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TARGETING METHYLGLYOXAL AND PPAR GAMMA TO ALLEVIATE NEUROPATHIC PAIN ASSOCIATED WITH TYPE 2 DIABETES

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TARGETING METHYLGLYOXAL AND PPAR GAMMA TO ALLEVIATE NEUROPATHIC PAIN ASSOCIATED WITH TYPE 2 DIABETES

DISSertation

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By
Ryan Benjamin Griggs
Lexington, Kentucky

Director: Dr. Bradley K. Taylor, Professor of Physiology
Lexington, Kentucky
2015

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TARGETING METHYLGLYOXAL AND PPAR GAMMA TO ALLEVIATE NEUROPATHIC PAIN ASSOCIATED WITH TYPE 2 DIABETES

Neuropathic pain affects up to 50% of the 29 million diabetic patients in the United States. Neuropathic pain in diabetes manifests as a disease of the peripheral and central nervous systems. The prevalence of type 2 diabetes is far greater than type 1 (90%), yet the overwhelming focus on type 1 models this has left the mechanisms of pain in type 2 diabetes largely unknown. Therefore I aimed to improve the current mechanistic understanding of pain associated with type 2 diabetes using two preclinical rodent models: Zucker Diabetic Fatty rats and db/db mice. In addition, I highlight the translational importance of simultaneous measurement of evoked/sensory and non-evoked/affective pain-related behaviors in preclinical models. This work is the first to show a measure of motivational-affective pain in a model of type 2 diabetes.

I used methodological approaches including: (1) immunohistochemical and calcium imaging to assess stimulus-evoked sensitization; (2) measurement nociceptive behaviors and evoked sensory thresholds as well as pain affect using novel mechanical conflict avoidance and conditioned place preference/aversion assays; (3) pharmacological and genetic manipulation of methylglyoxal, TRPA1, AC1, and PPARγ.

I hypothesized that the thiazolidinedione class of peroxisome proliferator-activated receptor gamma (PPARγ) agonists would reduce neuropathic pain-like behavior and spinal neuron sensitization in traumatic nerve injury and type 2 diabetes. As PPARγ is a nuclear receptor, and already targeted clinically to promote cellular insulin sensitization to reduce hyperglycemia, sustained changes in gene expression are widely believed to be the mechanism of pain reduction. In two separate research aims, I challenged this view and tested whether the PPARγ agonist pioglitazone would (1) rapidly alleviate neuropathic pain through a non-genomic mechanism and (2) reduce painful sensitization in nociceptive and neuropathic pain models independent from lowering blood glucose.

I aimed to investigate the contribution of the glucose metabolite methylglyoxal to painful type 2 diabetes. I tested the hypothesis that methylglyoxal produces nociceptive, evoked, and affective pain that is dependent on activation of the sensory neuron cation channel TRPA1 and the secondary messenger enzyme AC1. I also tested whether pioglitazone or the novel methylglyoxal scavenging peptide GERP10 could alleviate painful type 2 diabetes.
KEYWORDS: painful type 2 diabetes, pioglitazone, chronic neuropathic pain, methylglyoxal, TRPA1, PPAR gamma
TARGETING METHYLGLYOXAL AND PPAR GAMMA TO ALLEVIATE NEUROPATHIC PAIN ASSOCIATED WITH TYPE 2 DIABETES

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December 9, 2015
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Date
I dedicate this dissertation and the completion of my PhD to my father, Harry Milton Griggs, who attended the University of Kentucky in the 1960s as a PhD student in economics. To his lifelong regret, he remained ABD until his untimely death in 2012. Dad is missed and remembered daily, and he continually inspires me to be a better person. I also dedicate this dissertation to my beautiful and loving wife, Dr. Tracy Renee Butler, who completed her PhD in Psychology from UK in 2011.
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# TABLE OF CONTENTS

ACKNOWLEDGEMENTS ........................................................................................................ iii

TABLE OF CONTENTS ........................................................................................................ v

LIST OF FIGURES .............................................................................................................. x

CHAPTER 1: Background .................................................................................................. 1

1.1 Hypothesis and specific aims 1

1.2 Overview of Pain Physiology and Psychology 2

1.2.1 Terminology, or notes on the words and terms used to describe pain 2

1.2.2 The dimensions of pain 3

1.3 Painful diabetic neuropathy (aka painful diabetes) 5

1.3.1 The burden of chronic diabetic pain 5

1.3.2 Current treatments available for painful diabetes 8

1.4 Neuropathic pain sensitization 9

1.5 Specific mediators involved in the regulation of pain 11

1.5.1 Peroxisome proliferator-activated receptor gamma (PPARγ) 11

1.5.2 Transient receptor potential cation channel A1 (TRPA1) 13

1.5.3 Adenylyl cyclase, isoform 1 (AC1) 15

1.5.4 Methylglyoxal and glyoxalase 1 16

1.6 Modeling pain conditions in rodents 20

1.6.1 Nociceptive 21

1.6.1.1 Capsaicin 21

1.6.1.2 Methylglyoxal (acute administration) 23

1.6.2 Neuropathic 23

1.6.2.1 Traumatic (Spared) Nerve Injury 24

1.6.2.2 Diabetic neuropathic pain 24

1.6.2.2.1 Methylglyoxal (chronic administration) 25

1.7 Measuring preclinical "pain" in rodents 26

1.7.1 Stimulus-evoked measures 26

1.7.2 Stimulus-independent measures 28

1.7.2.1 Chemical-induced nociception 28

1.7.2.2 Mechanical conflict avoidance 28

1.7.2.3 Conditioned Place Preference/Avoidance 29

1.8 Preface 31

CHAPTER 2: Gabapentin alleviates affective pain after traumatic nerve injury ..........33

2.1 Abstract 33

2.2 Introduction 34

2.3 Methods 35

2.3.1 Animals 35

2.3.2 Spared Nerve Injury (SNI) surgery 35
2.3.3 Measurement of pain-like behavior and open field activity 35
2.3.4 Conditioned Place Preference 36
2.3.5 Experimental Design 39
2.3.6 Drugs 39
2.3.7 Statistical Analysis 39

2.4 Results 40
2.4.1 SNI produces evoked mechanical hypersensitivity 40
2.4.2 Gabapentin reverses SNI-induced evoked mechanical hypersensitivity 40
2.4.3 Gabapentin produces CPP in rats with SNI but not sham surgery 41
2.4.4 Gabapentin did not change locomotor activity in sham or SNI rats 43

2.5 Discussion 44

2.6 Supplemental Discussion 45
2.6.1 Gabapentin reduction of neuropathic pain in humans and rodents 45
2.6.2 Methodological considerations in CPP 47
2.6.2.1 Production of CPP to systemic gabapentin after injury 47
2.6.2.2 Constant experimental conditions are critical to yield consistent CPP results 48
2.6.2.3 Minimization of time spent outside the pairing chambers 49
2.6.2.4 Expression of CPP after systemic drug administration 50
2.6.3 Conclusions when experiments fail to produce CPP 51

