect signaling by neurotrophic factors enacts widespread inflammatory and cellular changes that make neurons more responsive to stimuli. Neurotrophic factors can cause the release of inflammatory mediators such as cytokines and interleukins, which contribute to OA-associated pain. The most potent signaling for neurotrophic factors occurs through tyrosine kinases, such as TrkA, which is the receptor for NGF. Nerve growth factor is the most well characterized neurotrophin,
particularly in connection with pain. Patients with a congenital mutation in NGF’s receptor TrkA, making the protein ineffective, have defective pain perception, which leads to injury (Indo, 2012). TrkA is selectively expressed by nociceptive sensory neurons with a high percentage also producing Substance P or CGRP (McMahon, 1996).

There are other tyrosine kinases that play similar roles in neurons such as RET, which is activated by the artemin (ARTN)/ glial-derived neurotrophic factor receptor (GDNF) alpha-3 (GFRα3) complex (Bespakov, 2007) and also by NGF (Tsui-Pierchala, 2002). The GDNF family appears to play a role in both neurotrophic support of neurons, as well as pain processing. Neurotrophic support of the peripheral nervous system, particularly the sympathetic subset of neurons seems to be due to the GDNF family and NGF family in overlapping roles (Baloh, 2000). One GDNF receptor, GFRα3, is mostly expressed in a subpopulation of nociceptive sensory neurons in the dorsal root ganglia (DRG) that coexpress RET, TrkA, and the thermosensitive channels TRPV1 and TRPM8 (Elitt, 2006). This cellular evidence and additional behavioral data (see below) points to a role for artemin in chronic pain signaling, and exploring this is the focus of this thesis.

GDNFs background

The GDNF family is a family of neurotrophic factors that was first recognized in the early 1990s (Lin, 1993). GDNF factor ligands (GFL) include GDNF (Lin, 1993), neurturin (Kotzbauer, 1996), persephin (Milbrandt, 1998), and artemin (Baloh, 1998). By 1997 enough of the GDNF family had been discovered that the nomenclature was changed to number their glycosyl-phosphatidyl inositol–(GPI-)anchored receptors as GFRα1-4 (GFRα Nomenclature Committee, 1997). Each GFL has a specific GFRα receptor that it has a high affinity for: GDNF binds to GFRα1 (Jing, 1996; Treanor, 1996); Neurturin binds to GFRα2 (Baloh, 1997; Buj-Bello
Artemin signals through GFRα3 (Balogh, 1998); Persephin binds to GFRα4 (Milbrandt, 1998). There has been shown to be some overlap between ligands and receptors, the most significant being overlap between GDNF and neurturin and their binding to GFRα1 and GFRα2 (Jing, 1997; Cacalano, 1997). Each GFRα1-4 directly interacts with the extracellular domain of the tyrosine kinase RET to cause RET to autophosphorylate (Sanicola, 1997). RET is the signaling part of this complex. RET is known to activate the phosphatidylinositol 3-kinase (PI3-kinase) and mitogenactivated protein kinase (MAPK) pathways along with other cell surface proteins (Airaksinen, 2002).

**Figure 1.1** Diagram of GDNF family signaling.

The current knowledge of GFL signaling indicates that it occurs with dimers of each molecule: a GFL homodimer binds symmetrically to two GFRα receptors and that complex recruits two RETs which are dimerized to become active. An alternative order has been proposed for artemin is that an artemin homodimer binds to GFRα3 and RET then that complex recruits another artemin/GFRα3/RET complex (Schlee, 2006). The formation of a new, symmetric surface between the binding of a GFL and a GFRα receptor seems to be essential for RET to bind and become activated (Wang, 2012).
All of the GFLs function as cell survival signals for neurons especially in the peripheral nervous system. Knock-outs of GFRα1, GFRα2, and GFRα3 all show peripheral nervous system abnormalities such as ptosis (drooping eye lids), and widespread decreases in the number of sensory neurons and amount of innervation (Balah, 2000). Mice lacking GFRα3, to which artemin binds, show a less extreme phenotype, but lack the proper migration of the superior cervical ganglia and have complete loss of innervation to the eye lid (Nishino, 1999). GFRα3 is only expressed in the central nervous system during early development, in maturity it is only expressed in the peripheral nervous system (Naveilhan, 1998). At maturity GFRα3 expression in the DRG has been reported to be between 20-24% of DRG neurons in rats and mice (Naveilhan, 1998; Orozco, 2001; Lippoldt, 2013). In both mice and rats GFRα3 expression is predominantly in nociceptive neurons in the DRG, as identified by co-expression of TRPV1, peripherin, RET, TrkA, and CGRP (Orozco, 2001).

In development, artemin guides axons of the sympathetic nervous system to innervate the periphery, this includes joint innervation. Artemin signaling through GFRα3 is essential for the proper development of the sympathetic nervous system, functioning primarily as a chemoattractant or guide for axonal outgrowth. Artemin-knock out mice as well as GFRα3 knock-out mice show deficient sympathetic nervous system innervation throughout their bodies. This improper innervation was found to be linked with decreased cell survival since the neurons that failed to innervate their target would not receive any survival factors from the target tissue. (Honma, 2002).

Post-development, artemin functions as a response to nerve-injury, stimulating nerves to recover normal function by acting as a survival and stimulatory factor. Artemin that was intrathecally infused was able to prevent changes seen in c-fiber nerve injury caused by sciatic
nerve transection, such as maintaining cell survival, conduction velocity, and substance P release (Bennett, 2006). Multiple doses of artemin delivered subcutaneously to mice after spinal nerve ligation, a model of nerve injury, were able to reduce pain behavior (Gardell, 2003), indicating that artemin in its capacity as a nerve growth factor can be protective against nerve injury-induced neuropathic pain. This along with GFRα3’s expression in nociceptive neurons has led to additional work investigating the role of artemin in various modalities of pain.

Artemin/GFRα3 in hypersensitivity and inflammatory pain

The expression of the GFRα3 receptor specifically in the sensory nociceptive neuron population indicates that artemin/GFRα3 signaling has a role in nociception. Experimental data indicate that artemin/GFRα3 signaling may play a role in a broad range of hypersensitivity conditions or states, including cold, heat, and possibly mechanical hypersensitivity states.

The precise mechanisms for how artemin/GFRα3 can induce hypersensitivity are not entirely known, though given the broad range of hypersensitivity that can be induced, it is likely that artemin/GFRα3 acts through regulating other channels, such as transient receptor potential (TRP) channels. Transient-receptor potential (TRP) channels, particularly TRPV1, TRPA1, and TRPM8, are responsible for conveying pain signals (Caterina, 1997; Story, 2003; Bandell, 2004; Bautista, 2007). Regulation of these channels by neurotrophins is essential for modulating pain signaling (Amaya, 2004; Malin, 2006; Kayama, 2017; Lippoldt, 2013).

Artemin has been shown to be essential for TRPM8-mediated cold pain. A hindpaw injection of artemin caused a greater response to the evaporative cooling assay (using acetone) in wild type mice. When this experiment was done on TRPM8 KO mice there was no difference between the artemin injected mice and the saline controls, indicating that TRPM8 is essential for artemin-induced hypersensitivity to cold (Lippoldt, 2013). One proposed mechanism for artemin-
TRPM8 mediated hypersensitivity to cold involves a subpopulation of about half of TRPM8+ neurons that are unmyelinated C-fibers that coexpress GFRα3, and largely express CGRP, a neuropeptide related to pain and migraine, and TRPV1. A RET independent pathway that GFLs are known to activate, NCAM, was found to coexpress with this population of TRPM8-GFRα3+ neurons (Lippoldt, 2013). Using GFRα3 knock-out mice as well as an anti-artemin antibody, Lippoldt et al (2016) showed that this system mediates cold hypersensitivity and not thermal or mechanical, however, it is unknown if these behavioral assays were performed blinded. Another proposed mechanism for artemin-induced cold hypersensitivity is increased expression of TRPA1, which may contribute to cold sensation (Story, 2003; Bandell, 2004). Cold hypersensitivity was also found in mice that were genetically modified to overexpress artemin in keratinocytes (skin cells). These mice had elevated expression of TRPA1 in the DRG, which overlapped with GFRα3 expression. Cold hypersensitivity was measured by withdrawal time of the mouse’s tail from an ethanol bath. Mice that overexpressed artemin had a lower threshold (i.e. faster withdrawal time) than wild-type mice (Elitt, 2006). In a model of bladder hyperalgesia TRPA1 upregulation was found to be due to artemin/GFRα3 signaling (Deberry, 2015).

Artemin/GFRα3 signaling might also have a role in thermal hypersensitivity to heat. Heat hypersensitivity has been reported from several laboratories in response to artemin. Hindpaw injections of artemin were found to decrease the latency to response to thermal (heat) stimuli (heat hypersensitivity) as measured by the Hargreaves apparatus (Lippoldt, 2013; Malin, 2006; Ikeda-Miyagawa, 2015). Arterin overexpression in mice led to increased mRNA for TRPV1, the receptor activated by noxious heat. These mice had shorter withdrawal times when tested with the Hargreaves apparatus and an increased response in both number of neurons and magnitude of the calcium-evoked transient to capsaicin, a TRPV1 agonist, as measured by
calcium imaging (Elitt, 2006). The evidence that artemin signaling causes increased activity of TRPV1 is strong. In separate calcium imaging experiments incubation with artemin potentiated the effects of capsaicin in cultured DRG neurons and caused some cells that had previously been unresponsive to respond to capsaicin indicating that TRPV1 was functionally upregulated after being incubated with artemin (Malin, 2006; Ikeda-Miyagawa, 2015). Multiple hindpaw injections of artemin also caused upregulation of TRPV1 in DRGs. (Ikeda-Miyagawa, 2015). The increase in TRPV1 in response to artemin provides a mechanism for artemin-induced thermal hypersensitivity.

Artemin/GFRα3 signaling might also have a role in mechanical hypersensitivity, which is of particular interest due to the prevalence of mechanical hypersensitivity in OA patients (Fingleton, 2015) – see Chapter 5. Neither a single artemin hindpaw injection (Lippoldt, 2013) or artemin overexpression (Elitt, 2006) caused mechanical hypersensitivity as measured by an electric Von Frey, however, repeated artemin injections over 5 days caused mechanical hypersensitivity (Ikeda-Miyagawa, 2015). The mechanism for this is unknown, but inflammatory pathways could be a contributing factor.

Artemin and GFRα3 have been found in inflammatory states. Mice given hindpaw injections of Complete Freund's Adjuvant (CFA) to induce an inflammatory state had significantly elevated GFRα3 expression at day 7 post-injection (Malin, 2006). Artemin mRNA was found to be upregulated in the skin (keratinocytes) at 3 hours, peaked at 24 hours, and then continued for 7 days after the initial CFA injection, much longer than the 24-hour time-course of NGF upregulation (Ikeda-Miyagawa, 2015).
Artemin and GFRα3 have a clear role in thermal hypersensitivity and a possible role in mechanical hypersensitivity and inflammation, all important components of the pathology of OA.

**Artemin/GFRα3 in musculoskeletal pain**

The concept that artemin/GFRα3 may be involved in musculoskeletal pain is very recent. The only work that has been done on the role of artemin/GFRα3 in musculoskeletal pain has used a model of inflammatory bone pain in rats meant to be representative of the pain experienced by patients with osteoarthritis. Nencini et al. (2018) injected artemin directly into the bone cavity and determined the bone pain phenotype by conducting electrophysiological and behavioral tests. *In vivo* electrophysiological recordings of bone afferent neurons found that artemin significantly increased neuron baseline activity and sensitized neurons to a ramping stimulus. Artemin applied to the medullary cavity also reduced weight-bearing in the injected limb for 2 hours as measured by an incapacitance meter. To determine whether this artemin-induced sensitization was relevant to inflammatory bone pain, such as osteoarthritis pain, rats were given a co-injection of CFA and anti-artemin antibody or the isotype control into the tibial medullary cavity and weight-bearing was evaluated. Rats given an anti-artemin antibody did not develop any reduced weight-bearing in the injected limb, while controls given CFA and an isotype control had significantly reduced weight-bearing on the injected limb (Nencini, 2018). To investigate the potential of artemin sequestration further Nencini et al. (2019) used a similar model where carrageenan, another inflammatory agent with a shorter time course, was injected into the bone cavity. In this model anti-artemin antibody reduced the electrophysiological pain phenotype (Nencini, 2019). This work indicates that artemin has a role in OA-associated pain, as CFA is often used as a quick model of longer-lasting inflammatory pain. However, the model
used here is not a true model of chronic pain or of joint deterioration. The sequestration of artemin has yet to be examined in a model of OA.

**Mouse Models of OA**

In order to study osteoarthritis (OA)-associated pain using rodents, a model of OA must be induced. These induced models attempt to mimic OA, but none are exactly equivalent to naturally occurring OA. Some models are probably more suited for studying joint pathology research, others for the study of inflammatory processes, and others for studying pathology-associated pain (Malfait, 2015). Induced mouse models of OA have a wide range of severity and progression rate of pathology allowing researchers to choose a model suited to their investigational needs (Poole, 2010). There are three main types of models of OA in mice: chemical, surgical, and traumatic injury. The primary chemical model of OA is involves using mono-iodoacetate (MIA). Surgical models include destabilization of the medial meniscus (DMM), anterior cruciate ligament transection (ACLT), and meniscectomy or meniscal/ligamentous injury (MLI). Traumatic injury models, meant to replicate post-traumatic OA (PTOA), use mechanical techniques to cause tibial compression overload and joint overload (Fang & Beier, 2018). Each model and induction method have different advantages and disadvantages and the ideal model for an experiment depends on the experiment being done. The two primary models that have been used to study OA-associated pain, rather than other aspects of OA such as joint pathology, are the MIA and DMM models (Inglis, 2008; Malfait, 2013).

The DMM model is considered to be one of the most representative of naturally occurring disease for studying OA in mice. In this surgery the medial meniscotibial ligament is transected causing meniscal instability and altered loading of cartilage, that gradually leads to cartilage erosion, subchondral bone sclerosis, and osteophyte formation. Histological changes
due to DMM can be observed as early as 2 weeks after surgery with more pronounced histological OA by 12-16 weeks (Fang & Beier, 2018). Studies looking at pain behavior post-DMM surgery have found mechanical hypersensitivity starting at 4 weeks and enduring until euthanasia at week 16 and reduced locomotion starting at 8 weeks post-DMM surgery (Miller, 2012). Malfait et al (2013) found mechanical hypersensitivity in DMM mice as early as 2 weeks post-surgery. In this same set of mice, no thermal hypersensitivity as measured by the Hargreaves apparatus or differences in weight-bearing as measured by the Catwalk system were detected out to 6 weeks (Malfait, 2013). However, weight-bearing deficits were observed at 8-10 weeks post-DMM surgery as measured by an incapacitance meter (Inglis, 2008). Methods to measure the presence of pain in the DMM mouse model include measures of mechanical hypersensitivity, cold allodynia, and thermal hypersensitivity. There has been concern over the reproducibility of studies using the DMM model because as a surgical model there is more variance in OA induction than non-surgical models. This concern has led to a general call to standardize methods for surgical induction of DMM (Ratneswaran, 2017).

The ACLT model of OA is induced by transecting the anterior cruciate ligament. Anterior cruciate ligament tears are a common human injury that can lead to OA due to the instability of the joint that cruciate transection induces. Physiological changes start occurring in the joint at 4 weeks, with full OA-like symptoms at 12 weeks. This model is not the most representative of human OA in terms of joint pathology as other joint structures and ligaments are often purposely damaged along with the transection of the ACL to increase the severity of the OA and decrease the time it takes OA to develop (Fang & Beier, 2018). ACLT transection is primarily preformed in the rat - mouse data for this procedure is limited (Pitcher, 2016).
Medial Ligamentous Injury (MLI), or meniscectomy surgery, is similar to DMM in that the joint is destabilized. The primary difference is in the surgery itself. MLI surgery is specifically designed to transect the medial collateral ligament, which is not as deep within the joint space. This model takes between 3 and 5 months to fully develop into a chronic pain state, which is analogous to the DMM model (Hamada, 2014). This model has been used to study OA-associated pain, but not as widely as the DMM model. This model causes a reduction in weight-bearing on the affected limb (5 weeks post-surgery), and decrease in latency due to evoked response to touch (1 week post-surgery), and cold stimuli (2 weeks post-surgery) (Knights, 2012).

There are noninvasive trauma models that have been described. Specialized equipment is used to mechanically induce a joint injury externally, which then progresses into OA. There are multiple types of joint injury that can be induced including intra-articular fracture of tibial subchondral bone, cyclic tibial compression loading of articular cartilage, and anterior cruciate ligament (ACL) rupture via tibial compression overload (Christiansen, 2015). Due to the fact that these models have only been described more recently, there have not been studies characterizing the progression of pain with the detail that has been applied to the DMM model. There have been multiple studies looking at the pathology of noninvasive trauma models and they seem to be a reasonable reflection of the disease. Noninvasive trauma models have been described as having the advantage of being highly reproducible, unlike the variation that can arise from surgery to induce OA in the DMM model (Kuyinu, 2016; Zhao, 2014). A disadvantage of these systems is that the progression of OA is much slower than surgical models (Kuyinu, 2016). These models vary a lot from each other depending on the methods used to compress the joint, including force and speed, that results in different disease phenotypes. Trauma models are mainly useful for
studying the early processes involved in the development of OA, rather than later-stage OA or OA-associated pain (Fang & Beier, 2018).

Chemically induced models, of which MIA is the most common, are easy to implement and minimally invasive. The MIA model of OA is induced via an intra-articular injection of mono-iodoacetate. This chemical, when injected into the joint space, interrupts glycolysis by inhibiting glyceraldehyde-3-phosphatase in chondrocytes, leading to extensive cell death. MIA can be toxic if it escapes the joint space, particularly at higher doses. The MIA model is commonly used to test pharmacological agents for the alleviation of OA pain as it is a robust, reproducible, and fast model of OA-associated pain (Pitcher, 2016). The induction of joint damage through chemical means is very different from the naturally occurring disease and the rate of progression is much faster than would naturally occur. However, MIA is considered an appropriate model to study OA-associated pain (Guzman, 2003).

Each model of OA has advantages and disadvantages and currently there is no ‘gold-standard’ model for OA (Fang & Beier, 2018). Clinically, OA is a heterogeneous disorder with different subset of patients progressing and responding differently to treatments depending on the root cause(s) of OA and the neurobiology driving the pain. Research is still ongoing to determine what factors and symptoms are important for designating subgroups in human patients. Currently, important designations are inflammation (local or systemic), overall progression of joint damage (early or late stage), joint alignment (correctly or misaligned), and, bone density (Bruyere, 2015). Presumably pain states and hypersensitivity will vary based on multiple patient factors. The MIA-induced model is arguably more representative of OA patients with established pain and sensitivity - those which would stand to benefit the most from novel drugs to alleviate OA-associated pain.
Here, the primary goal was to select a model that would be highly representative of OA-associated pain and hypersensitivity, develop this pain relatively quickly, and be reproducible with minimum variation between mice.

**Measuring OA-associated pain in rodent models**

According to the International Association for the Study of Pain (IASP), pain is “An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” (Cohen, 2018). In the quest to develop new therapeutics to alleviate pain this broad definition can present challenges since our current understanding of pain is of pain as an experience rather than as a distinct physiological phenomenon that can be directly quantitated. The experience of pain can vary widely between individuals and is influenced greatly by an individual’s environment and expectations making robust validations of pain scales and assays extremely important. Robust assays to measure pain become essential if basic science is to be successfully translated.

There are two main categories of assays for measuring pain in animals: reflexive assays to measure hypersensitivity and complex behavioral assays to measure the impact of spontaneous pain. Reflexive assays involve a stimulus that the animal responds to in some way, typically by withdrawing. More complex behavioral pain assays work by quantitating an animal’s behavior typically at a specific task such as walking or burrowing where impairment indicates pain. Examples of complex behavioral assays to measure spontaneous pain assays include static limb-use assays and grimace scales.

There are advantages and disadvantages to both categories as each can give information about different aspects of pain. The reflexive assays give information about hypersensitivity and are very repeatable with less variation in measurements and between mice. Complex pain assays
give more information about the affective aspect of pain or the “unpleasantness” by evaluating
an animal’s ability or willingness to perform an activity. The results tend to be more much
variable and require larger sample sizes to get robust data. Ideally studies evaluating the
effectiveness of potential analgesics would use a combination of reflexive, complex, and
spontaneous pain assays to obtain a more complete view of the drug’s effectiveness.

*Reflexive assays*

Reflexive assays tend to be used more commonly, particularly for rodent studies, though
there has been a push in the literature to use more complex behavioral assays in the pain
literature. Large animal models tend to incorporate more of the complex behavioral assays.
Reflexive assays are primarily measuring sensitivity, rather than actual pain. Sensitivity data is
still useful information that is thought to highly relate to pain, or at least to our ability to treat
pain. The primary stimuli that animals are sensitive to are heat, cold, and pressure (mechanical).
Each of these modalities are tested in different reflexive assays.

Mechanical sensitivity can be measured with tools such as Von Frey filaments or a
pressure application measurement (PAM) device (Martinov, 2013; Malfait, 2013). PAM devices
can be used to apply pressure directly to the knee or hindpaw to determine the mechanical
threshold (Malfait, 2013). Testing with Von Frey filaments is considered to be the gold-standard
for evaluating mechanical hypersensitivity in rodents. There are many different methods used
with Von Frey filaments (Martinov, 2013). The most robust methods for Von Frey testing are the
up-down method which uses different sized filaments to apply different amounts of force.
Filaments of increasing force are applied until the mouse responds with a withdrawal or paw
licking. At this point the stimulus force is decreased and then ramped up again. This is done to
determine the accuracy of the paw withdrawal threshold (Chaplan, 1994; Bonin 2014). The other
most common Von Frey technique used is the electric Von Frey or the dynamic plantar aesthesiometer. This is an automated system that uses a single filament and ramps up force until withdrawal (Martinov, 2013; Malfait, 2013).

Cold sensitivity in rodents has been measured by a variety of methods. Common assays include the acetone evaporation test (Yoon, 1994; Carlton, 1994), tail flick assay (Pizziketti, 1985), cold plate assay (Allchorne, 2005), two-temperature choice test (Dhaka, 2007), and cold plantar assay (Brenner, 2012). The acetone evaporation test uses the time mice spend flicking their paws after it is cooled with acetone (Yoon, 1994, Carlton, 1994). The tail flick assay measures how long until mice withdraw their tails from a cold-water bath (Pizziketti, 1985). In the cold plate assay mice are placed on a cold plate and their response recorded. Latency, number of jumps, and number of paw lifts are common outcome measures for the cold plate assay (Allchorne, 2005). The two-temperature choice test allows mice to choose between 2 plates of different temperatures with the time spent on each plate meant to indicate the mouse’s preference (Dhaka, 2007). The cold plantar assay measures withdrawal time from the cold stimulus of dry ice (Brenner, 2012). The lack of a standard cold withdrawal assay, compared to the Von Frey for mechanical and the Hargreaves for heat sensitivity, is a weakness in the field. The novel plantar cold assay is the most similar in methodology to the reflexive tests for the other modalities and thus could fill that gap.

Measuring sensitivity to heat in rodents has a similar variety of methods including tail withdrawal (Calo, 2009), the hot plate assay (Belknap, 1990), and the temperature choice test (Lee, 2005). However, unlike for measuring cold sensitivity, heat sensitivity has a gold standard assay – the Hargreaves apparatus (Malfait, 2013). The Hargreaves apparatus uses a light source
placed under the paw as a heat source. Withdrawal latency is measured to determine sensitivity (Hargreaves, 1988).

**Limb-use Assays**

Limb-use and gait analysis are useful and clinically relevant outcome measures for preclinical OA studies. Limb-use and gait analysis quantify the effects of pain and joint function on limb use. This is different from reflexive outcome measures, which measure sensitivity to a stimulus as an evoked response. In limb-use and gait analysis assays mice are often allowed freedom of movement and are not stimulated by the equipment in an effort to capture their natural behavior and limb-use. Gait patterns can be altered to protect an injured joint from loading and/or movement-induced pain. Common compensatory changes are shuffle-stepping and limping (Lakes & Allen, 2016).

Unfortunately, there is not enough consistency in the methods used for gait analysis in rodents nor parameters reported to fully understand the utility of measuring limb use in mice. There are many different methods and systems that have been used to measure gait changes in rodents – paws can be dipped in ink or complex systems can be used such as the catwalk, DigiGait (Dorman, 2013), GaitScan (Adams, 2016), and TreadScan (Dorman, 2013), which provide dynamic gait analysis. The catwalk system uses a camera to record the refracted light of rodent pawprints as they traverse along a lit glass walkway. The GaitScan is similar to the catwalk except it does not use refracted light. The DigiGait and the TreadScan both use cameras to record pawprints while the rodents are ambulating on a transparent treadmill. All of these systems automatically calculate an array of outcome parameters such as print area and intensity, which are the most reported gait analysis parameters for OA models (Lakes & Allen, 2016). Subtle changes in gait can be detected by analyzing multiple parameters together (Lakes &
Allen, 2016; Jacobs, 2014). It is not yet clear which gait changes are associated with functional/mobility problems of the joint and which are due to a fear of movement because of the associated pain (Jacobs, 2014). Additionally, spatiotemporal gait parameters are commonly reported and well-studied in larger quadrupeds and have more recently been applied to rats and mice. Spatial parameters use the mouse’s footprints, and calculate indices based on distances between footprints. Temporal parameters focus on how long the mouse spends on each foot and whether the gait is regular. The classically reported parameters are stride length, step length, stride length asymmetry, and step width for spatial and duty factor, temporal symmetry, stance time imbalance, and limb phase for temporal parameters. Differences in these parameters can be subtle and difficult to detect, especially in such small animals. A single gait measurement may not be enough as an outcome measure for limb-use. Since mice have four legs there are many different ways that they can compensate for a painful joint in a single limb and the altered gait pattern must be considered carefully (Lakes & Allen, 2016).

Relatively few studies using mice and models of OA have been published describing the use of the Catwalk system. These studies have used different parameters to examine changes in limb-use after OA model induction and have had variable results – indeed, only a few studies have found any significant differences between mice with OA and controls. The DMM model in mice has been used in a few Catwalk studies with varied results. One study found no difference in any parameters (Malfait, 2010). Another found differences in temporal parameters after 12 weeks (Muramatsu, 2014). One found significant difference in the log of the paw intensity between the contra- and ipsilateral paws at 10 weeks (Fang, 2018). The literature using the Catwalk system is even more limited for the MIA-induced model of OA in mice. Miyagi et al (2017) used the Catwalk as an outcome measure to determine the efficacy of an anti-NGF
growth factor as an analgesic for OA-associated pain in the MIA model. They found duty cycle, swing speed, and print area to be significant parameters (Miyagi, 2017). In contrast, a study using rats with MIA found significantly reduced intensity in the ipsilateral pawprints, analogous to peak vertical force (Ferreira-Gomes 2008).

Dynamic gait analysis promises to be a powerful tool to investigate limb-use in rodents, but until there is greater consensus on which parameters should be reported and which are truly meaningful and indicative of pain it has yet to reach its true potential.

Static weight-bearing in rodents, however, is very well established and straight-forward in methodology and analysis. Static weight-bearing is measured with an incapacitance meter, which has individual force plates for each hindpaw. The rodent is trained to stand on the force plates prior to the experiment. The weight placed on each paw is typically recorded over a 3-5 second period. Rodents with OA induced in a hindlimb put less weight on that limb (Malfait, 2013). One variation in measuring weight distribution with an incapacitance meter is the position of the mouse. Some systems have the mouse standing on all four paws, just measuring the hindlimbs, while other force the mouse into an upright position where the mouse is only placing weight on the hindlimbs. Horizontal incapacitance meters measures the weight-bearing in mice that are standing at rest in their normal position (Williams, 2019).

**Measuring Pain in Dogs with OA**

There has been interest in measuring pain in companion animals both from a translational and a quality of life standpoint. Companion animals share the same environment as humans and as such have the same risk factors for osteoarthritis and chronic pain that humans have including inactivity, weight gain, diabetes, and genetic predisposition. These companion animals can
provide a window into the mechanisms of chronic pain as models of naturally occurring disease while receiving quality care.

Measuring pain in companion animals is a complex endeavor and there have been many approaches. The most validated techniques are clinical metrology instruments (CMIs) and gait analysis. These quantify different aspects of pain. CMIs capture the affective component of chronic pain with scores for behaviors. Gait analysis is a way to evaluate how chronic pain impacts function. A dog will put less weight on a leg with an osteoarthritic joint and that force can be measured (Lascelles, 2019).

Four CMIs have been validated for canine OA: the American College of Veterinary Surgeons canine orthopedic index (COI) (Brown, 2014), the canine brief pain inventory (CBPI) (Brown, 2008), the Helsinki chronic pain index (HCPI) (Hielm-Björkman, 2009), and the Liverpool osteoarthritis in dogs (LOAD) (Hercock, 2009). These questionnaires cover a variety of areas including stiffness, gait, function, quality of life, pain severity, pain impact on function, chronic pain, and mobility. These areas can be used to monitor changes in a patient over time, such as when determining whether a potential analgesic is effective over the course of a study (Lascelles, 2019). The CBPI questionnaire was developed as an owner assessment, which can provide insight to a dog’s condition in their normal environment. Four questions regard pain severity and six focus on how pain interferes with normal activities. The answers from these categories are averaged to give a pain severity score and a pain interference score (Brown, 2008). The LOAD is another owner-completed questionnaire with 13 questions on lifestyle and mobility that provide an overall LOAD score (Walton, 2013). These questionnaires complement each other in that the CBPI can provide information on severity of pain and interference of pain with the performance of daily activities, while the LOAD probably gives more information on what is
classically thought of as ‘lameness’, and well as giving information on how well the activities of daily living are performed (Brown, 2008, Hercock, 2009). Indeed, the LOAD was shown to encompass multiple dimensions, whereas the CBPI shown to encompass only one. (Walton, 2013).

Objective gait analysis most often is based on the measurement of ground reaction forces using a force plate or pressure sensitive walkway. Dogs should be walked or trotted across the equipment at a consistent speed in a straight line in order to obtain usable data. The primary outcome measures for limb-use are peak vertical force (PVF) and vertical impulse (VI), though there are many different analyses that can be done with force plate data. Force plate analysis has been validated – the PVF and VI improved in OA dogs after administration of the analgesic carprofen (Brown, 2013). The most limiting factor for gait analysis in naturally occurring disease in dogs is that this system is best for single limb-OA. This allows researchers to compare an OA limb to a normal limb. If all of a dog’s joints are equally osteoarthritic then gait analysis won’t show changes in weight-bearing in a consistent manner (Lascelles, 2019).

Conclusion

There is an unmet need in our society for non-addictive and effective analgesics to treat OA-associated pain. In the following chapters, we explore the role of artemin and its receptor GFRα3 in OA pain. The current excitement around anti-NGF antibodies for treating OA-associated pain supports looking at the role of other neurotrophins in OA pain. Artemin and GFRα3 are prime candidates due to GFRα3’s specific expression in the nociceptive neurons in the DRG, their role in regulating TRPV1, TRPM8, and TRPA1 expression, and artemin’s ability to induce inflammatory bone pain as well as hypersensitivity to multiple pain modalities.
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CHAPTER 2

Correlation of artemin and GFRα3 with osteoarthritis pain: early evidence from naturally occurring osteoarthritis-associated chronic pain in dogs

Introduction

As mentioned in Chapter 1, the success of any drug development program will fail if the biological basis or rationale for the therapeutic is not sound. So far, the assumption has been that the mechanisms discovered in an induced rodent model of pain are relevant to the natural disease state in humans. An informative approach would be to ask ‘what mechanisms are altered in the natural disease state?’ Tissue is not always easily obtained from humans; however, peripheral tissue can be readily obtained from the millions of joint surgeries performed each year on pet dogs (Lascelles, 2009) and central nervous system tissues are available from the thousands of dogs with OA-associated pain that are euthanized each year. If the outcome measures described above are used to pain phenotype the pets, then the information garnered from such tissue can point to novel relevant pathways.

Therefore, in the work described here, we performed work to uncover potential pain-relevant pathways associated with OA pain. To determine potential targets for the treatment of OA-associated pain we used samples from naturally occurring OA in dogs. First, we evaluated the primary afferent TRPV1-colocalized pain receptors that appeared to be upregulated in naturally occurring OA in dogs. We achieved this by using DRG procured from very well pain-phenotyped dogs that had been euthanized. The results indicated that GFRα3, known to have a role in pain in rodents, was upregulated in the ipsilateral DRG, a result that warranted further exploration of whether GFRα3 was involved in OA-associated pain. This chapter describes that work, and the correlative relationship between GFRα3/artemin and OA-associated pain in dogs.
Correlation of Artemin and GFRα3 With Osteoarthritis Pain: Early Evidence From Naturally Occurring Osteoarthritis-Associated Chronic Pain in Dogs

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Arthritis, including osteoarthritis (OA) and other musculoskeletal-associated pain, is a worldwide problem, however, effective drug options are limited. Several receptors, neurotransmitters, and endogenous mediators have been identified in rodent models, but the relevance of these molecules in disease-associated pain is not always clear. Artemin, a neurotrophic factor, and its receptor, glial-derived neurotrophic factor (GDNF) family receptor alpha-3 (GFRα3), have been identified as involved in pain in rodents. Their role in OA-associated pain is unknown. To explore a possible association, we analyzed tissue from naturally occurring OA in dogs to characterize the correlation with chronic pain. We used behavioral assessment, objective measures of limb use, and molecular tools to identify whether artemin and GFRα3 might be associated with OA pain. Our results using banked tissue from well-phenotyped dogs indicates that artemin/GFRα3 play an important, and hitherto unrecognized, role in chronic OA-associated pain. Elevated serum levels of artemin from osteoarthritic humans compared to healthy individuals suggest translational relevance. Our data provide compelling evidence that the artemin/GFRα3 signaling pathway may be important in OA pain in both non-humans and humans and may ultimately lead to novel therapeutics.

Keywords: osteoarthritis, pain, DRG, GDNF, artemin, GFRα3, dogs, human

INTRODUCTION

A significant proportion of the economic burden of chronic pain is due to musculoskeletal pain, and yet treatment options remain limited and insufficient for the alleviation of osteoarthritis (OA)-associated pain, the major component of musculoskeletal pain (Burgess and Williams, 2010). Despite significant successes arising from basic pain research in terms of our understanding of
molecular mechanisms and the growing number of potential targets for new drug development, drug candidates are failing when they enter Phase II (efficacy) trials in humans due to limited knowledge of the cellular and molecular components involved in disease conditions. Diseases in companion animals have been suggested to be good models of certain human conditions, including OA (Mogil, 2009; Percie du Sert and Rice, 2014; Klink et al., 2017; Lascelles et al., 2018).

The glial cell line-derived neurotrophic factor (GDNF) family members (GDNF, neurturin, and artemin) sensitize nociceptors; in particular, artemin is an important effector in inflammatory hyperalgesia (Malini et al., 2006). Artemin is thought to be an important endogenous mediator for inflammation, migraine, burning mouth syndrome, and neuropathic cold allodynia (Lippoldt et al., 2013, 2016; Shinoda et al., 2015; Shang et al., 2016; Nencini et al., 2018). Recently, artemin signalling pathways have been shown to be involved in the pathogenesis of bone pain (Nencini et al., 2018). Another important role of artemin has been identified in the trigeminal ganglia (TG), where artemin regulates an inducible form of nitric oxide synthase (iNOS). The regulation of iNOS might be involved in the mechanism through which artemin participates in the trigeminal pain pathways (Shang et al., 2017). Furthermore, anti-artemin therapy has been shown to be effective in preventing and reversing ongoing bladder hyperalgesia in an animal model of cystitis (DeBerry et al., 2015). In contrast, artemin exerts an antinoceptive effect on herpes-related pain by modulating the dynorphin levels in the central nervous system of HSV-inoculated mice (Asano et al., 2006). Based on this evidence, peripheral artemin is involved in generating pain signals in rodents.