CHAPTER 3: Pioglitazone rapidly reduces neuropathic pain through astrocyte and non-genomic PPARγ mechanisms ................................................................. 53

3.1 Abstract 53
3.2 Introduction 54
3.3 Materials & Methods 56
3.3.1 Animals 56
3.3.2 Spared Nerve Injury (SNI) surgery 56
3.3.3 Pain-like behavior. 57
3.3.4 Motor coordination. 58
3.3.5 Intracerebroventricular cannulation. 59
3.3.6 Drugs 59
3.3.7 Drug Injections 60
3.3.8 Capsaicin-induced nociception. 60
3.3.9 Immunohistochemical quantification of Fos and GFAP in the dorsal horn. 61
3.3.10 Western blotting of lumbar spinal GFAP. 62
3.3.11 Statistical analysis. 63

3.4 Results 63
3.4.1 Systemic pioglitazone reduces mechanical and cold hypersensitivity 63
3.4.2 PPARγ in the spinal cord mediates the anti-hyperalgesic actions of systemic pioglitazone 64
3.4.3 PPARγ in the spinal cord mediates the anti-hyperalgesic actions of intrathecal pioglitazone 67
3.4.4 PPARγ activation does not produce motor deficits or analgesia 68
3.4.5 PPARγ agonists rapidly reduce nociceptive and neuropathic pain-like behavior 71
3.4.6 In vivo blockade of protein translation in the dorsal horn by intrathecal anisomycin 73
3.4.7 Anisomycin does not alter the anti-nociceptive effects of intrathecal pioglitazone 74
3.4.8 Spinal anisomycin does not change the rapid anti-hyperalgesic effects of pioglitazone 76
3.4.9 Acute pioglitazone reduces expression of GFAP after nerve injury 78

3.5 Discussion 81
3.5.1 Anisomycin to assess translation-independent activity of nuclear receptors in the spinal cord 81
3.5.2 Non-genomic PPARγ activity mediates the early anti-hyperalgesic effect of pioglitazone 81
3.5.3 Genomic PPARγ activity mediates the late anti-hyperalgesic effects of pioglitazone 82
3.5.4 The anti-hyperalgesic effect of pioglitazone is mediated by spinal PPARγ 83
3.5.5 Pioglitazone acutely inhibits astrocyte activation 84
3.5.6 Conclusions 86

CHAPTER 4: Pioglitazone inhibits the development of hyperalgesia and sensitization of spinal nocireponsive neurons in type 2 diabetes .................................................87

4.1 Abstract 87
4.2 Introduction 88
4.3 Materials and Methods 90
4.3.1 Subjects 90
4.3.2 Measurement of Blood Glucose & HbA1c 91
4.3.3 Pain-Like Behavior: Stimulus-Evoked 92
4.3.4 Pain-Like Behavior: Affective-Motivational 93
4.3.5 pERK Quantification via Immunohistochemistry 94
4.3.6 Quantification of Methylglyoxal-Derived Advanced Glycation End-Products (MG-AGEs) 96
4.3.7 Insulin Quantification via ELISA 96
4.3.8 Pioglitazone Incorporation into Food 96
4.3.9 Experimental Design: Characterization of PDN 97
4.3.10 Experimental Design: Pioglitazone Administration 97
4.3.11 Statistical Analysis 98

4.4 Results 99
4.4.1 The ZDF Rat is a Model of Progressive Painful Diabetic Neuropathy 99
4.4.2 Chronic Administration of Oral Pioglitazone Reduces Pathological Signs of Diabetes 106
4.4.3 Chronic Oral Pioglitazone Inhibits the Development of PDN and Reduces Evoked pERK 107

4.5 Discussion 109
4.5.1 ZDFs Develop Multiple Types of Pain-Like Behavior 109
4.5.2 ZDFs Develop Central Sensitization in Spinal Dorsal Horn Neurons 111
4.5.3 Pioglitazone Reduces Pathological Signs of PDN in ZDF by Actions at PPARγ 112
4.5.4 Pioglitazone Reduces PDN Independent of its Anti-Hyperglycemic Action

4.5.5 Conclusions and Future Directions

4.6 Supplemental Data & Discussion

4.6.1 Pioglitazone produces weight gain and hyperphagia.

4.6.2 Exploratory locomotor behavior in ZDF decreases as PDN develops

4.6.3 Mechanisms of PDN

CHAPTER 5: Methylglyoxal produces pain in type 2 diabetes via sensitization of TRPA1 and AC1

5.1 Abstract

5.2 Introduction

5.3 Materials and Methods

5.3.1 Subjects

5.3.2 Drugs & Injections

5.3.3 Pain-like Behavior

5.3.4 pERK quantification via immunohistochemistry

5.3.5 Calcium imaging

5.3.6 Quantification of Methylglyoxal-derived advanced glycation end-products (MG-AGEs)

5.3.7 Measurement of blood glucose & HbA1c

5.3.8 Data analysis and statistics

5.4 Results

5.4.1 Methylglyoxal produces pain-like behavior

5.4.2 Methylglyoxal activates spinal neurons to produce PDN

5.4.3 TRPA1 mediates spinal sensitization and pain in type 2 diabetes

5.4.4 AC1 facilitates methylglyoxal-induced hypersensitivity

5.4.5 Methylglyoxal scavenging reduces pain-like behavior in type 2 diabetes

5.5 Discussion

5.5.1 Methylglyoxal contributes to painful type 2 diabetes

5.5.2 Methylglyoxal requires TRPA1 to produce painful diabetes

5.5.3 MG induces cross-modality sensitization to heat via a TRPA1-AC1 pathway

5.5.4 Conclusions toward treatments for painful diabetes

CHAPTER 6: Overall Discussion and Supplemental Data

6.1 Summary

6.2 How does type 2 diabetes lead to painful neuropathy?

6.3 Methylglyoxal and glyoxalase 1 in painful type 2 diabetes

6.4 Mechanisms of PPARγ-related analgesia
6.4.1 The rapid effects on pain by PPARγ agonists 168
  6.4.1.1 pERK and Fos 172
  6.4.1.2 Cytokines and chemokines 173
  6.4.1.3 mitoNEET 174
  6.4.1.4 GPR40 174
  6.4.1.5 TRP channels 176
6.4.2 How does pioglitazone reduce painful type 2 diabetes? 176
6.5 Conclusions 180
APPENDIX ........................................................................................................ 183
REFERENCES ..................................................................................................... 186
VITA .................................................................................................................. 247
LIST OF FIGURES