The GDNF family of ligands mediate their effects through GDNF receptors, and, at this point, four different members of this family have been identified (GFRα1−4). GDNF binds to GFRα1, neurturin activates the GFRα2 receptor, and artemin binds to the GFRα3 receptor. All of the GDNF-receptors form a complex with the tyrosine kinase RET, and then, through RET, activate several intracellular signaling pathways (Baloh et al., 1998a,b). GFRα1 and GFRα2 receptor expression has been demonstrated in the non-peptidergic neuronal populations, but, in contrast, GFRα3 is predominantly expressed by a subpopulation of nociceptive sensory neurons that are peptidergic (Orozo et al., 2001; Elitt et al., 2006), some or all of which also express the Ret receptor tyrosine kinase, and by the transient receptor potential (TRP) ion channel proteins TRPV1 and TRPA1 (Orozo et al., 2001; Elitt et al., 2006; Goswami et al., 2014). Furthermore, the GFRα3 receptor is also expressed by transient receptor potential cation channel subfamily melanostatin (TRPM8) expressing neurons that are involved in encoding cold sensations (Lippoldt et al., 2013, 2016). Outside of the dorsal root ganglia (DRG), other reports suggest the expression of GFRα3 in non-neuronal cells (Yang et al., 2006; Thai et al., 2019). Another receptor sub-family, GFRα4, is not functionally expressed in the DRG (Lindahl et al., 2000; Luo et al., 2007). All evidence considered, it is clear that GFRα3 is exclusively expressed in nociceptive neurons in the DRG, suggesting that artemin/GFRα3 interaction is important in the transmission of pain and could be a potential target in the development of analgesia.

Overall, artemin/GFRα3 is involved in inflammation and bone pain in rodents, but its expression and functional correlation with pain in naturally occurring OA are unknown, to the best of our knowledge. By analyzing samples from dogs with naturally occurring OA, we aimed to identify relevant and translationally resilient pharmacological target(s). We employed this innovative approach to identify whether artemin/GFRα3 may play a role in OA-associated pain. We analyzed DRG from pet dogs with naturally occurring OA and found significantly increased GFRα3 in the DRG serving osteoarthritic joints. This led us to hypothesize that the expression of its ligand, artemin, would be increased in serum and that synovial fluid artemin may be related to joint pain in dogs with OA. Next, we explored the relationship between serum artemin and OA status in humans. In summary, our results provide the first evidence of a link between artemin/GFRα3 and OA-associated pain in the natural disease state.

**MATERIALS AND METHODS**

**Collection of Tissues and Fluids**

The serum and synovial fluid samples used in the work described here were collected from client-owned pet dogs that had been carefully evaluated and found to have OA-associated pain or found to be normal (i.e., pain-phenotyped animals), as a result of their involvement in veterinary clinical research studies (Muller et al., 2019) or ongoing translational pain research studies at NC State College of Veterinary Medicine. The breeds of dogs included were representative of the medium to large breeds that present for OA-associated pain. None of these dogs were receiving or had received analgesics for 4 weeks prior to sample collection. All dogs had undergone radiographic evaluation and examination by a specialist veterinary orthopedic surgeon and had clinical metrology instrument data available to define whether OA was present and whether pain was associated with it. In addition, these data were supported in some cases by force plate data on limb use. The characteristics of dogs used in various parts of this work are detailed in Table 1.

Peripheral nervous system tissue samples (lumbar DRG) were collected after euthanasia from well-phenotyped research animals of known pain and OA status (similar to a previous study (Little et al., 2016)). These were mixed-breed hounds, and they had not received any analgesic treatment. DRG (L4-L6) samples were collected within 30 min of euthanasia, with DRG identified by counting lumbar vertebras. All these dogs had radiographic confirmation of OA and force plate data verifying unilateral joint pain (Table 1, column 4). DRG samples for the pilot data (not shown here) were collected from pet client-owned dogs being euthanized due to unilateral OA pain that were examined and evaluated by a veterinarian prior to euthanasia.

All original studies and sample collection were conducted with informed and written owner consent and Institutional Animal Care and Use Committee (IACUC) approval. All samples were stored at −80°C within 2 h of collection, or 2 h of euthanasia in the case of DRG, and all the samples to be used in this study were collected within the last 5 years.
Measurement Ground Reaction Forces

Kinetic gait analysis of limb use was performed using dual in-series force plates (ATMI, Watertown, MA, United States). Dogs (Table 1, column 3) were specifically recruited for having single limb lameness, as demonstrated by a lower peak vertical force ([PVF] expressed as a percentage of body weight) compared to the contralateral limb, detectable pain on manipulation, and radiographic evidence of OA in one joint in the index limb. Dogs were trotted over the force plates, and trials were accepted as valid if the dog trotted across the force plate in a straight line and an observer confirmed foot strikes on the plates without noticing any unusual activity by the dog (pulling, visually detectable movement of the head from side to side), with a target velocity of 1.7-2.1 m/s and within acceleration changes of ±0.5 m/s². Gait velocity and acceleration were measured by means of five photocell switches (photocells 50 cm apart) connected to the computer analysis system. Five valid trials were collected from each dog during trotting, and data were collected for all limbs. A single handler and observer performed all gait analysis. specialized computer software (Sharon software, DeVitt, MI, United States) was used to calculate the ground reaction forces (GRFs) of the limbs. GRFs were expressed in percent body weight, and then symmetry indices were calculated for the index limb and the contralateral limb. Symmetry indices (SI) for PVF and vertical impulse (VI) were calculated by the use of the following equation:

\[ SI = \frac{(x_i - x_c)}{(1/2)(x_i + x_c)} \times 100 \]

where \( x_i \) is the mean of a given gait variable for the index limb, and \( x_c \) is the mean of a given gait variable for the contralateral limb. By using symmetry indices, data for fore and hind limbs can be combined.

Clinical Metrology Instrument Assessments of Pain and Mobility Impairment

Where applicable, caregiver (owner) assessments of pain, activity, and function were captured using validated clinical metrology instruments (questionnaires): the Liverpool Osteoarthritis in Dogs (LOAD) (Herrick et al., 2009; Walton et al., 2013) and the Canine Brief Pain Inventory (CBPI) (Brown et al., 2007). The LOAD is a 13-item instrument with all items reported on a five-point Likert-type scale. Each item is scored between 0 and 4, and the item scores are summed to give an overall instrument score. The LOAD covers three domains: activity/exercise, stiffness/limpiness, and the effect of weather (Walton et al., 2013). The CBPI is a two-part instrument based on the human Brief Pain Inventory; the pain severity score (CBPI PSS) is the arithmetic mean of four items scored on an 11-point (0 to 10) numerical scale, and the pain interference score (CBPI PIS) is the mean of six items scored similarly. It has been reported to measure two dimensions (pain and interference) (Walton et al., 2013).

Quantitative RT-PCR

Total RNA was extracted from DBS using Qiagen’s RNeasy kit and converted into cDNA. Quantitative real-time PCR was accomplished using commercially available TaqMan® primer sets. Samples were run in triplicate. Amplification efficiencies were normalized against GAPDH, a standard housekeeping gene (Wheeler et al., 2019). Individual \( \Delta C_T \) values were calculated using StepOne Software v2.2.2. Relative expression \( (\Delta \Delta C_T) \) of each gene was calculated with the following equation:

\[ \Delta \Delta C_T = C_{T,GAPDH} - C_{T,GOI} \]

where \( \Delta C_T \) is the gene of...
interest. ΔCt values were linearized using $2^{-\Delta \text{Ct}}$ and then multiplied by 1000 so that the y-axis is on a more intuitive scale. Standard deviation was calculated using the linearized ΔCt values and used in the SEM calculation: $\text{SEM} = \text{standard deviation} / \sqrt{n_{\text{technical}}}$, where $n_{\text{technical}}$ is the number of technical replicates. The Taqman probes for the dog genes studied were purchased from Thermofisher, Carlsbad, CA, United States: TRPV1 (C#27233943), GFRα3 (C#2673182), and GAPDH (C#04419463).

**Western Blot**

To extract total protein, DRG were homogenized using a tissue homogenizer in the presence of 100 μl of ice-cold RIPA buffer supplemented with protease inhibitor tablets (Pierce™). Total protein of lysates was measured using standard BCA (Bicinchoninic Acid Assay). Protein lysates were then denatured by heating at 95°C in Laemmli’s buffer containing 2% w/v SDS, 62.5 mM Tris (pH 6.8), 10% glycerol, 50 mM DTT, and 0.01% w/v bromophenol blue (Pitake et al., 2019). The lysates were cooled on ice and briefly micro-centrifuged. Aliquots of 35 μg of DRG protein were loaded onto a 4–12% SDS-PAGE gel, and subsequently electro-blotted onto PVDF membranes. Membranes were incubated in 15 ml of blocking buffer (20 mM Tris base and 140 mM NaCl, 5% bovine serum albumin, and 0.1% Tween-20) for 1 h. Membranes were then incubated with the desired primary antibody [rabbit anti-GFRα3 primary antibody (RA300177), 0.2 μg/ml; loading control mouse anti-GAPDH (Cat. sc-32233)] diluted in 10 ml of blocking buffer at 4°C overnight. The next day, the membrane was washed and incubated with an appropriate horseradish peroxidase-conjugated secondary antibody [secondary goat anti-rabbit-HP (from Santa Cruz (sc-2030); secondary goat anti-mouse IgG HRP (1:10000)] in 10 ml blocking buffer for 1 h at room temperature. Immuno-reactive proteins were revealed using enhanced chemiluminescence detection (Pierce ECLTM). Denitometry analysis was performed using open-source ImageJ software from NIH.

**Quantitative Measurement of Artemin**

Artemin was analyzed using a quantitative competitive enzyme-linked immunosorbent assay (ELISA). Canine serum samples from OA dogs and normal dogs (n = 25 OA; n = 11 normal) were stored at -80°C and thawed and vortexed before use. Canine serum samples were run according to the protocol provided by the kit (ABclonal Canine Artemin ELISA Kit CA0039). The stop solution changes color from blue to yellow; the intensity of the color was measured at 450 nm using a spectrophotometer (LabSystems Fluoroskan Ascent FL ELISA plate reader) and sample concentrations were determined by comparison to the standard curve.

Artemin levels from human serum were measured using the R&D Systems Human Artemin DuoSet ELISA (R&D, DYS259) and the associated protocol. Human serum samples were obtained from Reprocell (Beltsville, MD) after searching their database of available samples. All OA serum samples (n = 5) were from middle-aged (45–63), female patients who had endured chronic, multiple-joint OA pain for a minimum of 13 years. Non-OA control serum samples (n = 4) were obtained from the same company and matched for age and gender. Serum samples were stored at -80°C until used, at which point they were vortexed and aliquoted for the ELISA. All samples were run in triplicate. Spectrophotometer readings were at 450 and 570 nm. The 570 nm readings were subtracted from the 450 nm for the most accurate results. Sample concentrations were determined by comparison to the standard curve.

**Statistical Approach Including Sample Size Estimations**

Data are expressed as mean ± SEM. All assays were performed by individuals who were blinded to the metadata. Statistical analysis was performed in GraphPad Prism and JMP statistical software. For canine serum artemin, differences between the two groups were examined using a parametric Student’s t-test, with p < 0.05 considered significant and p > 0.05 considered nonsignificant. A formal sample size estimation was used for serum artemin concentrations, based on pilot data, and indicated that a total sample size of 28 would be required for a power of 0.8, at an alpha of 0.05. Canine serum artemin concentrations were compared to force plate data (symmetry index) and owner questionnaires using linear regression. Correlation coefficients (R-squared values) and p-values were generated using JMP-Pro software. The threshold to determine whether a correlation was significant was set at a p-value of 0.05. Similar sample size calculations for the RT-PCR pilot data indicated that a paired sample size of 7 was needed for a power of 0.8 at an alpha of 0.05. No other sample size estimations were performed. For Western blot assay, we performed a pairwise t-test and determined the statistical significance. Sample size for human serum samples was limited due to the availability of the samples; a 2-tailed Student’s t-test was used to evaluate the data statistically. Data were tested for normal or non-normal distribution, and appropriate statistical tests were used. Animals and data points were not excluded from the analysis. All relevant data are available from the authors.

**RESULTS**

**DRG Sensory Neurons Express GFRα3**

In early pilot work, we evaluated dog DRG tissue for multiple receptors that we had found to be expressed on TRPV1-expressing neurons in the dorsal root ganglia (DRG) in mice (Goswami et al., 2014). This pilot work indicated increased GFRα3 in DRG serving osteoarthritic joints in dogs. Therefore, we determined the expression levels of GFRα3 in the lumbar (L4-L6) DRG serving contralateral versus ipsilateral joints with associated pain within the same dog (n = 5; Table 1, column 4) by using quantitative RT-PCR. Our results showed an approximately 4-fold increase in GFRα3 expression in ipsilateral DRG serving OA joints compared to those serving normal joints (Figure 1A) suggesting a role of GFRα3 in OA pain. We also compared the expression of TRPV1 receptors, a well-known molecule involved in pain signal transduction (Mishra et al., 2011). Our results
demonstrated an increase in the expression of Trpv1 mRNA in the ipsilateral DRG compared to contralateral from OA dogs.

Next, we examined the expression of GFRα3 at the protein level in the DRG sensory neurons. Due to an absence of a dog-specific GFRα3 antibody and because of high homology between the dog and mouse protein sequences (76% identity for the GFRα3 receptor, we used an anti-mouse GFRα3 antibody for the Western blotting. In addition to overall sequence homology, the synthetic peptide corresponding to amino acids from 347 to 360 of the murine GFRα3 has 90% identity with the dog-predicted GFRα3 receptor. We performed GFRα3 antibody characterization using negative control (heart tissue) and secondary antibody control (data not shown). However, these methods still do not validate the specificity of the antibody, which needs to be further tested either in GFRα3 knockout mice or using pre-adsorption with GFRα3 synthetic protein/peptide. We used Western blotting to detect and quantify the presence of GFRα3 receptors in the DRG serving contralateral versus painful OA (ipsilateral) joints within the same dog. We identified GFRα3 protein in DRG from contralateral DRGs and found an increase in GFRα3-receptor protein in the ipsilateral OA DRGs, again suggesting a role of GFRα3 in pain sensitivity (Figure 1B).

Uprgulation of Serum Artemin in Osteoarthritic Dogs

The endogenous ligand for GFRα3 is artemin (Balogh et al., 1998a,b; Wang et al., 2006). Having found increased expression of GFRα3, we investigated whether circulating or local levels of artemin were increased in dogs with painful OA. Serum samples were collected from non-OA healthy controls and dogs with OA-associated pain at the time of screening, prior to their involvement in a clinical study (Mueller et al., 2019). The demographics of the dogs are tabulated in Table 1 (columns 1 and 2). The OA dogs were older, but otherwise there were no differences between the groups. We measured serum artemin concentrations using ELISA, which showed an increased concentration of artemin in dogs with OA-associated pain compared to pain-free healthy dogs (Figure 2A). Pain and disability in pet dogs with OA can be measured using validated clinical metrology instruments (CMIs), and we found that higher serum artemin concentrations were associated with higher scores on the CMIs and thus greater levels of pain and functional impairment. There was a significant relationship between LOAD index values and serum artemin concentrations ($R^2 = 0.11; p = 0.05$) and significant relationships between serum artemin and the Canine Brief Pain Inventory (CBPI) pain ($R^2 = 0.11; p = 0.049$) and CBPI interference ($R^2 = 0.16; p = 0.014$) subscales (Figure 2B).

Next, we evaluated local concentrations of artemin in synovial fluid and compared synovial fluid artemin concentrations with objectively measured limb use (a measure of joint pain). Synovial fluid samples were collected from dogs enrolled in a pilot study aimed at assessing the efficacy of a TNFα fusion protein injected intra-articularly (unpublished data). The demographics of the dogs are tabulated in Table 1. Synovial fluid artemin concentrations were normally distributed. There was a significant, negative relationship between synovial fluid levels and index limb use compared to contralateral limb, expressed as the symmetry index of PVP ($R^2 = 0.62; p = 0.02$) (Figure 2C), and
a similar pattern was seen for the symmetry index of vertical impulse (VI) (Supplementary Figure S1).

**Upregulation of Serum Artemin in Osteoarthritic Human Serum**

After finding upregulation of artemin in canine serum samples, we investigated whether this relationship held true in humans. Human serum samples were obtained from patients with well-characterized OA and from age- and sex-matched controls (Reprocell, Beltsville, MD). Samples were from female patients who had been suffering from OA for a minimum of 13 years (n = 5) and age/gender-matched controls (n = 4). Samples were analyzed for artemin with an ELISA. Our results showed a trend of an increased level of artemin in people with OA-associated pain compared to pain-free healthy individuals (Figure 2D, p = 0.1).

**DISCUSSION**

OA patients experience chronic pain, however, the molecular basis for chronic pain in these patients is still largely unknown (Eltner et al., 2017). Although it has recently been revealed that neurotrophic factors play an important role in pain transduction by activating the receptors present on the DRG...
sensory neurons in mice (Orozco et al., 2001; Elitt et al., 2006; Malin et al., 2006; Kashyap et al., 2018; Nencini et al., 2018), it is currently unknown whether artemin, an endogenous neurotrophic factor, and its receptor, GFRα3, play an important role in pain hypersensitivity in OA patients. Here, we combined molecular and immunological methods to explore the association of artemin and GFRα3 with OA-associated pain in dogs with OA. Using within-dog control tissues, we found increased expression of GFRα3 in DRG serving painful OA joints. We found a positive association between peripheral released artemin in the synovial fluid and joint pain, as measured by limb use. Although the work with both DRG and synovial fluid only involved small numbers of dogs, it does suggest that the artemin/GFRα3 axis is upregulated in OA pain states. Also, we found an association between serum artemin concentrations and OA, both in dogs and in humans. The positive correlation between artemin concentrations and owner questionnaire scores (LOAD and CBPI), albeit a weak correlation, is interesting. The correlations between the questionnaires and serum artemin were low but are most significant for the CBPI pain and interference subscales.

The CBPI focuses primarily on pain and the impact of pain on the ability to perform activities. The relationship between serum artemin and such scores should not be interpreted as evidence of a serum biomarker but rather as additional evidence of a potential role for artemin in the OA pain state. It must be cautioned that our data only show a correlation or association between OA pain and artemin/GFRα3, not causation. Regardless, we believe these data form a compelling rationale for investigating the role of artemin/GFRα3 in OA pain in rodent OA models.

Initially, glial cell line-derived neurotrophic factors (GDNFs), such as artemin, were studied for their role in development and neuronal survival (Luo et al., 2007), but recently there has been a shift to examining their role in the modulation of pain. There is already evidence that artemin and GFRα3 have a role in pain in rodents. Several researchers have found increased levels of artemin associated with tissue damage or inflammation in rodent pain models, such as CFA injection (Malin et al., 2006; Ikeda-Miyayawke et al., 2015; Lippoldt et al., 2016) and the nitrogen peroxide (NTG) migraine model (Shang et al., 2016). GFRα3 expression has also been tied to painful conditions such as cold allodynia and thermal hypersensitivity (Malin et al., 2006; Lippoldt et al., 2016). Other data indicate that GFRα3 knockout mice do not acquire the hypersensitivity normally seen with CFA injection, nerve injury, or chemotherapy (Lippoldt et al., 2016). GDNF, a ligand in the same family as artemin, has been implicated in pain sensitivity in dogs in a study by Plassais et al. (2016), which found that a mutation that decreased the expression of GDNF was responsible for pain insensitivity in dogs with self-mutilation syndrome. There has recently been an investigation into the role of GDNF in inflammatory bone pain using an acute CFA-induced model in rats. The investigators found that artemin was able to sensitize bone afferent neurons to mechanical stimulation, but they found no upregulation of GFRα3 (Nencini et al., 2018).

Part of the interest in the role of GFRα3 in pain comes from its co-localization and interactions with the TRPV1 ion channel. TRPV1 and GFRα3 are highly co-localized, and knockdown of GFRα3 results in a lack of anatomy-induced increase in TRPV1 expression (Jankowski et al., 2010). Further, investigations have shown that artemin injections can induce the upregulation of TRPV1 (Ikeda-Miyayawke et al., 2015) and can sensitize TRPV1, producing heat hyperalgesia (Malin et al., 2006). Interestingly, our results suggest similar findings, with GFRα3 expression increases occurring in conjunction with TRPV1 expression in dog DRG as measured by qPCR. In the future, it will be interesting to determine the different subpopulations of neuronal types in the dog DRG by developing some canine-specific antibodies, which will provide information about various subsets of neurons that have been identified in mouse DRG.

The exact cellular mechanisms for artemin’s potential actions in OA have not been investigated. OA is a disease characterized by hypersensitivity to hot, cold, and mechanical pain (Arendt-Nielsen et al., 2018), and this hypersensitivity is driven by neuronal changes in the DRG. This may be mediated in part by GFRα3. Data suggest that artemin/GFRα3 is upstream in a pathway that regulates TRP channels (Ikeda-Miyayawke et al., 2015). The GFRα3 signaling pathway acts through the activation of the tyrosine kinase RET, possibly the RET51 isoform, and downstream effects may be due to the ERK/MEK pathway (Li et al., 2009). A role in the development and maintenance of OA pain and sensibility, possibly through the ERK/MEK pathway, would fit in with our current knowledge of GFRα3/artermin. However, this needs to be investigated in the future.

Next, our results indicate that artemin/GFRα3 may play a hitherto unrecognized role in OA-associated pain and hypersensitivity. We have shown that artemin is related to various measures of OA-associated pain, which suggests a possible mechanism for broad thermal and mechanical hypersensitivity in OA patients. By using samples from naturally occurring OA in dogs, we were able to identify this potential target in the normal disease state. There is growing interest in this general approach—so-called reverse translation or multidirectional translation—with neurobiological evidence from the target condition being used to validate the model and inform mechanistic research (Dawes et al., 2011, 2014). Companion animals are particularly useful in this regard, as they share the same environment and habits as their human owners, making them ideal for investigations into diseases that affect both species, such as osteoarthritis. Future research will determine whether such a reverse translational approach is a good way to identify relevant targets for mechanistic evaluation in rodent models.

Osteoarthritis in pet dogs is very similar to human OA [biomechanically, structurally, histologically, genomics, and molecularly (Clements et al., 2006; Proven et al., 2012; McCoy, 2015)], and recent reviews have highlighted the potential of using pet dogs to inform the translational process, particularly for efficacy screening of putative analgesics prior to human clinical trials (Klinck et al., 2017; Lascelles et al., 2018). It has been proposed that using tissue from naturally occurring disease states will better inform the direction of basic research and the development of novel targets (Klinck et al., 2017; Lascelles et al., 2018). Interestingly, during the drafting of this manuscript, Reegeneron announced that it was advancing a fully human antibody to the GFRα3 neurotrophic factor receptor into clinical
studies for OA pain in humans. Some limitations of this study include a small sample size of human serum samples and an age discrepancy between the control and OA dogs. Our study highlights a clinically relevant avenue for further research to determine the role of artemin/GFRα3 in OA pain.

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 the North Carolina State University Institutional Animal Care and Use Committee (IACUC). All original studies and sample collection were conducted under-informed and written owner consent and IACUC approval. Written informed consent was obtained from the owners for the participation of their animals in this study. Protocols used under this study include: Dog Studies – 13-010-B, 15-163-O, and 16-094.

AUTHOR CONTRIBUTIONS

BL and SM conceived the idea and designed the experiments. LM, JW, MI, and SP performed the experiments. LM, JW, BL, and SM analyzed the data. LM, BL, and SM wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2020.00077/full#supplementary-material

FIGURE S1 | Correlations of artemin and limb use during pain. Plot of suprachiasmatic nucleus (pCN) against limb use, expressed as a symmetry index (SI) of vertical impulse (VI). Negative values of SI correspond to decreased limb use, and the plot shows that increased suprachiasmatic nucleus concentrations of artemin correspond to less limb use (R² = 0.44, n = 6, p = 0.074).

REFERENCES


Conclusion

In this chapter we found that the GFRα3 /artemin signaling pathway appeared to be related to pain behaviors in naturally occurring OA in dogs. This suggests that GFRα3 /artemin signaling has a role in OA-associated pain, but further work needs to be done to establish a causal relationship between GFRα3 /artemin and OA-associated pain. Importantly, this discovery was made using tissue from naturally occurring painful disease, and so we believe the results to be highly clinically relevant. This strongly justifies further work to test a causal relationship by using animal models. We therefore moved onto using a mouse model of osteoarthritis to explore the relationship in more detail.

References

CHAPTER 3
Defining the pain phenotype of MIA-induced osteoarthritis

Introduction

The overall goal of this work is to investigate the underlying neurobiology of, and to evaluate potential therapeutics for, OA-associated pain, with particular reference to the artemin/GFRα3 signaling pathway. To achieve this a mouse model needs to be selected that has minimal variance in induction and pain phenotype. This will allow for limiting animal use by minimizing the variation typically seen with behavioral data.

The MIA model of OA is commonly used in both mice and rats. More information on the pain phenotype of the MIA model exists in the rat compared to the mouse because the rat model has been used in investigations of pain and OA since 1985 (Kalbhen, 1985) while mice have only gained popularity in OA-research more recently because of the new commercial availability of transgenic and knock-out mouse strains commercially as well as the ability to generate genetically modified strains with relative ease. In the rat MIA has been shown to cause central sensitization (Abaei, 2016), rapid OA-like lesions in the joint (Guzman, 2003), decreased weight bearing (Vermeirsch, 2007; Thaker, 2012) and hypersensitivity to mechanical (Liu, 2011; Vermeirsch, 2007; Thaker, 2012; Kumari, 2015), thermal (hot) (Liu, 2011; Vermeirsch, 2007; Kumari, 2015; Thaker, 2012) stimuli.

Mice injected intra-articularly with MIA have been assessed for mechanical hypersensitivity (Harvey, 2009; Ogbonna, 2013; Ogbonna, 2015; Pitcher, 2016; Horvath 2016) using various Von Frey protocols including the up-down method (Harvey, 2009; Ogbonna, 2013; Ogbonna, 2015; Pitcher, 2016) and the dynamic aesthesiometer (Horvath, 2016). The MIA mouse model has robust mechanical hypersensitivity within a few days after induction (Harvey,
Hypersensitivity to heat was observed in one mouse study where they did not find any sensitization due to MIA (Harvey, 2009). Cold hypersensitivity has not been investigated in the mouse model of MIA.

Data on measuring limb use in the mouse MIA model is limited and there has been no consistency in the parameters reported. Miyagi et al (2017) used the Catwalk as an outcome measure to determine the efficacy of an anti-NGF growth factor as an analgesic for OA-associated pain in the MIA model. They found duty cycle, swing speed, and print area to be significant parameters in that they responded to the treatment with NGF (Miyagi, 2017). In contrast, a study using rats with MIA found significantly reduced intensity in the ipsilateral pawprints, analogous to peak vertical force, in hindlimbs where MIA had been injected into the stifle joint (Ferreira-Gomes, 2008). It has been previously noted that the parameters reported for dynamic weight-bearing analysis, particularly using the Catwalk system are not consistent (Lakes & Allen, 2016). Further work is needed to determine if changes in limb use in relation to intra-articular MIA can be reliably and consistency measured in mice.

In contrast to the dynamic weight-bearing data, the MIA model of OA has been shown to have reduced static weight bearing in the affected limb out to 28 days post-injection by multiple investigators (Ogbonna, 2015; Pitcher, 2016). Both of these studies used incapacitance meters that forced the mice into a standing position with their entire body weight being supported by their hindlimbs (Ogbonna, 2015; Pitcher, 2016). Static weight bearing needs to be used in conjunction with dynamic weight-bearing to confirm a loss of limb-use using a robust outcome measure.

After establishing the behavioral pain phenotype in wild-type mice, it should also be established in TRP knock-out mice as a foundation for future work investigating the role of TRP
channels in GFRα3/arternin signaling. Limited work has been done with TRP knock-out mice using the MIA model. TRP channels are known to have a role in pain and hypersensitivity, but their importance in OA is still to be determined. TRPA1 knock-out mice have been used with the MIA model of OA. These TRPA1 KO mice still had hypersensitivity to mechanical stimuli after MIA induction, though they experienced some protection compared to wild-type mice (Horvath, 2016). The MIA model of OA has not been induced in any other TRP channel knock-out mice. The phenotype of MIA-induced OA pain in TRPV1 knock-out mice is still unknown.

Behavioral data can vary based on study conditions, the type of mice used, and the specific paradigm of the assay. As such, this first section of mouse work is focused on establishing methods to assess evoked and non-evoked pain behavior in the MIA-induced OA model.

**Methods**

*Mice:* Male, 4-week-old C57BL6 mice (Jackson Labs) were used in these experiments. Mice were housed in groups of 4 and kept on a 12-hr light-dark cycle. All knock-out mice were on a C57/Bl6 background. TRPV1 knock-out (Jackson Labs; Stock No: 003770) and TRPA1 knock-out (Jackson Labs; Stock No: 006401) mice were obtained from Jackson labs and cross-bread to obtain TRPV1-A1 knock-out mice. KO mice were used for the experiments when they were 4 weeks old.

*Preparation and injection of MIA:* MIA induction was preformed according to the method published by Pitcher et al (2016). MIA (Sigma-Aldrich, I2512) was dissolved in 10 µl of sterile saline and injected intra-articularly using a zero-dead space needle (Hamilton). Control mice were injected with 10 µl of sterile saline. Mice were anesthetized during the injection using a nose-cone delivering 5% isoflurane carried in oxygen. The injection area (right knee) was
cleaned with surgical scrub (70% Ethanol) and iodine solution 3 times prior to the injection. To reduce inflammation and abrasions, we did not shave the injection area, deviating from the published protocol (Pitcher). Instead, the surgical scrub was used to flatten and part the fur by the injection site. The joint space was identified by flexing the leg and creating an indent with the needle (Pitcher et al). Mice were under anesthesia for no longer than 5 minutes.

**Behavior:** All behavioral assays were performed blinded by the same researcher to maintain consistency. Behavioral assays were conducted at the same time of day (afternoon) for each timepoint.

**Von Frey:** Mechanical sensitivity was measured using the Ugo Basile Dynamic Plantar Aesthesiometer, which shall be referred to as the electronic Von Frey. Mice were placed in testing chambers on top of a mesh floor, allowing for the probe to contact the mouse’s hindpaws. Mice were acclimated to the testing environment for 5 minutes before testing began. Five measurements were taken on each hindpaw using the ramping force setting. There were at least 5 minutes between each measurement.

**Hargreaves:** Thermal (hot) sensitivity was tested using Plantar Test Instrument (Ugo Basile), which shall be referred to as the Hargreaves apparatus. Mice were placed in testing chambers on a glass plate and given 5 minutes to acclimate. The thermal stimulus was positioned under the hindpaw at a light intensity that produces a hot thermal temperature of 42 degrees Celsius and withdrawal time was automatically recorded. Five measurements were taken on each hindpaw with at least 5 minutes between each measurement.

**Cold Assay:** Thermal (cold) sensitivity was tested using the Dry Ice Method described by (Brenner), which shall be referred to as the cold test. Dry ice was crushed into a cylinder shape, which was then applied to the mouse’s hindpaw under the glass plate and withdrawal time was
manually recorded with a stopwatch. Five measurements were taken on each hindpaw with at least 5 minutes between each measurement.

**SHIM Incapacitance Meter:** Static weight bearing was measured using a SHIM incapacitance meter (IC Meter) connected to a system 8000 micro-measurements tool (Williams, 2019). Mice were trained to walk into the instrument and stand on the two force plates – one for each hindpaw, which measured the weight on them in grams. Five measurements were taken on each mouse. Measurements were recorded at a rate of 10 per second for a total of 5 seconds. Measurements were only included if the mouse was still on the plates for the entire 5 seconds. Mice were trained to enter and stand still on the IC meter on two separate occasions prior to the beginning of data collection. Training for mice allowed them to explore the IC meter and then after they were acclimated, encouraged them to enter the IC meter. This was done by the experimenter picking up the mice every time they left the IC meter and placing the mice at the entrance again. The mice appeared to learn that inside the IC meter they would not be touched. Exclusion criteria for measurements was based on behaviors that deviated from the mouse standing still on the force plates. Measurements were excluded when mice moved off the plates before 5 seconds, mice pushed against the top of the meter, mice urinated or defecated on the plates, and mice braced their foot against the chamber wall rather than on the plate. Strainsmart software was used to collect data, which was then exported to excel for analysis.

Raw data of the average grams on each hindlimb were converted to percent weight on each hindlimb using these equations:

\[
\text{Percent weight on Ipsilateral Limb} = \frac{I_g}{T_g} \times 100
\]
\[ \text{Percent weight on Contralateral Limb} = \frac{C_g}{\frac{T_g}{I_g} + \frac{C_g}{T_g}} \times 100 \]

Where \( I_g \) is the grams on the ipsilateral hindlimb, \( C_g \) is the grams on the contralateral hindlimb, and \( T_g \) is the total mass of the mouse.

**Catwalk:** Gait-analysis was performed using the Catwalk XT system (Noldus) and the provided software. The Catwalk system visualizes footprints as the mouse freely transverses a glass walkway. Mice were familiarized with the equipment by allowing them to roam freely on the walkway for 10 minutes one day prior data collection. Mice were trained to walk back and forth across the walkway to their home cage. When data was being collected the mice where acclimated to the dark room and walkway for a minimum of five minutes. Five compliant runs were collected for each mouse at each timepoint. Runs were compliant if the mouse maintained less than 80% variation in velocity and transversed the walkway in less than ten seconds. This eliminated behavior like rearing and stopping to sniff from the dataset. Mice moved freely on the walkway until five compliant runs were collected. Runs were automatically classified to label footfalls using the Catwalk software and then manually verified for accuracy. The Catwalk system generated data for all parameters. Symmetry Indexes were generated for two parameters of interest: max contact max intensity and max contact area.

\[ \text{SI} = 100 \times \frac{X(\text{control}) - X(\text{injected})}{0.5 + (X(\text{injected}) + X(\text{control}))} \]

**Statistical Analysis:**

Data were analyzed using GraphPad Prism. Two-way ANOVAs and multiple t-tests with corrections for multiple comparisons were with significance set at an alpha of 0.05 for all experiments. ANOVA with repeated measures was used to compare MIA and control over time.
That comparison was made for day 7 through day 28 to quantitate the effects after the model was induced and stable. For multiple t-tests, statistical significance determined using the Holm-Sidak method, with alpha = 0.05. Data was tested for normalcy with the Shapiro-Wilk test. All data was normal. Graphs were produced using GraphPad Prism.

Results

MIA dose-response

We performed an experiment with 3 different concentrations of MIA (0.1, 0.5, or 1 mg of MIA dissolved in 10 ul of sterile saline) to determine the appropriate dose of MIA for subsequent experiments. A small sample size was used primarily because the main goal was to determine the highest dose of MIA that would produce robust hypersensitivity and visible lameness.