Figure 1-1. Publications and prevalence of diabetes...................................................... 7
Figure 1-2. Biochemical pathways of methylglyoxal production and degradation........19
Figure 1-3. Methods to measure MG-related entities....................................................20
Figure 1-4. Intraplantar capsaicin evokes pERK in the spinal cord dorsal horn...........22
Figure 2-1. Experimental timeline and diagram of the conditioning place preference (CPP) apparatus...........................................................................................................38
Figure 2-2. Gabapentin reverses evoked mechanical hypersensitivity associated with nerve injury.................................................................................................................41
Figure 2-3. Gabapentin attenuates affective pain associated with traumatic nerve injury. ......................................................................................................................................42
Figure 2-4. Gabapentin does not alter locomotor activity..............................................43
Figure 2-5. Morphine produces conditioned place preference in rats.........................48
Figure 2-6. Baseline preferences with the addition of a chapstick olfactory cue............49
Figure 3-1. A single systemic injection of pioglitazone (Pio) dose-dependently reduced behavioral signs of neuropathic pain. .................................................................65
Figure 3-2. Anti-hyperalgesic actions of systemic pioglitazone are mediated by spinal PPARγ........................................................................................................................................67
Figure 3-3. Spinal PPARγ mediates pioglitazone anti-hyperalgesia...............................68
Figure 3-4. Pioglitazone did not produce ataxia or changes in transient nociception.....70
Figure 3-5. PPARγ agonists rapidly reduce mechanical hyperalgesia.........................72
Figure 3-6. Anisomycin reduces translation of capsaicin-evoked Fos without blocking the rapid anti-nociceptive actions of pioglitazone.........................................................75
Figure 3-7. Early but not late pioglitazone anti-hyperalgesia is independent of translation. ......................................................................................................................................77
Figure 3-8. Acute PPARγ activation reduces GFAP expression.................................79
Figure 3-9. SNI is required for pioglitazone reduction of pain and astrocyte activation...

Figure 4-1. ZDF rats develop a type 2 diabetes phenotype.................................

Figure 4-2. ZDF rats develop hyperalgesia............................................................

Figure 4-3. Diabetes increases the avoidance of mechanical probes. .................

Figure 4-4. Diabetes exacerbates noxious pressure-induced activation of dorsal horn neurons.................................................................

Figure 4-5. Pioglitazone reduces pathological signs of type 2 diabetes. .............

Figure 4-6. Pioglitazone attenuates pain-like behavior........................................

Figure 4-7. Pioglitazone attenuates dorsal horn neuron activation......................

Figure 4-8. Pioglitazone increases food consumption and weight in both ZL and ZDF.

Figure 4-9. Pioglitazone effect on weight gain in Sprague-Dawley rats after traumatic nerve injury..............................................................................................

Figure 4-10. Locomotor activity decreases with age in ZDF...............................

Figure 4-11. Immunohistochemical quantification of microglial and astrocyte markers.

Figure 5-1. Exogenous methylglyoxal produces spontaneous, evoked, and affective pain-like behavior.................................................................

Figure 5-2. Methylglyoxal activates nociceptive neurons in the spinal cord dorsal horn to produce pain-like behavior.................................................................

Figure 5-3. TRPA1 mediates methylglyoxal-induced spinal nociceptive neuron activation and hyperalgesia in painful diabetic neuropathy.................................

Figure 5-4. AC1 mediates methylglyoxal-induced hyperalgesia in painful diabetic neuropathy.................................................................

Figure 5-5. The methylglyoxal scavenger GERP10 reduces painful diabetic neuropathy.................................................................

Figure 6-1. Preliminary preconditioning results in db/db mice using CPP..............

Figure 6-2. TRPA1-dependent calcium response in DRG neurons from ZL and ZDF.
Figure 6-3. Nociceptive behavior after MG administration in type 2 diabetic mice........160
Figure 6-4. Diabetic measures after chronic administration of methylglyoxal........164
Figure 6-5. Pain-like sensitivity is unchanged by MG minipump administration......166
Figure 6-6. Chronic pioglitazone administration reduces light touch-evoked pERK.....170
Figure 6-7. Acute pioglitazone does not reduce evoked spinal pERK or Fos in SNI rats.
....................................................................................................................................171
Figure 6-8. Pioglitazone produces conditioned place preference in sham but not SNI rats. ......................................................................................................................................................172
Figure 6-9. Effect of acute systemic pioglitazone on heat hyperalgesia and blood glucose in type 2 diabetes models.................................................................177
Figure 6-10. Pioglitazone reduces MG-evoked pain-like behavior.........................178
Figure 6-11. Overall schematic of the current results and future directions..............181
CHAPTER 1: Background

1.1 Hypothesis and specific aims

My overall hypothesis is that type 2 diabetic neuropathic pain is alleviated by either administration of thiazolidinedione PPARγ agonists or inhibition of the methylglyoxal-TRPA1-AC1 pathway. To test this hypothesis I designed and performed many experiments to investigate the following specific aims:

1) Determine whether the known analgesic gabapentin alleviates motivational-affective pain in the spared nerve injury model of neuropathic pain. The purpose of this aim was to validate the use of conditioned place preference testing to assess a preclinical measure of affective pain.

2) Test whether the rapid antihyperalgesic action of TZDs is dependent on a non-genomic mechanism.

3) Determine whether astrocyte mechanism are involved in rapid pioglitazone antihyperalgesia.

4) Characterize the neuropathic pain phenotype of the Zucker Diabetic Fatty rat model of type 2 diabetes and determine whether spinal nocireponsive neuron sensitization is associated with pain-like behavior in this model.

5) Determine whether pioglitazone alleviates pain and spinal sensitization in type 2 diabetes independent from its reduction of hyperglycemia.

6) Test whether methylglyoxal is sufficient to produce nociceptive, evoked, and affective pain-like behavior and determine whether the methylglyoxal scavenging peptide GERP10 alleviates type 2 diabetic hyperalgesia.

7) Determine the contribution of TRPA1 and AC1 to methylglyoxal-mediated pain in type 2 diabetes.
1.2 Overview of Pain Physiology and Psychology

The phenomenon of pain is a complex physiological and psychological experience defined by the International Association for the Study of Pain (IASP) as “An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage.” Importantly, pain can exist in the absence of any noticeable injury and as such, current medical practice treats a patient’s pain, even when there is no obvious injury. This fact alone makes the treatment of pain in humans very difficult, because in many cases physiological symptoms of pain may not exist or are not detectable and caregivers must rely on subjective reports only. In this line of thinking, the ability of preclinical researchers to utilize animal (non-human) models to develop treatments (i.e. drugs, or pharmacological agents that act on one or multiple biological targets to effect physiological change) for psychological pain is virtually impossible because animals lack the ability to verbally self-report. However, I argue that there are very similar ways for both patients and preclinical rodent models (it should be recognized that there are other animal models used in pain research such as dogs, rabbits, and non-human primates) to self-report the presence or absence of the sensory-discriminative and affective-motivational dimensions of pain (see 1.7.2 and Chapter 2: ).