Sensitivity to mechanical, thermal, and cold stimuli were evaluated in mice that received injections of saline (control), 0.1 mg, 0.5 mg, or 1 mg of MIA. None of the doses chosen proved to be lethal as all mice survived to day 28. All mice that received MIA showed hypersensitivity compared to control mice. Mice that received 1 mg of MIA showed the most hypersensitivity to mechanical, hot, and cold stimuli at day 28. Due to the small sample size of two mice per group chosen for this preliminary work, statistical power was limited. There was significant difference between control mice and 1 mg MIA at day 28 for all three behavioral assays (two-tailed t-test assuming unequal variances; Von-Frey p<0.05; Hargreaves p<0.05; cold p<0.01). These data indicated that a dose of 1 mg of MIA would achieve the most hypersensitivity in mice. All subsequent injections were using the 1 mg of MIA in 10 ul volume.
Figure 3.1 Hypersensitivity in response to multiple doses of MIA. Control (n=2), 0.1 mg MIA (n=2), 0.5 mg MIA (n=2), and 1 mg MIA (n=2) mice were tested for hypersensitivity to mechanical (A), heat (B), and cold (C) stimuli using the Von Frey, Hargreaves, and cold assays through 28 days. Data are represented as mean ± SD. There was significant difference between control mice and 1 mg MIA at day 28 for all three behavioral assays (two-tailed t-test assuming unequal variances; Von Frey p<0.05; Hargreaves p<0.05; Cold p<0.01). ANOVA with repeated measures for day 7 through day 28 found that all MIA doses were significantly different from control mice after MIA induction for all three assays (p<0.05). D) Depiction of the intra-articular injection of MIA.
Thermal and mechanical sensitivity changes in the MIA-induced OA model in mice

Mice injected with either MIA (1mg in 10 µL) or saline (10 µL) and were tested for mechanical, heat, and cold hypersensitivity to further profile the sensitivity of MIA mice at this dose. Sensitivity of the contralateral limb did not differ from the contralateral or ipsilateral limbs of the saline injected mice (data not shown). The ipsilateral (injected) limb of MIA mice was hypersensitive to mechanical, hot, and cold stimuli for the 4 weeks tested compared to the ipsilateral limb of the saline-injected controls.

Figure 3.2 Hypersensitivity to mechanical, thermal, and cold stimuli after MIA induction. MIA (n=5) and control (saline injected; n=6) mice were tested for hypersensitivity to mechanical (A), heat (B), and cold (C) stimuli using the Von Frey, Hargreaves, and cold assays through 28 days. Data are represented as mean ± SD. Repeated measures ANOVA detected a
significant difference between MIA and control mice across all sensitivity testing (p<0.05). D) Depiction of the Von Frey, Hargreaves, and Cold test assays demonstrating how all three assays target the plantar hindpaw and measure paw withdrawal.

Dynamic measures of limb-use

The Catwalk system and associated software have been used previously in mouse models of OA, but with mixed results, as explained in Chapter 1 (Malfait 2010, Muramatsu 2014, Fang 2018, Miyagi 2017). Thus, all parameters were collected and analyzed with particular attention given to parameters previously reported in mouse OA experiments as well as parameters analogous to ground reaction forces, particularly peak vertical force, which is considered an indicator of OA-associated pain in dogs (Budsberg 2001). Even with analyzing all parameters, none were significantly different between the ipsilateral (injected) and contralateral limb in MIA mice at day 28.

Two particular parameters of interest were Max Contact Max Intensity (MCMI) and Max Contact Area (MCA). MCMI was the parameter closest to peak vertical force and had the greatest difference of any parameter, but it was not significant. MCA was given close attention due to the observation that MIA mice would curl their paws in an attempt to protect that limb while walking. In the acute phase of MIA around day 7 post-injection MCMI was significantly different from baseline and MCA showed no difference.

However, in a longer study the difference MCMI and MCA were found to disappear after the day 7 timepoint and did not reappear out to day 28. While these parameters could be potentially useful in the acute phase, their lack of stability needs to be taken into account.
Figure 3.3 Evaluation of dynamic weight-bearing in MIA using the Catwalk system. Max Contact Max Intensity (A, C) and Max Contact Area (B, D) are illustrated. A higher symmetry index indicates less use of the MIA limb. At day 7 (n=5) there was a significant difference in MCMI (two-tailed t-test; p<0.05), but not in MCA. However, subsequent data (n=9) showed the asymmetry had disappeared by day 8.

Static bodyweight distribution

Static weight bearing as measured with a SHIM incapacitance meter was assessed as the percent of hindlimb supported body weight placed on each hindlimb (Williams, 2019). Mice placed significantly less weight on the injected limb starting at Day 2 and continuing to Day 28
(two-tailed paired t-test; p<0.5). Bearing less body weight on the MIA hindlimb is an indicator that the MIA model of OA is causing reduced limb-use and an indicator of OA-associated pain.

**Figure 3.4 Static weight-bearing after MIA induction.** A) MIA (n=8) mice were tested for static limb-use (weight bearing) for 4 weeks after MIA induction, comparing the ipsilateral and contralateral limbs. Data are presented as the percent of total hindlimb supported body weight put on each hindlimb. The MIA-injected hindlimb had significantly less weight put on it than the control contralateral limb from day 2 onward (two-tailed t-test; p<0.05) Data are represented as mean ± SD. ANOVA with repeated measures comparing MIA to control between day 7 through day 28 found that they were statistically significant (p<0.01). B) Static weight-bearing data represented as a symmetry index. More asymmetry in weight bearing was observed after MIA-induction compared to baseline, following the same pattern of being significantly different from baseline starting at day 2 after MIA-induction (two-tailed t-test; p<0.05).

**The MIA-induced OA pain phenotype in TRP channel knock-out mice**

Since the induction of MIA in wild-type mice caused hypersensitivity to heat, cold, and mechanical stimuli, this was further investigated in these knock-out lines to determine if MIA-induced hypersensitivity was through either the TRPV1 or TRPA1 channels.
For sensitivity to heat, TRPV1 knock-out mice had an increased withdrawal latency compared to wild-type mice at baseline; i.e. the TRPV1 knock-out mice were less sensitive to heat. After MIA was induced, TRPV1 knock-out mice had significantly shorter withdrawal latencies (more sensitive to heat), though not as short as wild-type MIA mice (Figure 5B).

TRPA1 KO mice had a significantly longer withdrawal latency to cold stimuli at baseline compared to wild-type mice, but no difference from wild-type on day 28 after MIA induction (Figure 5C). Dual TRPV1-A1 knock-out mice showed the same, expected longer withdrawal latencies for heat (Figure 5B) and cold (Figure 5C) at baseline. At day 28 TRPV1-A1 mice did not have a significant difference from wild-type mice at day 28 for either heat (Figure 5B) or cold (Figure 5C). This is probably due to the inherit variability of behavioral data and a small sample size. Neither TRPV1 or TRPA1 knock-out mice showed any difference from WT mice in mechanical sensitivity.
Figure 3.5 MIA induced hypersensitivity in knock-out mice. All data for wild-type MIA (n=5), wild-type control (n=6), TRPV1 KO mice with MIA (n=4), TRPA1 KO mice with MIA (n=5), and TRPV1-A1 KO mice with MIA (n=7) are represented as mean ± SD. A) Mechanical sensitivity in WT controls, WT MIA and KO MIA mice. Both WT and KO mice given MIA showed a significant reduction in withdrawal force (g) at day 28 (two-tailed paired t-test; WT p<0.01; TRPV1 KO MIA p<0.01; TRPA1 KO MIA p<0.01; TRPV1-A1 KO MIA p<0.01).

Control WT mice demonstrated no change in sensitivity at day 28 (two-tailed paired t-test; p>0.05). KO MIA mice were similar to WT mice following MIA injections at 28 days post-MIA injection. TRPV1 KO mice with MIA were actually significantly more sensitive at 28 days than WT mice (two-tailed t-test; p>0.05 at baseline and p<0.01 at Day 28). There was no difference at day 28 between WT MIA and TRPA1 KO MIA and TRPV1-A1 KO MIA mice (two-tailed t-test; TRPA1 KO MIA p>0.05; TRPV1-A1 KO MIA p>0.05). B) Hot thermal sensitivity in WT controls, WT MIA and KO MIA mice. TRPV1 and TRPPV1-A1 KO mice had significantly longer withdrawal latencies (less sensitive to heat) at baseline than WT mice (two-tailed t-test; TRPV1 KO p<0.01; TRPV1-A1 KO p<0.05). TRPA1 KO mice were not different from WT mice at baseline (two-tailed t-test; p>0.05). At day 28 TRPV1 KO MIA mice had less hypersensitivity to heat than WT MIA mice (two-tailed t-test; p<0.01), unlike TRPA1 and TRPV1-A1 KO MIA mice (two-tailed t-test; TRPA1 KO MIA p>0.05; TRPV1-A1 KO MIA p>0.05).

C) Cold sensitivity in WT controls, WT MIA and KO MIA mice. TRPA1 and TRPV1-A1 KO mice had a higher tolerance for cold at baseline (two-tailed t-test; TRPA1 KO p<0.01; TRPV1-A1 KO p<0.01). TRPV1 KO mice were no different from WT mice (two-tailed t-test; p>0.05). There was no difference from any of the knock-out MIA mice at day 28 compared to controls (two-tailed t-test; TRPV1 KO p>0.05; TRPA1 KO p>0.05; TRPV1-A1 KO p>0.05).
A  Mechanical Sensitivity in Knock-out MIA Mice

B  Thermal Sensitivity in Knock-out MIA Mice

C  Cold Sensitivity in Knock-out MIA Mice
Discussion

The MIA-induced OA model has been widely accepted as a model of OA, often used to investigate potential therapeutics or the pathology of the disease. More recently disease modifying therapeutics are being investigated in other OA models, such as the DMM model, with more representative joint pathology. The MIA-model remains a robust model for testing potential analgesics (Pitcher, 2016). However, until now, knowledge of the pain phenotype of MIA-induced OA mouse model was limited. Here, for the first time we phenotyped the MIA-induced OA mouse model for mechanical, heat, and cold hypersensitivity as well as limb-use and weight bearing using consistent methods with a single researcher conducting every behavioral assay. We are only the second group to evaluate the MIA-model in mice with an electric Von Frey (Horvath, 2016), though mechanical hypersensitivity has been shown repeatedly in the MIA model (Harvey, 2009; Ogbonna, 2013; Ogbonna, 2015; Pitcher, 2016; Horvath, 2016). This is the first time hypersensitivity to heat and cold has been demonstrated in mice with MIA-induced OA (Malfait, 2013). For heat, our results were contrary to the only other study investigated heat sensitivity (Harvey, 2009) and for cold, this is the first time cold sensitivity has been tested in mice with MIA-induced OA.

Interestingly, we showed that in mice with MIA-induced OA there is increased mechanical, heat, and cold hypersensitivity compared to control. Changes in heat, cold and mechanical sensitivity occur in association with OA pain in human subjects (Fingleton 2015) and dogs with naturally occurring OA (Knazovicky, 2016) showing that the MIA model has some fidelity to naturally occurring OA-associated pain. Hypersensitivity to all three modalities supports the use of the MIA model for studying OA-associated pain, because approximately 70% of human patients with knee OA experience hypersensitivity in at least one modality –
mechanical, hot, or cold (Wylde 2012). It is believed that the sensitivity changes are a result of OA-associated pain, and that measuring changes in sensitivity relates to pain (Aranda-Villalobos, 2013). Reflexive assays that measure sensitivity are commonly used when screening analgesics as reflexive tests are more high-throughput and sensitive than more complex behavioral assays (Gregory, 2013).

Measurements of bodyweight distribution as measured on the SHIM IC meter indicated that static weight bearing was reduced in the ipsilateral limb of MIA mice, considered to be a measure of joint pain. However, the measurement of kinetic limb use indices using the Catwalk system did not detect any differences between control mice and mice which had had MIA injected into the right stifle. this finding was unexpected as Miyagi et al (2017) used the Catwalk as an outcome measure when evaluating the efficacy of an anti-NGF growth factor in the MIA model and found duty cycle, swing speed, and print area to be significantly negatively altered by MIA, and significantly improved with anti-NGF mAb (Miyagi 2017). The ability of the Catwalk to quantitate gait in mice is not certain. Malfait et al’s (2010) study using DMM mice also detected no differences between baseline and post-surgery tests in any gait parameters using the Catwalk system. Other studies have reported a wide variety of parameters as significantly altered in the pain model, but there appears to be little consistency across the studies (Muramatsu, 2014; Fang, 2018; Miyagi, 2017). In the three studies using the catwalk in mice with OA, one study reported a difference in intensity (Fang 2018), two in temporal gait patterns (Muramatsu 2014, Miyagi 2017), and one in print area (Miyagi, 2017). Fang et al. (2018) and Muramatsu et al. (2014) both used the DMM model and found different parameters to be significant with no overlap. Further work needs to be done to standardize the parameters reported for the Catwalk and to validate it as a measure of limb-use in mice with induced-OA.
The roles of both TRPV1 and TRPA1 in pain and sensitivity associated with MIA-induced OA have been previously investigated. One study that attempted to block each channel with an antagonist and found that neither antagonist improved weight-bearing or ongoing pain (Okun, 2012). Another study compared induced MIA OA in TRPA1 knock-out mice and wildtype mice. TRPA1 knock-out mice had less mechanical hypersensitivity and a lesser reduction in weight bearing compared to wild-type mice (Horvath, 2016). Here, to determine whether TRPV1 or TRPA1 had a role in MIA-induced hypersensitivity knock-out mice for these channels had MIA-OA induced in the knee joint and the mice tested for sensitivity for 28 days. The loss of function of TRPV1 was partially protective for thermal hypersensitivity induced by MIA. On the other hand, the loss of TRPA1 was not protective for either cold or mechanical hypersensitivity from MIA, though it is thought to partially signal for those modalities (Story, 2003; Bandell, 2004; Petrus, 2007). This could be due to the nature of the cold assay where the response time was so short that differences in hypersensitivity would be difficult to detect. It could also be that TRPM8 is more important for MIA-induced cold hypersensitivity. TRPM8 knock-out mice should be tested in the future to determine if TRPM8 is a mediator of cold hypersensitivity in the MIA model of OA. The lack of protection for TRPA1 knock-out mice with MIA for mechanical sensitivity was contrary to Horvath et al.’s (2016) data, which showed some protection in the initial phases of MIA-induced mechanical hypersensitivity. The differences between wild-type mice and TRPA1 KO mice with MIA disappeared by day 10 in that study (Horvath, 2016) and because our study went out to 28 days, no significant differences were seen. We cannot rule out that TRPA1 contributes to hypersensitivity in OA based on these data, just that there are other channels involved as well. These data indicate that some TRP
channels play a partial role in MIA-induced hypersensitivity and the mechanisms involved here are more complex than a single channel being responsible for a hypersensitivity modality.

There are many TRP channels and though their involvement has been shown in chronic pain states, therapeutics targeting specific TRP channels have failed to pass clinical trials (Mickle, 2016). This is likely because TRP channels do not act in isolation, especially in a chronic pain state. TRPV1 is the most well studied TRP channel for signaling noxious heat, but TRPV3 (Smith, 2002; Moqrich, 2005) and TRPV4 (Todaka, 2004) also signal at high temperatures. Noxious cold sensation is transmitted through both TRPA1 (Story, 2003; Bandell, 2004) and TRPM8 (Bautista, 2007) and mechanical hypersensitivity involves a whole slew of receptors beyond TRPA1 (Petrus, 2007), including Piezo1 and Piezo2 (Coste, 2010) and potassium channels such as TRAAK, TREK1, and TREK2 (Brohawn, 2015). A potential analgesic would need to target multiple channels, either directly or by regulating them on a higher level as seen with neurotrophins such as NGF (Ji, 2002; Eskander, 2015; Zhang, 2005; Diogenes, 2007; Kayama, 2017) and artemin (Elitt, 2006; Deberry, 2015; Ikeda-Miyagawa, 2015).

It should be noted that there are some clear limitations of this study. Only male mice were used as pain sensitivity can be impacted by sex (Wiesenfeld-Hallin, 2005). The best practice for inclusion of female mice in a chronic pain study is to monitor their estrous cycle, which was not undertaken for this initial work in establishing the model and pain phenotype. This work should be repeated using female mice. A further limitation lies in the blinding – the mice with MIA were so obviously impacted by the injection that there was no way to truly disguise which mice were in each group.
Here, we have established a phenotype for pain behavior in MIA model of OA in mice. These mice had mechanical, heat, and cold hypersensitivity as well as reduced weight bearing in the ipsilateral hindlimb. These outcome measures can now be used with this model to evaluate potential analgesics for their ability to alleviate hypersensitivity for these modalities in OA-associated pain.
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CHAPTER 4

Characterization of GFRα3/Artemin in the MIA model of OA in mice

Introduction

While it is likely that GFRα3 and artemin have an important role in pain signaling the precise mechanisms and sites of action are unclear. Here we seek to determine the mechanisms of GFRα3/Artemin signaling in the MIA model of OA by quantitating peripheral expression of GFRα3 in the DRG and artemin in the joint and serum. In pain GFRα3’s position on the sensory neuron afferents is clearly it’s site of action (Elitt, 2006). However, when it comes to artemin, the site of action and the site of production is less well defined and has yet to be investigated in a model of OA.

After neurodevelopment GFRα3 is expressed almost exclusively in DRG neurons with no expression in either the brain or spinal cord (Yang, 2006). GFRα3 is primarily expressed in peripheral sensory neurons (Orozco, 2001). There is some GFRα3 expression in non-neuronal tissues including pancreas (Nivlet, 2016), kidney (Yang, 2006), and epithelial cells of the reproductive and digestive systems (Yang, 2006). The function of GFRα3 in these different cell types is not entirely known and it may not be functionally important (Nivlet, 2016). At maturity GFRα3 expression in the DRG has been reported to be between 20-24% of DRG neurons in rats and mice (Naveilhan, 1998; Orozco, 2001; Lippoldt, 2013) and predominantly in nociceptive neurons (Orozco, 2001). GFRα3 expression in the DRG has yet to be evaluated in a chronic pain state, even though it is known to co-express and interact with nociceptive channels (Malin, 2006; Elitt, 2006; Ikeda-Miyagawa, 2015).

GFRα3 is co-expressed with both TRPV1 and TRPA1 in the DRG – 68.5 ± 5.4% of TRPV1 positive neurons and 84.1 ± 2.6% of TRPA1 expressing neuron also expressed GFRα3.
mRNA (Ikeda-Miyagawa, 2015). Artemin/GFRα3 signaling have been shown to contribute to TRPV1 and TRPA1 regulation (Malin, 2006; Elitt, 2006; Ikeda-Miyagawa, 2015). Artemin causes increases in the mRNA for GFRα3, TrkA, TRPV1, and TRPA1 (Elitt, 2006) and, in reverse, an anti-artemin antibody was shown to block upregulation of TRPA1 (DeBerry, 2015) and knockdown of GFRα3 with siRNA blocked upregulation of TRPV1 in a nerve injury model (Jankowski, 2010). Regulation of TRP channels would be a mechanism that would explain GFRα3/artemin signaling contributing to hypersensitivity in multiple modalities, such as the hypersensitivity seen in the MIA model of OA, which has yet to be investigated for GFRα3 and artemin expression.

Artemin has been shown to be produced in skin cells, including keratinocytes (Elitt, 2006; Hidaka, 2016) and fibroblasts (Murota, 2012), glia, including Schwann cells, (Balogh, 1998), cancer cell lines, including pancreatic (Gao, 2015; Ceyhan, 2006) and breast cancer (Wu, 2013), and arteries (Damon, 2007; Honma, 2002). As a neurotrophic factor artemin has primarily been shown to increase neural afferent density, particularly in pain (Elitt, 2006) and cancer (Ceyhan, 2010; Gao, 2014). Artemin has also been shown to have a role in angiogenesis in cancer (Banerjee, 2012). Artemin has been shown to increase GFRα3 neural afferent density (Elitt, 2006), which could be a mechanism for chronic joint pain. First, artemin expression in the MIA model of OA, particularly locally in the joint, must be determined.

In previous work we showed that both GFRα3 and artemin were upregulated in naturally occurring OA (Minnema, 2020). GFRα3 was upregulated in the DRG as measured with qPCR and artemin was present at a higher concentration in the serum of OA dogs. Here, mice with MIA-induced OA where analyzed for *de novo* expression of GFRα3. Analysis of the expression
of GFRα3 in the MIA model will also determine if this model holds fidelity to the increased expression of GFRα3 in found in naturally occurring OA in dogs.

Further, we seek to learn more about the role of artemin in OA by determining the presence of artemin at two sites – systemic artemin in the serum and local artemin in the joint capsule. Systemic artemin might not be entirely produced by the vasculature and the transport of artemin from a local site to a peripheral site is unknown. The joint capsule contains neural afferents, which would be a potential location for artemin/GFRα3 signaling to occur. The joint capsule is made up of dense, fibrous tissue with an inner layer of synovium, which contains synoviocytes that produce synovial fluid (Ralphs, 1994). In previous work (Chapter 2) artemin was found in the synovial fluid and the concentration of artemin was found to correlate to limb-use (Minnema, 2020). This makes the synoviocytes and, by extension the joint capsule, a likely source of artemin within the joint.

Methods

Mice: Male, 4-week-old C57BL6 mice (Jackson Labs) were used in these experiments. Mice were housed in groups of 4 and kept on a 12-hr light-dark cycle. Mice were injected with MIA or saline as per the method described in Chapter 3.

Tissue Collection: Mice were euthanized with either CO₂ asphyxiation or an overdose of avertin delivered via IP injection.

DRG: Lumbar DRG (L1-L6) were collected from both mice injected with MIA and from mice injected with saline into the intra-articular space. DRG were collected from both the ipsilateral and contralateral sides. They were removed using a ventral dissection approach in which the spine column was removed, the ventral bone of the column was cut up and lifted, and the DRG were removed from the gaps between each vertebra. Vertebra were counted from the
end of the ribs to ensure that lumbar DRG were being removed. DRGs were trimmed of any excess fiber and fresh frozen in Tissue-Tek O.C.T. Samples were stored at -80 degrees Celsius prior to sectioning.

**Serum**: Blood was collected from mice via a cardiac puncture. After collection the blood was kept at room temperature for 1 hour to clot. After clotting the blood was spun down at 100 g for 10 minutes to separate out serum. Serum was transferred over to a clean vial and stored at -80 degrees Celsius until used for ELISA analysis.

**Joint capsule**: Joint capsule was collected from both the ipsilateral (MIA) and contralateral (control) joints of mice injected with MIA as well as from naïve mice. The leg was severed from the mouse and the skin and muscle was removed to increase visibility of the joint capsule. The joint was partially transected at the patellar ligament and the joint capsule removed from the center down to where it connected with bone. Removed joint capsule was placed in vials with RNAlater (Invitrogen; cat: AM7021) and stored at -80 degrees Celsius until mRNA extraction was performed.

**Whole Joints**: The hindlimbs of MIA mice were collected with the skin removed and taped into a straight position. They were then refrigerated for 24 hours in 4% PFA. The hindlimbs were washed and transferred over to a 10% EDTA solution (PH 7.4) to de-ossifying the bone for two weeks. Fresh EDTA solution was used for each week. After 2 weeks the joint was cut in half on the frontal plane to expose the inside of the joint. The two halves of the joint were put in fresh EDTA solution for an additional 24-48 hours, until the entire bone was soft and did not offer resistance when prodded. The hindlimbs were trimmed around the joint and frozen in tissue tech for sectioning.
Immunohistochemistry (IHC) of DRG: DRG were sectioned on a cryostat at 12 um thickness and collected on slides for IHC. Slides were immersed in 4% PFA for 10 minutes and then washed with PBS before blocking for 1 hour with 5% BSA blocking solution. Primary antibodies were diluted (1:500 each) in 5% blocking solution and added to the slides. The two primary antibodies used were TUJ (Abcam; Cat: ab7751), a neuronal marker, and GFRα3 (Neuromics, Cat: GT15123,). The GFRα3 antibody was validated for specificity using heart as a negative control (supplementary data) as heart tissue has been shown to be negative for GFRα3 expression in rats and humans (Yang 2006). Slides were kept at 4 degrees overnight with the primary antibodies. Slides were washed with PBS the next morning and Alexa Fluor conjugated secondary antibodies (Invitrogen; 488, Cat: A20181; 546, Cat: A20183) were applied in 2% blocking solution for 1 hour each, washing slides with PBS in between secondaries. Slides were washed, dried, and finalized with mounting media that contained DAPI. DRG were imaged using a Leica microscope with filters for GFP (488) and Cy3 (546) channels. DRG images were analyzed with the countered blinded to metadata. DRG were analyzed for total number of neurons, which were stained with TUJ, and for total number of neurons expressing GFRα3, which were stained with TUJ and GFRα3 antibodies. Both ipsilateral (MIA) and contralateral DRG were analyzed and compared.

ELISA: Mouse serum artemin concentrations were tested using the R&D Systems DuoSet ELISA kit (Cat No. DY1085-05). Samples were run in duplicate. Spectrophotometer readings were at 450 nm and 570 nm. The 570 nm readings were subtracted from the 450 nm for the most accurate results. Sample concentrations were determined by comparison to the standard curve.

qPCR: Joint capsules were analyzed for artemin and GFRα3 expression using qPCR. mRNA was extracted using the Qiagen RNeasy fibrous tissue kit (Cat: 74704). The optional
steps involving the addition of Proteinase K were used since joint capsule is a highly fibrous tissue. mRNA concentrations were measured on a nanodrop (thermo scientific) and the 260/280 and 260/230 ratios were recorded. cDNA was generated using 200 ng of mRNA. Quantitative real-time PCR was accomplished with commercially available Thermofisher TaqMan primer sets for GAPDH (Cat: 4331182) and artemin (Cat: 4331182). Equal amounts of cDNA were used in triplicates and amplification efficiencies were validated and normalized against GAPDH, fold increases were calculated using the comparative threshold cycle method. Individual CT values were calculated using StepOne Software v2.2.2. Relative expression (ΔCT) of each gene was calculated with the following equation: \[ \Delta C_T = C_{T,GAPDH} - C_{T,GOI} \], where GOI is the gene of interest. ΔCT values were linearized using \[ 2^{\Delta CT} \] and then multiplied by 1000 so that the y-axis is on a more intuitive scale. Standard deviation was calculated using the linearized ΔCT values. All values shown are Average ± SD. Paired t-tests were used when comparing contralateral versus ipsilateral GFRα3 and artemin expression levels.

**Immunohistochemistry (IHC) of Joint Capsules:** Joints were sectioned on a cryostat at 16 um thickness and collected on slides for IHC. Slides were immersed in 4% PFA for 10 minutes and then washed with PBS before blocking for 1 hour with 5% BSA blocking solution. The primary antibody, anti-artemin (R&D, Cat: MAB1085) was diluted (1:500) in 5% blocking solution and added to the slides. This antibody still requires validation experiments as it has not been reported in the literature for IHC yet. Most of the few anti-artemin antibodies that have been used are no longer commercially available. Slides were kept at 4 degrees overnight with the primary antibody. Slides were washed with PBS the next morning and an Alexa Fluor conjugated secondary antibody for 546 (Invitrogen, Cat: A20183) was applied in 2% blocking solution for 1 hour. Slides were washed, dried, and finalized with mounting media that contained
DAPI. Joints were imaged using a Leica microscope with filters for DAPI and Cy3 (546) channels. Images were analyzed with the experimenter blinded to metadata. Both ipsilateral and contralateral joints were analyzed for n=3 mice with MIA-induced OA. Joints were analyzed for intensity using ImageJ using the Analyze and measure tools. Three rectangular boxes were drawn around the synovium and mean, max, and background intensity were recorded. Synovium was identified by finding the connections of the joint capsule to the bone and then following it until a distinct inner layer of cells was evident. Not all slides produced had easily identifiable synovium and were excluded. Mean and max intensity were averaged across 3-5 slides for each joint and the average background was subtracted to yield mean and max intensity for each joint for each mouse. Values for the contralateral and ipsilateral joints were averaged and compared.

Statistics

Data were analyzed using Microsoft Excel and GraphPad Prism. The Excel data analysis tool-pack was used for all single comparison t-tests. Each data set was first analyzed for normalcy and outliers. All data were normal. Outliers were removed from the mouse serum artemin concentrations dataset as identified by the ROUT method. Significance set at an alpha of 0.05 for all experiments. Graphs were produced using GraphPad Prism.

Results

GFRα3 expression in the DRG of mice with MIA-induced OA

Previously, we determined there to be upregulation of GFRα3 in the DRG of osteoarthritic dogs using qPCR. Here, we sought to determine whether the same change held true for the MIA-model of OA in mice using immunohistochemical techniques, which allowed us to investigate whether there was de novo expression of GFRα3. The expression of GFRα3 in the
ipsilateral DRG of MIA-injected joints doubled to approximately 40 percent of neurons, increasing significantly from both the contralateral side and from controls that received saline, both of which had GFRα3 expressed in about 20 percent of neurons, which matches the reported percentage of GFRα3 expression (Orozco, 2001). This data shows that GFRα3 is expressed in an increased number of neurons in the DRG in a chronic pain state.

**Figure 4.1 De novo expression of GFRα3.** IHC on mouse DRG staining for TUJ (red) and GFRα3 (green). DRG are from 3 sham and 3 MIA mice. 10 sections from each side were counted for number of neurons (express TUJ) and number of neurons expressing GFRα3. This was expressed as a percentage of neurons in the DRG expressing GFRα3. There was no difference in between the ipsilateral and contralateral sides in control mice (saline injected) and the percent of GFRα3 found corroborates the published percentage. MIA mice had significantly (two-tailed t-test; p=0.016963) more GFRα3 expression in ipsilateral DRG compared to contralateral. There was no difference between saline controls (ipsilateral and contralateral) and contralateral MIA DRG (two-tailed t-test; p=0.84 and p=0.90).
Serum artemin concentrations in MIA-injected mice

To further our comparison to naturally occurring OA in dogs, mouse serum concentrations of artemin were determined. Contrary to expectations, mice with MIA-induced OA did not have elevated serum artemin compared to naïve mice. Naïve mice had a very wide range of serum concentrations, while the MIA-injected mice had a lower, narrower range. Serum artemin was measured at both 7 days after MIA injections and 28 days from different cohorts of mice. At day 7 there was no difference between MIA-injected mice and controls. There was a significant difference between MIA-injected mice at day 28 compared to controls, though here it was the that the day 28 MIA-injected mice had much lower serum artemin concentrations than controls.

Figure 4.2 Artemin serum concentrations in the MIA-model of OA. An ELISA kit was used to analyze mouse artemin serum concentrations. Serum from MIA-injected mice at Day 7 (n=12) and Day 28 (n=13) and control mice (n=24) were analyzed for artemin concentration. No difference was detected between MIA Day 7 and control mice (two-tailed t-test; p>0.05). Serum artemin concentrations between control mice and MIA Day 28 were significantly different (two-tailed t-test; p<0.01). Data are represented as individual data points graphed over the mean ± SD.
Artemin expression in the joint capsules of MIA-injected and control mice

Joint capsule tissue was analyzed for artemin mRNA to determine whether artemin was upregulated locally in the joint capsule after the MIA model had reached 28 days. No upregulation was found in MIA-injected mice compared to naïve controls (Figure 3).

![Artemin mRNA expression in the joint capsule](image)

**Figure 4.3 qPCR analysis of artemin in the joint capsule of MIA-injected mice.** The fold change of artemin mRNA between the ipsilateral and contralateral joint capsules was determined for mice with MIA-induced OA (n=6) and for naïve control mice (n=4). Data is represented by the $2^{ΔCT} ± SD$. There was no difference between the groups (t-test; p>0.05).

Joint capsule IHC of MIA-injected mice

To further investigate whether an increase of artemin in the joint capsule was occurring in any way after MIA injection, joints were stained with an anti-artemin antibody and intensity was measured. There was no observed difference in either average pixel intensity or average max pixel intensity between the contralateral (control) and ipsilateral (MIA-injected) joint capsules (Figure 4).
Figure 4.4 Comparison of contralateral and ipsilateral joint capsule for artemin expression.

Comparison of staining intensity of artemin in synovium between contralateral (control) and ipsilateral (MIA-injected) joints. Both mean intensity (A) and max intensity (B) were compared using a paired t-test to compare within mice (n=3). Neither outcome measure had any significant difference (p>0.05). Data are represented as mean ± SD. Example images of joints stained for artemin with the synovium selected as measured on ImageJ are shown (C, ipsilateral; D, contralateral).
Discussion

The role of GFRα3/artemin in pain has been partially established through work showing GFRα3/artemin signaling in cold pain (Lippoldt, 2013), bladder pain (Deberry, 2015), and inflammatory bone pain (Nencini, 2018). This is the first study to examine the role of GFRα3/artemin in a model of chronic pain. Here we determined that a chronic pain model induces de novo expression of GFRα3 in the DRG, doubling the number of neurons that express GFRα3. This expansion of GFRα3 expression could have important functional roles should as interactions with other nociceptive proteins that are expressed in the expanded population of neurons. Increased GFRα3 expression could prove to be a mechanism that contributes to pain signaling and potentiation. Further work needs to be done to determine the co-expression of other nociceptors in the population of neurons that have de novo expression of GFRα3 in a chronic pain state.

Part of the basis of this work was clinical data obtained from dogs with naturally occurring OA, which showed elevated artemin in the serum of OA dogs compared to normal dogs. The MIA model of OA in mice did not have elevated serum artemin compared to naïve mice. There are some key differences in the two samples, OA dogs and MIA-injected mice, that might explain this different result. Naturally occurring OA takes much longer to develop than the OA model induced by MIA. The dogs used in Chapter 2 had fully developed OA and associated pain, meaning that had been suffering from OA from a long time, much longer than the 28 days of the MIA-injected mice. Perhaps artemin does not become elevated systemically until later on in the disease and this could not be replicated with a mere 28 days post-injection timepoint.

The lack of systemic artemin increases prompted us to investigate artemin expression locally in the joint tissue. Neither mRNA or protein appear to be elevated in the ipsilateral MIA-
injected joint compared to the contralateral joint. These experiments were done on mice with MIA-induced OA for 28 days. Again, it could be that the 28 days was sufficient for the upregulation of GFRα3, but not artemin. This provides an insight into the pathology of GFRα3/artemin signaling upregulation – the receptor is impacted first according to this data.

It could also be possible that mice do not have the same increase in artemin that we observed in the dog, after all the MIA model is different from naturally occurring disease. It could also be that we are not looking in the right place. Artemin has been shown to be produced by a wide variety of tissues, including blood vessels, keratinocytes, and glia cells – perhaps the joint tissue we investigated here was not relevant and artemin is being produced by another cell type, such as chondrocytes, glia, or immune cells. This should be investigated further to determine what specific cell type is producing artemin in OA as this could be therapeutically relevant and allow for targeting of anti-artemin antibodies to a specific region, such as the joint space.

Here we determined that GFRα3 has de novo expression in the DRG serving the affected limb in mice with MIA-induced OA. This indicates that our model has some fidelity with the naturally occurring disease in dogs, in which we found an upregulation of GFRα3 in OA DRGs. The lack of artemin upregulation does not match what was observed in the naturally occurring disease, however, the lack of serum artemin increase might just be a function of time and not an invalidation of this model for studying GFRα3/artemin in chronic OA-associated pain. The hypersensitivity to all three modalities established in Chapter 3 along with the increase in GFRα3 expression in the DRG found here make the MIA model of OA well suited for testing the potential of an antibody targeting GFRα3/artemin signaling to relieve OA-associated hypersensitivity.
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and TRPA1 in cutaneous sensory neurons and leads to behavioral sensitivity to heat and cold. *Journal of Neuroscience*, 26(33), 8578-8587.


CHAPTER 5
The functional role of GFRα3/artemin in hypersensitivity

Introduction

The role of artemin in inducing sensitivity is only partially known. Artemin hindpaw injections have been shown to induce heat hypersensitivity (Lippoldt, 2013; Malin, 2006; Ikeda-Miyagawa, 2015), however, the verdict is still out on whether artemin can directly induce cold or mechanical hypersensitivity. Cold hypersensitivity is likely, but has only been evaluated using the evaporative cooling assay (Lippoldt, 2013), which is not as comparable to the Von Frey or the Hargreaves as the more robust plantar cold assay, which mirrors these other assays being placing the noxious stimulus directly to the plantar hindpaw and measuring withdrawal time (Brenner, 2012). Mechanical hypersensitivity has been found after repeated injections of artemin (Ikeda-Miyagawa, 2015), but not after a single injection (Lippoldt, 2013) and mechanical hypersensitivity has not been seen in mice that overexpress artemin (Elitt, 2006). There is yet to be a study that evaluates sensitivity to all the pain modalities – heat, cold, and mechanical – after artemin hindpaw injection in the same mice using consistent methods. Reflexive assays can be subjective, which makes study design and blinding essential. Some of the aforementioned studies did not state whether or not behavioral testing was conducted by blinded individuals (Lippoldt, 2013; Elitt, 2006).