1.2.1 Terminology, or notes on the words and terms used to describe pain

It is difficult to rationalize using the term pain to apply to an unpleasant experience in rodent models used in pain research, as only an inference can be made whether or not the animal is experiencing unpleasantness. I should note that many times I ascribe pain to conclusions that I make from this research in discussion; but, the use of the term pain in this case is simply an inference. To avoid confusion, and to help delineate discussion of human patients and rodent models, several terms defined by IASP exist to define the
phenomena we can measure and study in rodents. These terms can be found in the Appendix.

Several terms defined by IASP are inherently flawed if used to describe rodent research because they include the word “pain”. However, if “pain” is replaced with “response” then terms such as allodynia, hyperalgesia, and neuropathic pain can be reasonably applied to describe a pain-like experience in rodents. We have used the words allodynia, hyperalgesia, and hypersensitivity almost interchangeably, but it is important to note that each term describes a slightly different aspect of somatosensation. Here, allodynia refers to when an animal responds to a stimulus (e.g. von Frey filaments or acetone) that does not produce a response prior to injury in that animal or in control animals. Hyperalgesia is used to describe either a decrease in the response threshold or an increase in the magnitude of a response to a given stimulus. Hypersensitivity is a term that encompasses both allodynia and hyperalgesia while escaping the inherent flaws in the definitions ascribing pain to preclinical models.

1.2.2 The dimensions of pain

The emotional and unpleasant experience of pain begins with the sensory-discriminative, or the ability to sense a potentially tissue damaging stimulus and to discriminate its location on the body. The somatosensory system has the ability to distinguish both the quality (e.g. chemical, thermal, mechanical) and quantity (e.g. non-noxious versus noxious) of the stimulus. Several types of primary afferents containing nerve endings innervate the skin, viscera, internal organs, and blood vessels to function in a sensory-discriminative capacity. These include C, Aδ, and Aβ fibers that are distinguished by their neurophysiological properties including conduction velocity, degree of myelination, size (cross sectional area), length, action potential threshold, and expression of receptors. Reception is the process of detecting an exogenous (such as
mechanical touch or thermal changes) or endogenous (such as cytokines, chemokines, or lipid metabolites) stimulus. This typically occurs via protein receptors expressed on the plasma membrane of afferents with free nerve endings or by larger, more complicated structures such as Merkel discs, Meissner’s, or Pacinian corpuscles. Transduction is the process of converting that extracellular stimulus to an intracellular message, which typically occurs by depolarization of the neuron. A stimulus that is great enough to overcome the activation threshold of the neuron results in the initiation of an action potential. Transmission is the process by which the neuron propagates the action potential along its axon toward the first synapse at the junction between the primary afferent and the second order neuron in the central nervous system. Second order neurons for the limbs in humans and rodents are located in the spinal cord, which is the focus of most of the work herein, because typical behavioral tests in rodents involve measuring the sensitivity of hindpaws to mechanical, chemical, and thermal stimuli. Integration is the process by which CNS neurons compile signals from one or many primary afferents and relay this information to third and fourth order neurons that transmit signals through spinal and brainstem pathways to the thalamic integration center and cortical areas involved in the motivational-affective and cognitive-evaluative dimensions of pain. It should be noted that the stimulus-evoked signals can be modulated at several locations of the transmission process by inhibitory neurons, other excitatory neurons, neurons integrating other simultaneous stimuli, and descending modulation that is regulated by specific areas of the brain. These will not be investigated in the current work.

Motivational-affective describes the unpleasantness (affect) and the inherent drive to avoid pain (motivation). Motivational-affective pain can be independent from a sensory experience. This is evident by the fact that even the threat of pain can be perceived as painful and produce negative affect and avoidance. Conversely, the negative
reinforcement of ongoing pain is rewarding and therefore subjects will seek this pleasant experience. This extension of the pain experience beyond its sensory component increases the complexity of studying pain, as objective research is attempted to define a subjective experience. We and others report that some therapies may benefit the sensory aspect of pain (i.e. evoked hypersensitivity) without reducing the subjective affective component in preclinical models. This is highlighted by the small number of effective therapies developed by preclinical research, beyond the repurposing (e.g. gabapentin) or refinement (e.g. opioids) of “old” drugs. Here we describe and validate conditioned place preference, a preclinical assay used as a tool to assess the motivational-affective dimension of pain in rodents (1.8 ) and apply this tool to assessing affective pain in diabetes (section 5.4.1 ). For review of these dimensions of pain see (Auvray et al, 2010).

1.3 Painful diabetic neuropathy (aka painful diabetes)

1.3.1 The burden of chronic diabetic pain

According to the World Health Organization, almost 350 million people or ~5% of the population worldwide have diabetes. In the U.S. the prevalence is a bit greater at 9% (Center for Disease Control), or 29 million people. The cost of diabetes in the U.S. in 2012 was estimated to be $245 billion, with $176 billion in direct costs and $69 billion in lost productivity. Type 2 diabetes accounts for the majority of diabetes cases at 90%. This is shocking given the fact that the gold standard for type 2 diabetes prevention and treatment is diet and exercise.

Approximately 50% of diabetics will experience peripheral neuropathy at some point during the disease natural history (Vincent et al, 2011). Peripheral neuropathy is likely due to microvascular complications associated with the metabolic deficits and inflammation that result in insulin insensitivity, hyperglycemia, decreased motor and
sensory nerve conduction velocities, macrophage infiltration, loss of intraepidermal nerve fibers, and altered somatosensation, including painful hyperalgesia/alldynia as well as painless or insensate neuropathy. Here we focus on the neuropathic pain that occurs in approximately one-third of diabetic patients (Davies et al, 2006; Abbott et al, 2011; Lee-Kubli et al, 2014), a condition commonly referred to as painful diabetic neuropathy (PDN) (Vincent et al, 2011). As the current work focuses only on the pain-related behaviors associated with type 2 diabetes and not necessarily the overt dysfunction of sensory and motor neurons that is neuropathy, we suggest that the term painful diabetes is a suitable substitute for painful diabetic neuropathy. The pathogenesis of pain in rodent models of diabetic neuropathy is reviewed by several groups (Kapur, 2003; Tirabassi et al, 2004; Calcutt and Backonja, 2007; Obrosova, 2009).

The majority of preclinical painful diabetes studies focus on the streptozotocin (STZ) model of type 1 diabetes (Calcutt et al, 2009). However, as we further discuss below, STZ is an inappropriate model to study PDN because it produces a natural history of diabetes that does not model the human condition (Fox et al, 1999). Instead, we use two genetic models of type 2 diabetes: Zucker Diabetic Fatty rats (ZDF) and db/db mice (db/db). Figure 1-1A illustrates the dearth of pain publications in these two models of type 2 diabetes, compared to the explosion of STZ pain publications within the past couple decades. Figure 1-1B further illustrates the disparity between the very few numbers of preclinical studies investigating painful type 2 diabetes compared to exponential increases in publications on type 2 diabetes pain in patients. Figure 1-1C shows that the prevalence of diabetes in the United States has quadrupled in the past 15 years. Clearly more work is needed to benefit the growing problem of type 2 diabetes and associated pain.
I performed a Pubmed search on September 22, 2015 and quantified the number of publications per year from 1973-2015. The number of publications are shown using the following search terms. (A) Preclinical models of type 1 diabetes pain (“streptozotocin pain”) and type 2 diabetes pain (combined publications from “zucker diabetic fatty rat pain” and “db/db pain” searches). (B) Clinical condition descriptors of “type 1 diabetes pain” and “type 2 diabetes pain”. (C) The number of diagnosed diabetes (type 1 and type 2 combined) cases per year from 1980-2012.
1.3.2 Current treatments available for painful diabetes

Analgesic treatments are only effective in a small subset of PDN patients (Smith and Argoff, 2011). Maintaining blood glucose in the normal range is the first line of defense against PDN (Veves et al., 2008), and this can be accomplished with a lifestyle change to incorporate diet and exercise. However, in late-stage type 2 diabetes obesity and pain may prevent adequate management by diet and exercise. Therefore adjuvant pharmacotherapies may be needed to manage painful diabetes. Only duloxetine, a selective serotonin and norepinephrine reuptake inhibitor, and pregabalin, a gabapentinoid anxiolytic, are approved by the Food & Drug Administration for diabetic pain treatment (Smith and Argoff, 2011), but are only moderately effective in a subset of diabetic patients. PDN treatment is complicated by the fact that diabetes decreases the efficacy of opioid analgesics. The function and antinociceptive effects of opioids are diminished in both db/db mice (Kamei et al., 1997; Takeshita and Yamaguchi, 1998) and ZDF rats (Otto et al., 2011) as well as in STZ (Shaqura et al., 2013).

Ongoing clinical trials are testing the efficacy and safety of new pharmacotherapeutics but treatment options for PDN are still lacking (Javed et al., 2015). A non-exhaustive list of potential therapies for PDN include: the lipophilin ligand FK1706 (Yamazaki et al., 2012), taurine (Li et al., 2006), aldose reductase inhibitors (Schemmel et al., 2010), Poly-ADP-ribose inhibition (Drel et al., 2010), and TRPA1 antagonists (Wei et al., 2009; Wei et al., 2010; Barrière et al., 2012; Eberhardt et al., 2012; Koivisto et al., 2012; Andersson et al., 2013). One potential problem with the future success of these therapies for the treatment of painful type 2 diabetes is that they were developed using the preclinical STZ model. Here we use mouse and rat models to investigate both the mechanisms of and potential treatments for painful type 2 diabetes to combat the growing problem of diabetic neuropathic pain.
1.4 Neuropathic pain sensitization

Sensitization is defined as the increased responsiveness and/or decreased threshold of activation of peripheral or central neurons. Peripheral and central sensitization in rodents and humans is a major contributor to the transition from normal, protective, evolutionarily advantageous pain to pathological pain that hinders function and quality of life. It is the prevailing belief that peripheral sensitization mechanisms contribute to central sensitization (Baron et al., 2013) and mediate the shift between acute pain, which typically has a distinguishable stimulus (e.g. tissue or nerve injury, exogenous stimulus), and chronic pain, which may or may not have a recognizable cause. Our goal is not to re-review or redefine the mechanisms on central sensitization, but only to provide a brief overview as rationale for investigating the spinal mechanisms of painful diabetes and PPARγ-directed therapies for neuropathic pain.

Central sensitization is characterized by both functional studies and changes in the expression of sensitization markers. One prevailing theory is that peripheral injury leads to central sensitization in the form of long term potentiation (LTP) in the spinal cord dorsal horn (Sandkuhler and Gruber-Schoffnegger, 2012). A recent study showed that this plasticity that contributes to chronic pain could be reduced by reconsolidating the nociceptive event, effectively erasing the pain memory (Bonin and De Koninck, 2014). In addition, neuropathic pain models result in increased responses to peripheral input in dorsal horn neurons (Ji et al., 2003; Doolen et al., 2012), increases in the expression of astrocyte (Svensson and Brodin, 2010) and microglial (Beggs and Salter, 2007; Zhuo et al., 2011) markers, and exaggerated phosphorylation of extracellular signal-regulated kinase (pERK) and Fos (Gao and Ji, 2009), which acts as a marker for neuronal activation. Indeed, the above mechanisms are shown to contribute to spinal sensitization in models of type 2 diabetes (Cheng et al., 2010; Liao et al., 2011; Dauch et al., 2012; Xu
et al, 2014; Schuelert et al, 2015). For further review of painful central sensitization generalized to many types of pain see (Ji et al, 2003; Woolf, 2011; Baron et al, 2013).

Stimulus evoked phosphorylation of extracellular signal-regulated kinase (pERK) is a marker of central sensitization (Ji et al, 1999). Activation of peripheral nociceptors increases pERK in the spinal cord dorsal horn (Ji et al, 1999). Light-touch stimulation of the hindpaw in a traumatic nerve injury model of neuropathic pain leads to increased pERK in the dorsal horn of injured subjects (Zhuang et al, 2005; Morgenweck et al, 2013). Noxious hindpaw stimulation also induces pERK in spinal neurons (Ji et al, 2002). In injured animals, ERK is activated (i.e. phosphorylated) within ten to thirty minutes, indicating a relatively rapid increase in the responsivity of spinal neurons in response to peripheral stimulation, making pERK is a suitable marker for central sensitization (Gao and Ji, 2009). Further supporting the role of spinal ERK activation in neuropathic pain, intrathecal delivery of pERK inhibitors such as U0126 reduces hypersensitivity in diabetic pain models (Daulhac et al, 2006; Tsuda et al, 2008; Daulhac et al, 2011; Xu et al, 2014).

The mechanisms of central sensitization in painful type 2 diabetes are not well known, as only a few preclinical studies have investigated central sensitization in painful type 2 diabetes. Type 2 diabetic subjects exhibit increased expression of markers of central sensitization, including pERK (Daulhac et al, 2011; Dauch et al, 2012; Xu et al, 2014) and the astrocyte marker GFAP (Liao et al, 2011; Ren et al, 2012). To our knowledge, Schuelert et al were the first to show functional electrophysiological evidence of central sensitization in type 2 diabetes, indicating that spinal neuron responses to peripheral non-noxious mechanical stimulation were increased (Schuelert et al, 2015). Here we evaluate stimulus-dependent pERK in type 2 diabetic rats and mice to investigate the role of spinal nocireponsive neurons in type 2 diabetes. We also
test the hypothesis that sensitization of these neurons contributes to the pathogenesis of PDN.