By definitively answering whether artemin can induce heat, cold, and/or mechanical hypersensitivity in a blinded fashion with a single person conducting all assays, the role of artemin in OA-associated pain, particularly neuropathic pain components will be further illuminated.
Anti-artemin monoclonal antibodies have been shown to be effective as analgesics in multiple inflammatory pain models (DeBerry, 2015; Nencini, 2018; Thornton, 2013). Based on this, it is possible that they will be effective at alleviating OA-associated pain, as there is a significant inflammatory component to OA-associated pain. A subset of patients with OA also have a neuropathic component to their pain that leads to various symptoms of hypersensitivity to modalities such as heat and cold (Thakur, 2014; Hochman, 2011). This subset would stand to benefit the most from the development of novel analgesics as neuropathic pain is not effectively managed by NSAIDs (Hochman, 2011). The extensive hypersensitivity to heat, cold, and mechanical stimuli seen in mice with MIA-induced OA makes this model ideal for evaluating potential analgesics for their ability to alleviate neuropathic pain in OA.

Currently, the closest model to OA-associated pain that anti-artemin has been tested in is a model of inflammatory bone pain induced by injecting carrageenan into the bone space in rats. This model caused decreased weight bearing on the injected limb and sensitized nociceptive neurons to mechanical stimuli. Treatment with the anti-artemin antibody reduced carrageenan-induced changes in mechanical sensitization (Nencini, 2018). Similarly, in the most common model of inflammatory pain, an intraplantar injection of Complete Freund’s Adjuvant (CFA), anti-artemin antibody was able to improve weight bearing on the injected paw in mice (Thornton, 2013).

Finally, an anti-artemin antibody has been used to reduce hyperalgesia in a model of interstitial cystitis/painful bladder syndrome induced by cyclophosphamide. Nociceptive behavior was measured by evaluating electromyographic responses to compressed air in the bladder. Anti-artemin was able to both prevent and recover the increased electromyographic responses that correlated with hyperalgesia (DeBerry, 2015). These three experiments that show
anti-arteimin’s efficacy in relieving inflammatory pain indicate that it should be able to alleviate at least the inflammatory aspect of OA-associated pain and their efficacy in reducing sensitization in nociceptive neurons (Nencini, 2018) indicates that it might be effective at relieving the neuropathic aspect of OA-associated pain.

Here, arteimin will be evaluated for its role in inducing hypersensitivity directly, which has yet to be established for all three modalities – heat, cold, and mechanical. Arteimin’s ability to induce hypersensitivity will also be investigated using TRPV1 and TRPA1 knock-out mice, which will determine whether arteimin-induced hypersensitivity is mediated through either channel for each modality. While application of arteimin has been shown to upregulate TRPV1 and TRPA1 (Elitt, 2006), it has not been shown in vivo whether this upregulation is functionally important for inducing sensitivity. Finally, to determine whether GFRα3/arteimin signaling can be targeted to alleviate pain, antibodies blocking GFRα3/arteimin signaling will evaluated for their ability to alleviate hypersensitivity in the MIA-model of OA.

Methods

Mice: All aged matched, 4-week-old, adult male C57BL6 mice (Jackson Labs) were used in these experiments. Average mouse weight was 25 grams. Mice were housed in groups of 4 and kept on a 12-hr light-dark cycle. All knock-out mice were on a C57/Bl6 background. TRPV1 knock-out (Jackson Labs; Stock No: 003770) and TRPA1 knock-out (Jackson Labs; Stock No: 006401) mice were obtained from Jackson labs and cross-bred to obtain TRPV1-A1 knock-out mice.

Behavior: Mice were tested using the same protocol as in chapter 3 for the Von Frey, Hargreaves, plantar cold assay, and IC meter. Mice were given a minimum of 5 minutes to acclimate to all behavioral equipment before testing began. For the reflexive assays, mice were
tested 3 times on each hindpaw to reduce the stress of multiple assays and timepoints and to avoid hypersensitivity that could be induced by over-testing the mice.

Assessment of the hyperalgesic effects of artemin: Male C57/bl6 mice received hindpaw injections of artemin to determine whether artemin can induce hypersensitivity. A 200 ng injection of Artemin (R&D Biosystems; Cat. 1085-AR/CF) was delivered in 10 µl of sterile saline using an insulin syringe to the subcutaneous layer of the left hindpaw while mice were conscious and restrained. Control mice received 10 µl of sterile saline. Mice were assessed for weight bearing and for mechanical, heat, and cold sensitivity and underwent a single behavioral test per injection to avoid overstressing them as they were being tested at 1, 2, 4, 6, and 24 hours post-injection. Mice had a minimum of one week of washout between hindpaw injections.

Artemin hindpaw injections were also conducted in the same manner on TRPA1 and TRPV1-A1 knock-out (KO) mice. Due to the small number of KO mice tested, all KO mice received artemin and no blinding occurred.

Assessment of the analgesic effect of intra-articular anti-GFRα3 monoclonal antibody: Mice underwent baseline testing for weight bearing with the SHIM incapacitance meter prior to MIA injections following the same training and testing protocol described in Chapter 3. After MIA injections as per the methods in Chapter 3, mice were tested weekly to confirm reduced weight bearing in the ipsilateral limb out to day 28. After day 28 mice received an intra-articular injection of 10 µg in 10 ul of a polyclonal anti-GFRα3 antibody (abcam; Cat. ab2028) or IgG isotype control (R&D; rabbit IgG) dissolved in PBS. An intra-articular injection was chosen as it allowed for a lower dose per mouse. Mice were anesthetized with isoflurane and injected with anti-GFRα3 antibody using the same protocol as MIA injections (Chapter 3). After administration of the antibody mice were tested for weight bearing at 1, 2, 4, 6, and 24 hours.
post-administration (see Figure 1 for timeline). Mice were tested in two separate batches, both with n=4 per group.

**Figure 5.1 Timeline of the assessment of the analgesic effect of an intra-articular injection of an anti-GFRα3 antibody.**

*Assessment of the analgesic effect of a systemic anti-artemin monoclonal antibody:* Mice underwent baseline testing for mechanical and cold sensitivity prior to MIA injections (see Chapter 3 Methods). After MIA injections mice were tested weekly to confirm hypersensitivity in the model out to day 28. Mice were also tested for weight bearing using the IC meter to confirm that less weight was placed on the ipsilateral (MIA) limb post-day 28. After day 28 mice received a 100 ul IP injection of either PBS or 25 ug of anti-artemin monoclonal antibody (R&D, cat: MAB10851-500) dissolved in PBS, as determined by their randomly assigned groups. Mice were tested for sensitivity to mechanical and cold stimuli at 2, 4, 6, and 24 hours post intraperitoneal injection. One week after anti-artemin was injected, the antibody administration was repeated to test the effect on thermal heat hypersensitivity which was measured at a MIA baseline and then 2, 4, 6, and 24 hours post IP injection (see Figure 2 for timeline).
Figure 5.2 Timeline of the assessment of the analgesic effect of an intraperitoneal injection of an anti-artemin antibody.

**DRG cell culture:** Normal mouse DRG were isolated from 4-week-old C57BL6 mice and cultured. Two biological replicates were used for the dose-response to artemin. DRGs were dissociated in 1 mL of media containing 2.5 U/mL of Dispase (Fisher, 07913) and 2.5 mg/mL of collagenase (Sigma, C0130). After dissociation, the cells were washed with complete media (DMEM with 10% FBS (VWR, 97068-085) and 1% PenStrep (VWR) and pelleted at 1800 rpm for 20 minutes. The cell suspension was plated on 18mm round glass slides with a coating of laminin (Sigma, L2020) and poly-L-lysine (Sigma, P4707) and incubated for 1.5 hours. Afterwards, 1 mL of complete media was added and the cells were incubated overnight. All incubation steps were done at 37 °C with 5% CO2.

**Calcium imaging:** DRG neurons were cultured according to the above method and were imaged within 24 hours of isolation. Artemin (R&D Biosystems; Cat. 1085-AR/CF) was dissolved in 1x PBS at 250 ng/mL, 1 μM capsaicin (Sigma, M2028) in 1X PBS was generated
via a 1:1000 dilution from a 1 mM capsaicin stock in ethanol, and KCl (1 mM) was dissolved in 1X PBS. For all slides, 100 μL of each compound was added to each slide tested and a minimum of two minutes was allowed to elapse between artemin and capsaicin applications.

Before imaging, cells were incubated in 350 µL of complete media containing 4.2 µM Fura-2 AM (Enzo, ENZ-52006) for 30 min at 37 °C with 5% CO2. Additionally, cells were incubated with 15 nM rabbit IgG isotype control (abcam, ab172730) or 15 nM rabbit anti-GFRα3 (abcam, ab2028) for 30 minutes before imaging. During imaging, the cells were perfused with Locke buffer containing the following: 135 mM sodium chloride, 3.2 mM potassium chloride, 2.5 mM magnesium chloride, 2.8 mM calcium chloride, 667 µM monobasic sodium phosphate, 14.2 mM sodium bicarbonate, and 10.9 mM D-glucose (all VWR) with a pH between 7.00 and 7.40. The buffer and the holding plate were kept at 37 °C while imaging. Imaging data was collected on a TE200 inverted microscope using NIS Elements software (Nikon). Cells were exposed to 340 nm and 380 nm wavelengths for 100 ms and the A340/A380 ratio was calculated. Traces were analyzed using Excel and responses greater than 10% of the baseline were counted. Error bars represent ± SEM.

**Statistical Analysis**

All behavioral data was collected by a researcher blind to the groups. Data was analyzed using Microsoft excel and GraphPad Prism. Two-way ANOVA and multiple t-tests with corrections for multiple comparisons were done using GraphPad Prism. For multiple t-test, Statistical significance determined using the Holm-Sidak method, with alpha = 0.05. Data was tested for normalcy with the Shapiro-Wilk test. All data was normal. Graphs were produced using GraphPad Prism. Significance was set at an alpha of 0.05 for all experiments.
Results

Assessment of the hypersensitivity induced by artemin in wild type mice

To determine if artemin can induce hypersensitivity for mechanical, thermal, and/or cold stimuli, we injected artemin in the hindpaw of mice. Mice were hypersensitive in all three modalities after artemin injection (Figure 5.3), indicating that artemin can directly induce acute hypersensitivity to mechanical, hot, and cold stimuli. Thermal hypersensitivity to heat (Figure 5.3A) and cold (Figure 5.3B) lasted from 1 to 4 hours post-injection while mechanical hypersensitivity (Figure 5.3C) had a slightly delayed onset, lasting from 2 to 6 hours post-injection. All mice returned to baseline by 24 hours post-injection.

As a separate measure of mechanical hypersensitivity mice were placed on the IC meter after artemin hindpaw injections to evaluate static weight bearing. Artemin injected mice had reduced weight bearing on the injected hindpaw (Figure 5.3D), further indicating a role for artemin in mechanical hypersensitivity.
Figure 5.3 Artemin-induced hypersensitivity to heat, cold, and mechanical stimuli. Mice that received artemin had significantly reduced withdrawal latencies to heat and cold (A,B) compared to mice that received saline at 1 hr (p<0.05), 2 hr (p<0.01), and 4 hr (p<0.01) post-injection (multi-comparison t-test; n=9 per group). Mechanical withdrawal force (C) was significantly decreased at 2 hr (p<0.05), 4 hr (p<0.01), and 6 hr (p<0.01) post-injection with artemin (multi-comparison t-test; n=8 per group). Significantly less body weight on the injected paw at 2 hr post-injection in artemin injected mice (n=4; p<0.05) compared to controls (n=5) was determined with the SHIM IC Meter. A two-way ANOVA was significant for a difference between groups for the IC meter (p<0.05). Data are represented as mean ± SD.
Assessment of the hypersensitivity induced by artemin in TRP channel knock-out mice

Since it has been previously reported that artemin/GFRα3 signaling can upregulate both TRPV1 (Elitt, 2006; Ikeda-Miyagawa, 2015) and TRPA1 (Elitt, 2006; DeBerry, 2015; Ikeda-Miyagawa, 2015), we evaluated the functional role of these ion channels in artemin-induced hypersensitivity in TRPA1 and TRPV1-A1 double knock-out mice. These knock-outs were then compared to the wild-type mice used in Figure 3. TRPA1 alone and TRPV1-A1 double knock-out mice were pooled together for data analysis for cold and mechanical assays as TRPA1 has been shown to contribute to cold and mechanical modalities (Story, 2003; Bandell, 2004; Petrus, 2007), while TRPV1 is exclusively activated by heat (Caterina, 1997) and should not alter the results for the cold and mechanical hypersensitivity. For the analysis of heat sensitivity in TRPV1-A1 double knock-out mice the loss of TRPA1 should not have any effect on thermal sensitivity and any changes in sensitivity to heat can be attributed to the lack of TRPV1.

When analyzing the raw values, TRPA1 knock-out mice did not have cold hypersensitivity in response to artemin, indicating that the artemin-induced cold hypersensitivity could be mediated through TRPA1. Withdrawal latencies in TRPA1 knock-outs were reduced from baseline following injection with artemin, but were not significantly different from wild-type mice injected with saline at any timepoint post-injection. However, this reduction from baseline indicated these TRPA1 KO mice were still experiencing some artemin-induced cold hypersensitivity. When analyzed as a change from baseline (calculated here as subtracted from baseline), there is no difference in artemin-induced cold hypersensitivity between TRPA1 KO and wild-type mice. Mechanical hypersensitivity was not found to differ between wild-types and TRPA1 knock-out mice injected with artemin. TRPV1-A1 KO mice were not protected from artemin-induced hypersensitivity to heat.
Figure 5.4 Artemin-induced hypersensitivity in TRP channel knock-out mice. Artemin hindpaw injections in TRPA1 and TRPV1-A1 KO mice. A) TRPA1 KO mice were not significantly different from WT controls for the cold plantar test (multi-comparison t-test; p>0.05), indicating protection from artemin-induced hypersensitivity. B) When analyzed as change from baseline the TRPA1 knock-out mice show no difference from WT mice for artemin-induced cold hypersensitivity (multi-comparison t-test; p>0.05) C) Mechanical hypersensitivity was still present in TRPA1 KO mice as they were not significantly different from WT artemin injected mice at any timepoint after injection (multi-comparison t-test; p>0.05). D) TRPV1-A1 KO mice were hypersensitive to heat after artemin hindpaw injection. There was no significant difference between TRPV1-A1 KO and WT artemin injected mice (multi-comparison t-test; p>0.05) for their cold and mechanical responses. Data are represented as mean ± SD.
Pharmacological inhibition with an anti-GFRα3 antibody

In vitro blockade of GFRα3

To determine whether it was possible to block the artemin-GFRα3 pathway as a possible therapeutic intervention, we first examined the functional blockade of anti-GFRα3 antibody on DRG neurons in culture. We incubated mouse DRG neurons with anti-GFRα3 antibody and the percentage of neurons responding to artemin was evaluated in response to change in calcium levels. We evaluated the dose-response of artemin and found a dose of 250 ng/mL artemin activates approximately 17% of neurons (Figure 5.3A), which approximately validate the number of GFRα3 expressing neurons in the mouse DRG. Pre-incubation of neurons with 15 nM of rabbit anti-GFRα3 significantly decreased the percentage of neurons responding to artemin when compared to neurons incubated with a rabbit IgG isotype control (Figure 5.3B). The significant decrease in number of cells responded to artemin strongly suggests that blocking artemin’s ability to bind GFRα3 could be a viable treatment for OA pain.

**Figure 5.5 In vitro blockade of GFRα3.** A) Artemin dose response. Cultured DRG neurons treated with artemin to determine dose using calcium imaging. A minimum of 300 neurons were tested at each concentration. Data are represented as ±SEM. B) Percent responding neurons to artemin and capsaicin with/without anti-GFRα3. Data are represented as mean ± SEM. Significance determined by using students t-test (p<0.01).
In vivo blockade of GFRα3 in MIA-injected mice

To determine whether this anti-GFRα3 antibody would be a viable treatment option in vivo, MIA mice were administered 10 µg intra-articularly. Intra-articular injections have been used before in mice to test drugs to relieve OA-associated pain (Kras, 2015). Mice were injected with MIA and then after day 28 administered either anti-GFRα3 or an isotype control. There was no difference in weight bearing in mice that received anti-GFRα3 compared to controls.

![Graph showing weight bearing of MIA mice injected with anti-GFRα3 or isotype control](image)

**Figure 5.6 Intra-articular injection of anti-GFRα3 antibody in MIA mice.** No significant difference was found between MIA mice injected with anti-GFRα3 compared to isotype control (multi-comparison t-test; p>0.05). Two-way ANOVA uncovered no difference between groups (p>0.05). Data are represented as mean ± SEM.
Efficacy of anti-arthemin monoclonal antibody in reversing MIA-induced hyperalgesia

To further examine the role of artemin in MIA induced pain and hypersensitivity, artemin, an anti-arthemin antibody was tested for efficacy at alleviating OA-associated hypersensitivity as induced by MIA. This antibody was administered via an IP injection of 25 ug of anti-arthemin to systemically sequester artemin. Mice that received the antibody demonstrated reduced hypersensitivity compared to controls between 2 and 6 hours post injection (Figure 7). These positive findings indicate that anti-arthemin is effective at relieving OA-associated pain and in particular, the neuropathic components of OA-associated pain. This indicates that therapies targeting artemin could fill the current analgesic gap for OA patients with neuropathic pain.
Figure 5.7 Efficacy of an anti-artemin antibody in alleviating hyperalgesia in the MIA model of OA. Anti-artemin alleviates MIA-associated hypersensitivity for heat (A), mechanical (B), and cold (C). Anti-artemin was effective for relieving thermal hypersensitivity to hot and cold stimuli at 2, 4, and 6 hr after antibody administration and at 2 and 4 hr post antibody for mechanical hypersensitivity (t-test; p<0.05). Data are represented as mean ± SD. The mechanism of action of the anti-artemin antibody is depicted (D) where artemin is unable to bind to GFRα3, because it is bound to the antibody.
These positive findings indicate that anti-artemin is effective at relieving OA-associated pain and in particular, the neuropathic components of OA-associated pain. This indicates that therapies targeting artemin could fill the current analgesic gap for OA patients with neuropathic pain.

Discussion

Here artemin-induced hypersensitivity was established for heat, cold, and mechanical stimuli. The data shown here indicates that a single injection of artemin can induce hypersensitivity to thermal, cold, and mechanical stimuli as well as decrease weight bearing on the injected paw. This was the first study to evaluate heat, cold and mechanical hypersensitivity in response to artemin. This was the first time mechanical hypersensitivity has been demonstrated in response to a single injection of artemin. Both positive (Ikeda-Miyagawa, 2015) and negative (Lippoldt, 2013; Elitt, 2006) results have been reported previously for artemin induced mechanical hypersensitivity, but as mentioned in the introduction, these previous experiments were not necessarily performed blinded and the subjective nature of the reflexive assays could have allowed for unconscious biases to effect the results. This study used very consistent protocols and comparable reflexive assays entirely focused on paw withdrawal to measure sensitivity. Blinding and carefully conducted assays for measuring hypersensitivity are essential for pain research, which can be heavily impacted by experimenter bias (Andrews, 2016).

Here, for the first time, hypersensitivity in response to artemin was tested in TRP knock-out mice. It has been shown that exposure to artemin can upregulate TRP channels (Elitt, 2006) and some in vitro calcium imaging data even indicates that exposure to artemin can increase neuronal response to TRP channel agonists (Malin, 2006; Ikeda-Miyagawa, 2015). Here, for the
first time, we have described the hypersensitivity responses to artemin *in vivo* in TRPV1 and TRPA1 KO mice. Neither TRPV1 or TRPA1 knock-outs were protected completely from artemin-induced hypersensitivity. TRPA1 knock-out mice had some protection against artemin-induced cold hypersensitivity, however, this protection disappears when the data is analyzed as a change from baseline measurement. Thus, TRPA1 KO mice had the same decrease in withdrawal time seen in wild-type mice. Our data do not indicate an exclusive role for TRPA1 or TRPV1 in artemin induced thermal hypersensitivity. This is surprising considering numerous studies have found co-localization of GFRα3 with both TRPA1 and TRPV1 (Orozco, 2001; Ikeda-Miyagawa, 2015) and upregulation of TRPA1 and TRPV1 in response to artemin (Elitt, 2006). It could be that artemin induces hypersensitivity to heat through TRPV1 and additional channels, such as TRPV4. Under this hypothesis, the difference in response in TRPV1 KO mice would be difficult to detect as TRPV4 could compensate for TRPV1 in those mice. The same mechanism of other channels compensating could explain the results for TRPA1 KO mice as well, however, artemin-induced cold pain has been shown to be due to TRPM8 activation (Lippodlt, 2013). Despite its upregulation in response to artemin (Elitt, 2006), this data does not support a role for TRPA1 for artemin-induced cold hypersensitivity.

This data supports a broader hypothesis that GFRα3/artemin regulate multiple nociceptive targets and that blocking artemin/GFRα3 signaling will provide effective analgesia, because it is upstream, regulating these nociceptive channels. We tested two separate antibodies for blocking artemin/GFRα3 signaling. The first, an anti-GFRα3 antibody, was effective in vitro and showed no difference in vivo. This is probably due to the route of administration, as IP-injections of this antibody were cost-prohibitive, and this antibody was delivered via an intra-articular injection. There are many aspects of this approach that could have potentially affected
our results including the after-effects of anesthesia, additional joint damage from the needle, and the antibody not reaching the site of action. The second antibody used, an anti-artemin antibody, showed efficacy in blocking hypersensitivity in the MIA model. These promising results indicate that an anti-artemin therapy should be investigated further for relieving OA-associated pain and in particular, the neuropathic component of OA-associated pain.
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CHAPTER 6

Conclusion and future directions

Summary of current work

Here we determined that GFRα3/artemin signaling has a role in OA-associated pain. First, potential pharmacological targets for OA-associated pain were found by screening clinical samples from dogs with naturally occurring OA. In these dogs, concentrations of artemin in the serum and synovial fluid correlated to the severity of pain behaviors and limb-use. After identifying these targets in dogs, we explored the system further using a mouse model of OA, the MIA model. This model, while popular, previously had not been fully characterized for all three modalities of hypersensitivity that are found in OA patients – hot, cold, and mechanical – in the same study. We established that mice injected intra-articularly with MIA had intense, consistent, and prolonged hypersensitivity to all three modalities, making this model useful for both exploring how GFRα3/artemin could be mechanistically influencing hypersensitivity and testing potential therapeutics for their ability to alleviate hypersensitivity.

We found de novo expression of GFRα3 in the peripheral DRG neurons serving the MIA-injected joint, which could serve as a potential mechanism for increased GFRα3/artemin signaling causing increased hypersensitivity. We did not find any upregulation of artemin in these mice, which deviates from the naturally occurring disease in dogs. However, we were still able to link artemin to hypersensitivity in mice through hindpaw injections of artemin, which caused hypersensitivity to hot, cold, and mechanical stimuli. This hypersensitivity mirrored that seen in the MIA model indicating artemin/GFRα3 signaling has role in OA-associated pain. Finally, by blocking GFRα3/artemin signaling with an anti-artemin antibody in mice with MIA-
induced OA, we determined that blocking this signaling can alleviate hypersensitivity to hot, cold, and mechanical stimuli.

This work all indicates the GFRα3/artemin are potential pharmacological targets for treating OA-associated pain. Further work needs to be done to determine the feasibility of targeting this system for human patients by evaluating the safety and effectiveness of repeated and chronic doses of anti-artemin biologics and by determining the specific biological mechanisms at work that make blocking GFRα3/artemin effective. Currently the tissue producing artemin in OA patients is unknown. Determining which tissue is producing artemin would contribute to drug design and administration techniques to ensure that anti-artemin biologics are reaching the site of action.

**Importance of using naturally occurring disease in OA research**

The initial screening of clinical samples from dogs with naturally occurring OA was arguably the most important part of this work. By utilizing this reverse translational approach, we were able to determine that our work was clinically relevant before the mouse work was even conducted. Mouse models are extremely useful for characterizing disease mechanisms and testing pharmacological interventions, but they are still models and will never capture the full scope and complexity of the naturally occurring disease. By determining that GFRα3/artemin signaling potentially had a role in the OA-associated pain in dogs with naturally occurring disease first, we were able to ensure the relevance of the mouse work and improve the probability that this work will be relevant for treating OA-associated pain in dogs and, ultimately, humans.

The standard procedure of drug discovery is not working for pain medications. Potential analgesics are failing when they get to clinical trials and are thus failing to translate. The success
rate for analgesics from 1991 to 2000 to get from phase 1 to passing phase 3 clinical trials was only 17% and many of those “successes” were simply reformulations of already approved compounds (Mogil, 2010; Yekkirala, 2017; Kissin, 2010). Very few new pain drugs have entered the market, with the primary reason for most failures being a lack of efficacy causes by a lack of translation from mice to humans (Mogil 2010).

It’s difficult to tell why drugs are failing to translate from mice to humans, with one notable failure being neurokinin 1 antagonists, which block the receptor for Substance P. These drugs were efficacious in rodent models, yet failed in human trials (Hill, 2000). Other failures include sodium channel blockers (Wallace, 2002) and glycine antagonists (Wallace, 2002). Other potential analgesics, such as TRP channel antagonists are still under investigation, despite many unexpected set-backs in clinical development. TRP channel antagonists are still entering drug pipeline, but many have been abandoned due to either lack of efficacy and/or safety concerns. Some TRPV1 antagonists, such as Amgen’s AMG517, failed in Phase 1 clinical trials after causing hyperthermia. Newer second-generation TRPV1 antagonists, such as Glenmark–Eli Lilly’s GRC-6211, which was being tested for the relief of OA-associated pain, have managed to overcome the hyperthermia side-effect, but have still been abandoned for undisclosed reasons. Many TRPV1 antagonist raise the threshold for heat sensation, which can lead to injury, though this may be lessened in the second-generation drugs. Clinical studies are also ongoing for a TRPV3 antagonist, which has potential. Antagonists for TRPA1 and TRPM8 are still in the preclinical research stage (Brederson, 2013). While pain researchers are still hopeful about developing analgesics based on TRP channel antagonism, the hurdles that have appeared in clinical trials indicate that while the basic mechanisms of nociception are conserved, targeting human disease and predicting efficacy and safety in human patients is very difficult to do in the
pain field. While TRP channel antagonist avenue of research may eventually produce a viable analgesic, the growing cases of chronic pain and OA-associated pain in America won’t be getting a TRP channel antagonist from their doctors anytime soon.

Part of problem might be the difficulty of measuring pain in animals, which are nonverbal. Pain is a subjective experience and the perception of pain centrally can differ greatly from what is occurring in the periphery. The affective component in pain cannot be directly measured in animals and behavioral assays that might capture some of that experience are not as common as they should be. For the most part, when testing new analgesics, efficacy is evaluated using reflexive assays since they are highly standardized, easy to replicate, and have less variable results. This is advantageous for high-throughput screening of compounds, but since these assays are testing hypersensitivity rather than the affective component of pain, they might not translate to humans for the purpose of long-term overall pain relief (Mogil, 2009).

A different part of the problem might be the mouse models that are used. Mice are inbred and this loss of genetic variability is great for controlling for extra variables in a study but does not accurately reflect genetic variability seen in a natural population (Mogil, 2009). While the broad mechanisms of neurobiology and pain transmission are broadly conserved between mice and humans, a large proportion of chronic pain cases in humans have undetermined underlying causes, which, since unknown, cannot be directly modelled in mice. We need mouse models to understand the various disease mechanisms for chronic pain, but need to keep in mind the diversity of the disease they we seek to treat (Bruyere, 2015). Lower back pain is fundamentally different from knee osteoarthritis and an analgesic might work for one condition and not for the other because of these differences. The differences in pain pathology might extend deeper than
that as well – two patients, both with knee OA, might respond differently to an analgesic due to pathological differences that have yet to be elucidated.

In an effort to tailor drug development to discover elegant solutions to specific chronic pain conditions, we need to start relying on specific pain phenotyping to determine which populations of patients with OA a potential analgesic would be efficacious in and then tailor preclinical models to those phenotypes (Malfait, 2013). Not all OA patients present with hypersensitivity (Fingleton, 2015) and the extent to which a patient’s pain is primarily driven by central compared to peripheral mechanisms can be determining factors for whether or not an analgesic will work (Mease, 2011).

**Studies to determine the feasibility of continuous inhibition of artemin**

Here we determined that an IP-injection of anti-artemin antibody was able to alleviate hypersensitivity for around 4 hours. This is obviously too short of a timespan for serious pain-relief in human OA patients, which means that biologics targeting artemin need to be refined to have a longer half-life or need to be evaluated for long-term use at high doses. Currently anti-artemin has only been used single administration (Deberry, 2015; Nencini, 2019) and the potential effects of chronic administration are unknown.

The only other biologic targeting a neurotrophin is anti-NGF, which has limited side effects from long term use. The most concerning adverse event that was caused by anti-NGF was the development of rapidly progressive OA in a very small number of patients (<1.5%) (Mullard, 2018). Anti-artemin should also be investigated for the same adverse effects as anti-NGF since they are both neurotrophins and have similar mechanisms. Currently the cause behind the rapidly progressive OA seen a very small number of OA patients treated with anti-NGF is unknown. As such, it is possible that anti-artemin could also cause rapidly progressive OA and that should be
monitored if anti-artemin ever makes it to clinical trials. It is known that the rapidly progressive OA caused by anti-NGF is dose-dependent (Miller, 2017). Even if anti-artemin produces the same adverse event it could potentially have a wider therapeutic window than anti-NGF.

**Mechanisms to uncover for GFRα3/artemin signaling in OA pain**

Further work needs to be done to elucidate the specific mechanisms of how GFRα3 and artemin contribute to hypersensitivity and pain. Understanding the specific mechanisms is important for determining the potential side effects and risks of an anti-artemin biologic and for advancing our knowledge of how chronic pain works in the periphery.

*Signaling through TRP channels*

While GFRα3 is known to be highly co-localized with TRP channels, including TRPV1, TRPA1, and TRPM8, little is known about how GFRα3 can interact with these TRP channels. Here we’ve shown that TRPA1 knock-out mice are partially protected from MIA-induced cold hypersensitivity and artemin induced cold hypersensitivity when just analyzing raw values. The specific method by which GFRα3/artemin regulates or acts through TRPA1 has yet to be determined.

There many different ways that TRP channels have been shown to be regulated including alterations in phosphorylation (Joseph, 2019), upregulation of mRNA (Ikeda-Miyagawa, 2015, and interactions with other membrane proteins (Patil, 2019). Here we showed that in a chronic pain state there is *de novo* expression of GFRα3. We did not investigate the co-expression of TRP channels in this population of neurons with *de novo* expression of GFRα3. If GFRα3 signaling and pain potentiation requires TRP channels, and from our data TRPA1 least seems to downstream of GFRα3 signaling, then we would anticipate that the neurons the expanded
population of GFRα3-positive neurons would co-express other nociceptive channels and receptors.

The specifics of which TRP channels are co-expressed with GFRα3 in a chronic pain state should be determine to inform functional experiments for how GFRα3 can modulate TRP channels, whether it’s by upregulation, phosphorylation, interactions, or some combination of these mechanisms.

*Signaling through RET*

Once activated by artemin, GFRα3 then activates RET. Whether RET would be an effective analgesic target as well has yet to be determined. Artemin can signal through neural cell adhesion molecule (NCAM) as well, though to a lesser extent, and this could be responsible for artemin-induced cold hypersensitivity (Lippodlt, 2016). The co-expression of both RET and NCAM should be investigated in DRGs from animals with chronic pain. Calcium imaging experiment could be use for initial investigations of which signaling pathway, GFRα3 or NCAM, is activated for each pain modality by incubating primary culture DRG neurons with RET or NCAM inhibitors are measuring the responses to capsaicin, AITC, and menthol.

RET has been shown to be important for hypersensitivity in irritable bowel syndrome (Russell, 2019) and neuropathic pain (Sidorova, 2017). It has yet to be investigated in OA-associated pain. RET is a signaling complex that can interact with many other proteins and signaling molecules, which may include TRP channels or other nociceptive molecules. RET can also be regulated by other molecules as well that indicate it could contribute to OA-associated pain. The NGF receptor, TrkA can contribute to RET phosphorylation and lipid raft localization, which are important for downstream signaling (Tsui-Pierchala, 2002). RET could be a part of regulatory overlap between NGF and artemin.
Co-signaling with NGF

When NSAIDs are ineffective at controlling OA-associated pain it is presumably due to the majority of the pain being either neuropathic or centrally generated, rather than inflammatory. It is for this population of patients that aren’t responding to the typical analgesics that the anti-NGF biologic tanezumab is set to be approved (Pfizer and Lilly announce tanezumab BLA accepted). The primary factor keeping anti-NGF from being widely available is that in a very small subset of patients it can cause rapidly progressive joint deterioration, meaning those patients will be more likely to require joint replacement surgery.

Artemin/GFRα3 signaling shares similarities to NGF/TrkA signaling. Based on the data presented here, we anticipate that anti-artemin would fill a similar need as anti-NGF, though perhaps with a better safety profile and wider efficacy range.

Potential of ‘dual therapy’ with other neurotrophin inhibition – anti-NGF

A major challenge in treating chronic pain is that targeting one system is often not enough – there are biological redundancies in place to ensure a pain signal endures. Chronic pain, such as with OA, occurs in part due to changes in primary afferent (sensory neuron) sensitivity and receptor expression, such as with transient-receptor potential (TRP) channels. Malin et al (2006) have shown that NGF and artemin, when injected together, have a synergistic effect – increasing thermal hypersensitivity to a greater extent than either could individually. This suggests there may be cross-talk by which NGF and ARTN can enhance each other’s activity on a cellular level. A possible mechanism for this synergistic effect is through RET. Artemin binds to its receptor GFRα3 and that complex then acts on the tyrosine kinase RET (Wang, 2006). NGF phosphorylates RET through TrkA and the MAPK pathway. Glial factor ligands (GFLs) (which includes artemin) have faster kinetics for RET phosphorylation, but a
shorter duration. Of interest as a potential mechanism for NGF and artemin having a synergistic effect, is that when NGF phosphorylates RET, RET does not move from lipid-disordered membrane regions into highly lipid-ordered lipid rafts, but GFL stimulation does translocate RET into lipid rafts, which is important for downstream signaling (Tsui-Pierchala, 2002). A combination of NGF and artemin could phosphorylate RET and translocate it creating a synergistic effect.

The downstream effects of RET are not fully known, but upregulation of artemin or NGF also leads to increased expression of TRPV1 and TRPA1 (Elitt, 2006; Ji, 2002; Eskander, 2015; Zhang, 2005; Meents, 2017). Due to the apparent overlap in pathways between GFRα3 and TrkA it is highly likely that they are biological redundancies of each other for initiating a chronic pain state; the sequestration of only one can’t provide complete pain relief. Thus, it is hypothesized that crosstalk between NGF and ARTN, possibly through RET, is a biological redundancy with synergistic effects in chronic pain signaling. Thereby, targeting both parts of the pathway through a dual therapeutic of anti-ARTN and anti-NGF or a RET inhibitor will be more effective for alleviating hypersensitivity.

**Conclusion**

Here we here laid the foundation for the role of GFRα3/artemin in OA-associated pain by demonstrating that this system is upregulated in naturally occurring disease, can directly cause hypersensitivity, and can be blocked to alleviate hypersensitivity in the MIA model of OA. Further work needs to be done to fully understand how GFRα3/artemin potentiates chronic pain and how they interact with other nociceptive proteins and signaling molecules.
REFERENCES