1.5 Specific mediators involved in the regulation of pain

1.5.1 Peroxisome proliferator-activated receptor gamma (PPARγ)

Peroxisome proliferator-activated receptor gamma (PPARγ) is a nuclear receptor that regulates the gene expression related to insulin signaling, glucose and fatty acid metabolism, adipogenesis, neurological disease, and pain (Feinstein, 2003; Evans et al, 2004; Kota et al, 2005; van Neerven and Mey, 2007; Maeda and Kishioka, 2009; Ahmadian et al, 2013; Yonutas and Sullivan, 2013). The endogenous ligands for PPARγ include 15-Deoxy-Delta-12,14-prostaglandin J2 (15d-PGJ2), poly-unsaturated fatty acids (PUFA), and arachidonic acid and some of its metabolites. The exogenous ligands for PPARγ include thiazolidinedione (TZD), non-TZD molecules, and natural derivatives. TZDs include older molecules such as ciglitazone and troglitazone and newer molecules such as rosiglitazone and pioglitazone, in addition to some TZD-derivatives under development. Non-TZD PPARγ agonists include MDG548 (Lecca et al, 2015), PAM-1616 (Kim et al, 2010), MBX-102 (Gregoire et al, 2009), FK614 (Minoura, 2007), and other partial agonists such as PEA (Costa et al, 2008). Ligands are also derived from natural sources such as the compound monascin (Hsu et al, 2013). Studies using antagonists of PPARγ such as T007, BADGE, and GW9662 (2-chloro-5-nitrobenzanilide) suggest PPARγ-dependent effects of agonists. However, the excellent review from Luconi et al indicate a multitude of ways PPARγ agonists could exert their effects, including both PPARγ-dependent and independent pathways (Luconi et al, 2010). Germline PPARγ knockout is embryonic lethal but several conditional or tissue specific transgenic mutations have been reported in mice (Sarruf et al, 2009; Wu et al,
including a nestin-Cre neuronal knockout (de Guglielmo et al., 2014) that could prove very useful in future studies investigating PPARγ analgesia.

The FDA has approved rosiglitazone (Avandia®) and pioglitazone (Actos®) for the treatment of type 2 diabetes. These TZDs act as insulin sensitizers to lower blood glucose. It should be noted that their safety in humans has come under scrutiny. For example, pioglitazone may contribute to the development of bladder cancer following long-term administration (Lewis et al., 2011). The FDA has released safety precaution warnings for both TZDs with regard to cardiovascular risks; however, both drugs are still used in mono and combination therapy for the treatment of type 2 diabetes.

Over the past thirteen years, a growing body of preclinical evidence indicates that the PPARγ agonists 15d-PGJ2, rosiglitazone, and pioglitazone reduce central (Park et al., 2007), inflammatory (Taylor et al., 2002; Morgenweck et al., 2010; Hasegawa-Moriyama et al., 2013), nociceptive (Oliveira et al., 2007; Napimoga et al., 2008; Pena-Dos-Santos et al., 2009; Griggs et al., 2015), and neuropathic (Churi et al., 2008; Costa et al., 2008; Iwai et al., 2008; Maeda et al., 2008; Fehrenbacher et al., 2009; Jain et al., 2009; Jia et al., 2010; Takahashi et al., 2011; Hasegawa-Moriyama et al., 2012; Jain et al., 2013; Jain et al., 2013; Jia et al., 2013; Morgenweck et al., 2013; Paragomi et al., 2014; Zanardelli et al., 2014; Griggs et al., 2015) pain. PPARγ is expressed in key areas of pain processing including the brain (Cullingford et al., 1998; Cristiano et al., 2001; Zander et al., 2002; Dello Russo et al., 2003; Moreno et al., 2004; Park et al., 2004; Victor et al., 2006; Sarruf et al., 2009), spinal cord (Diab et al., 2002; Moreno et al., 2004; Churi et al., 2008; Maeda et al., 2008; Shibata et al., 2008), and peripheral nerves (Yamagishi et al., 2008). PPARγ agonists reduce many factors associated with behavioral hypersensitivity including: (1) proinflammatory TNFα, IL-1β, and NF-κB in the spinal cord and peripheral nerve (Park et al., 2007; Maeda et al., 2008; Jia et al., 2010; Jain et al., 2013; Jia et al., 2013); (2) M1 macrophage polarization with simultaneous promotion of M2 in peripheral nerve (Park et

1.5.2 Transient receptor potential cation channel A1 (TRPA1)

Subtypes of the transient receptor potential (TRP) superfamily of cation channels include ankyrin (A), vanilloid (V), melastatin (M), and canonical (C). TRP receptors are can be polymodal, responding to chemicals found in food as well as hot, cold, and mechanical stimulation. Here we use capsaicin, a compound found in chili peppers, to activate TRPV1 in order to model nociception in the rodent primary afferent. In addition, we investigate the activation of TRPA1 by the glucose metabolite methylglyoxal. See review on TRP channels in pain for more information on these well-studied receptors (Julius, 2013).

TRPA1 is activated by cold (Story et al, 2003) and mechanical (Kwan et al, 2009) stimuli, as well as chemicals (Bautista et al, 2005; McNamara et al, 2007; Trevisani et al, 2007; Eberhardt et al, 2012; Gregus et al, 2012) such as methylglyoxal (MG). Sensitization of TRPA1 in peripheral neurons is suggested to fine tune mechanical sensitivity (Kwan et al, 2009; Brierley et al, 2011). In mice, TRPA1 is expressed in small, unmyelinated, peptidergic peripheral DRG neurons (C-fibers) and predominantly colocalizes with TRPV1 (Story et al, 2003; Bautista et al, 2005; Kobayashi et al, 2005; Garcia-Anoveros and Duggan, 2007). The proportion of DRG neurons that express TRPA1 varies depending on the species used (rat or mouse) and between publications,
with a range of 20-40% in rat and 3-56% in mouse (Kobayashi et al, 2005). Functional studies indicated that MG produces a calcium response in 72% of capsaicin (prototypical TRPV1 agonist) responsive DRG neurons (Andersson et al, 2013), suggesting that 72% of TRPV1 fibers also express TRPA1. This expression and functional evidence suggests that TRPA1 may play a role in mediating diverse stimulus modalities, and supports our investigation of spontaneous nociceptive as well as both mechanical- and heat-evoked hypersensitivity by the novel agonist methylglyoxal (Eberhardt et al, 2012).

Important to note, there is little evidence to support the expression of TRPA1 on postsynaptic neurons in the dorsal horn, suggesting that presynaptic TRPA1 contributes to the activation of these nocireponsive neurons in the spinal cord.

TRPA1 is implicated in several pain conditions. The TRPA1 antagonist A-967079 reduces nocireponsive behavior after administration of mustard oil (TRPA1 agonist) as well as attenuating cold alldynia in the monoiodoacetate model of osteoarthritis. Inhibition of TRPA1 may reduce pain-related behaviors by blocking CGRP release in the DRG (Chen et al, 2011). Interestingly, A-967079 did not inhibit mechanical hypersensitivity in nerve injury (CCI/SNI) or inflammatory (CFA) pain models (Chen et al, 2011). However, an alternative TRPA1 antagonist (which we have used in the current studies) HC-030031 attenuated nocireponsive behavior after AITC administration (TRPA1 agonist) as well as mechanical hypersensitivity in both nerve injury (SNL) and inflammatory (CFA) pain models (Eid et al, 2008). Neither of these antagonists affected locomotor coordination, body temperature, heart rate, or naïve heat sensitivity (Eid et al, 2008; Chen et al, 2011). A third antagonist, Chembridge-5861528, reduces mechanical hypersensitivity after peripheral administration of TRPA1 agonists, in models of neuropathic pain such as STZ-induced type 1 diabetes or SNI, and in a post-operative pain model (Wei et al, 2009; Wei et al, 2010; Wei et al, 2012; Wei et al, 2013). Antagonist studies further implicate the contribution of spinal TRPA1 to peripheral
hypersensitivity in various pain models (Wei et al, 2011). Here we investigate the role of spinal and/or peripheral TRPA1 in the maintenance of pain in type 2 diabetes.

The structural-functional relationships (Paulsen et al, 2015) of the TRPA1 channel as well as the new development of selective antagonists (Preti et al, 2015) provide valuable information as to the mechanism of TRPA1 activation by endogenous and exogenous agonists. The mode of activation of TRPA1 by putative (functional) agonists may be due to covalent modification of specific, thermodynamically available, reactive protein residues such as cysteines (Macpherson et al, 2007). In various pain models, endogenous TRPA1 agonists are produced (Stucky et al, 2009) and this may mediate ongoing hypersensitivity associated with these models. Indeed, functional characterization of TRPA1 in DRG neurons indicated that an endogenous agonist activates TRPA1 via modification of cysteines (Takahashi et al, 2008). The release of endogenous TRPA1 agonists in a pathological pain condition (i.e. injury) or after peripheral stimulation (such as by mechanical stimulation of the hindpaw (McGaraughty et al, 2010) helps to explain the putative contribution of spinal TRPA1 to pain-related behaviors. In other words, our current results and other studies indicate that spinal administration of a TRPA1 antagonist blocks peripherally evoked hypersensitivity; therefore, either the pain model or the stimulation itself must result in the release of TRPA1 activators at the first synapse in the dorsal horn. This is explored in section 5.4.3.

1.5.3 Adenylyl cyclase, isoform 1 (AC1)

Calcium activated adenylyl cyclase 1 (AC1) (Xia and Storm, 1997) is a key signal transduction protein and modulator of pain plasticity in the central nervous system (Zhuo, 2012). AC1 (and AC8 to some extent) acts to facilitate N-methyl-D-aspartate receptor (NMDAR) mediated nociceptive transmission in the spinal cord dorsal horn, an
effect that is modulated by both 5-HT activity and forskolin-stimulated cAMP production (Wang and Zhuo, 2002). AC1 knockout and systemic administration of the AC1 inhibitor NB001 inhibited the late acute response and chronic hypersensitivity associated with administration of intramuscular formalin, while the immediate nocireponsive effects were unchanged by modulation of AC1. Similarly, knockout of AC1 does not reduce ERK activation in spinal neurons evoked by glutamate, capsaicin, or formalin in naïve animals, but does reduce persistent spinal long term potentiation (LTP) (Wei et al., 2006). These results suggest that AC1 mediates central sensitization of spinal nociceptive neurons but not rapid/acute responses to noxious stimulation in a naïve state. Supporting an AC1 role in central sensitization, a potentiated AC1 system in the spinal cord is revealed by the reinstatement of pain after recovery from an inflammatory injury (Corder et al., 2013). We hypothesized that AC1 mediates persistent hyperalgesia in type 2 diabetes that is dependent upon activation of TRPA1.

1.5.4 Methylglyoxal and glyoxalase 1

Methylglyoxal is a highly reactive cellular metabolite of glycolysis that accumulates in type 2 diabetic hyperglycemia (Bierhaus et al., 2012; Kender et al., 2014; Kong et al., 2014; Allaman et al., 2015; Dornadula et al., 2015). Figure 1-2 illustrates the biosynthetic and biodegradation pathways of methylglyoxal. Methylglyoxal contains both aldehyde and ketone moieties that result in protein modification at lysine/arginine residues and facilitates cysteine-cysteine disulfide bonds (Rabbani and Thornalley, 2012). Methylglyoxal forms other protein adducts such as advanced glycation end-products (AGEs) (Shipanova et al., 1997; Oya et al., 1999; Berner et al., 2012), which result in diabetic complications (Westwood and Thornalley, 1995; Desai and Wu, 2007; Matsui et al., 2010; Sohn et al., 2011). Figure 1-3 illustrates two methods of measuring
methylglyoxal-derived AGEs or free methylglyoxal in biological samples and depicts the structure of methylglyoxal.

Methylglyoxal is metabolized by the glyoxalase (GLO) system involving both GLO1 and GLO2 enzymes and requiring reduced glutathione (GSH) as an oxidizing agent. An excellent review of the history of discoveries related to MG and GLO1 is available (Rabbani and Thornalley, 2014). Potential polymorphisms in GLO1 can affect enzyme function (Peculis et al, 2013), thereby rendering an organism more susceptible to changes in glucose metabolism. This is exemplified in different mouse strains, where BALB/cByJ mice are protected from STZ-induced diabetic neuropathy compared to BALB/cJ mice, in which GLO1 expression is decreased tenfold (Jack et al, 2012). Both aging (Fleming et al, 2013) and diabetes (Jack et al, 2011; 2012; Skapare et al, 2013) are associated with reduced GLO1 activity thereby rendering greater risk for the detrimental effects of MG. Studies overexpressing GLO1 implicate methylglyoxal in: the production of anxiety-related behavior (Distler et al, 2012), reduction of seizures after administration of epileptic seizure agents (Distler et al, 2013), and inducing hyperalgesia in type 1 diabetes (Bierhaus et al, 2012). Conversely, chronic inhibition of GLO1 by Sr-p-Bromobenzylglutathionecyclopentyl diester (BrBz) produces evoked behavioral hypersensitivity (Andersson et al, 2013). This indicates that the endogenous levels of MG, which are inversely correlated with GLO1 activity (Thornalley, 1996), regulate pain sensitivity. We propose that overexpression of GLO1 activity in type 2 diabetes could reduce PDN as a future study (section 6.3.2).

Methylglyoxal is implicated in the pathogenesis of several neurological disease conditions. Methylglyoxal induces the release of glutamate in human neuroblastoma SH-SY5Y cells that is dependent on NMDA-R activation and results in the depolarization of neocortical pyramidal cells producing neurotoxicity through necrotic and reactive oxygen species mechanisms (de Arriba et al, 2006). Both calcium imaging and patch clamp
electrophysiology experiments indicate that DRG neurons are activated by methylglyoxal (300 μM), suggesting methylglyoxal might modulate peripheral nociceptor sensitivity to produce pain (Koivisto et al, 2012). Methylglyoxal generates anxiogenesis (Hovatta et al, 2005; Distler et al, 2012) and epileptic seizure activity that is regulated by GLO1 activity (Distler et al, 2013). Furthermore, methylglyoxal may be the common factor linking associations between Parkinson’s and type 2 diabetes (R. Hipkiss, 2012) and GLO1 variations are loosely associated with autism spectrum disorder (Kovac et al, 2014). Methylglyoxal is reported to activate ion channels involved in pain processing such as GABA_A (Distler et al, 2012), Nav1.8 (Bierhaus et al, 2012), and TRPA1 (Eberhardt et al, 2012; Koivisto et al, 2012; Ohkawara et al, 2012; Andersson et al, 2013; Koivisto et al, 2013). Here we investigate the activity of MG at TRPA1 receptors to produce painful type 2 diabetes.
Methylglyoxal is produced from (A) glycolysis and (B) lipid and protein metabolism and is (C) detoxified (i.e. metabolized) by the glyoxalase system using glutathione (GSH) redox cycling coupled to (D) NADPH production from pentose phosphate.

Figure 1-2. Biochemical pathways of methylglyoxal production and degradation. 
Adapted from (Allaman et al, 2015).
Figure 1-3. Methods to measure MG-related entities.
(A) Methylglyoxal-derived advanced glycation end-products (AGEs) can be measured using a competitive ELISA that recognizes hydroimidazolone MG-H1 protein residues where methylglyoxal covalently modifies arginine residues. (B) Free methylglyoxal (chemical structure is shown) circulating through the blood or within tissues can be measured by high-pressure liquid chromatography followed by tandem mass spectrometry. A representative mass spectrometry trace is shown for vehicle or methylglyoxal (MG) infusion. Mass spectrometry data courtesy of Dustin Carroll.

1.6 Modeling pain conditions in rodents

In order to study the human condition of pain we need preclinical models. Bioethical and practical considerations preclude the ability of the researcher to perform experimental manipulations on humans. For example, it is not feasible or reasonable to expect a patient to allow a researcher to transect a piece of their sciatic nerve in order to study the pain of traumatic nerve injury. However, as described later in section 1.6.2.1 we have used this technique to investigate neuropathic pain mechanisms in rats.
Animal models are important because they can be readily manipulated by the experimenter and constant variables (e.g. genetics, environment, time, location, etc.) can be well-controlled. In addition, reproducibility of results can be tested across experiments, labs, and institutions. Common animal models in the field of research are mice and rats, of which there are thousands of different strains and genetic backgrounds available through commercial vendors. The past twenty years of research has advanced biotechnology and genetic engineering so that specific pieces of DNA can be manipulated to create transgenic or knockout mice or rats. In this work we have utilized germline knockout mice to investigate the role of specific proteins involved in painful diabetes.

Manipulations other than genetic changes are also useful in pain research. The following sections describe two categories of animal pain models utilized in this research: Nociceptive, which we define as chemical activation of primary afferent nociceptors to produce immediate pain-like behavior that lasts only about one hour; and Neuropathic, which we define as a direct lesion to the peripheral (and to some extent, central) nervous system that results in pain-like behavior that lasts days to weeks to even an indefinite duration.

1.6.1 Nociceptive

1.6.1.1 Capsaicin

The prototypical agonist of TRPV1 is capsaicin, a compound found in chili peppers that contributes to the taste of spicy foods (Caterina et al, 1997). Intraplantar injection of capsaicin activates TRPV1-positive primary afferents (i.e. nociceptors) that are mostly small, unmyelinated C-fibers. These C-fibers typically respond to chemical (e.g. capsaicin) or thermal (e.g. heat) stimuli. TRPV1 is a cation channel whose open probability increases when a ligand such as capsaicin is bound. Increased open
probability allows a greater number of cations, such as sodium and calcium, to enter the neuron (as compared to the resting state with no ligand) resulting in depolarization of the neuron. When the stimulus is great enough, as is the case with injection of even low doses of the potent capsaicin agonist, the action potential threshold is reached and the nociceptor begins to relay “pain” information to the first synapse within the spinal cord dorsal horn. This activation of nociceptors by the capsaicin chemical stimulus leads to nociceptive responses in the form of paw licking, flinching, and lifting.

Intraplantar capsaicin injection has been used as a model of nociceptor activation. For example, capsaicin activates nociceptive neurons in the spinal cord dorsal horn as evidenced by both superfusion in physiological slice studies (Wei et al, 2006) and immunohistochemical analysis of the dorsal horn after peripheral administration (Griggs et al, 2015). As such, it is convenient to label these dorsal horn neurons as nocireponsive, since they respond to the stimulation of nociceptors. In addition, capsaicin also induces expression of both Fos and pERK in the dorsal horn, which provides for a relatively simple quantifiable measurement of nocireponsive neuron activation after peripheral nociceptor stimulation (Griggs et al, 2015).

Figure 1-4. Intraplantar capsaicin evokes pERK in the spinal cord dorsal horn. Intraplantar capsaicin (50 μg in 50 μl) was injected beneath the plantar skin of the left hindpaw in Sprague-Dawley rats. Animals were perfusion-fixed 10 min following intraplantar injection and spinal cords were harvested for immunohistochemical analysis. Representative images of the ipsilateral lumbar dorsal horn after (A)
vehicle or (B) capsaicin administration. (C) Quantification of the number of cell profiles positive for pERK (n=3-4). ★ p<0.05 vs. VEH.

1.6.1.2 Methylglyoxal (acute administration)

Akin to activation of TRPV1, evidence is building that methylglyoxal (MG) activates TRPA1-positive nociceptors to produce spontaneous nociceptive behavior (Eberhardt et al., 2012; Koivisto et al., 2012; Andersson et al., 2013; Koivisto et al., 2013; Oguri et al., 2014). Similar to activation of TRPA1 afferents by formalin (McNamara et al., 2007) or 15d-PGJ2 (Cruz-Orengo et al., 2008), we tested the hypothesis that intraplantar administration of MG would induce nociception and activation of spinal neurons (see Figure 5-2). This model of chemical induced nociception allowed us to further investigate the role of TRPA1 and spinal sensitization in MG-mediated nociceptive pain.

1.6.2 Neuropathic

The IASP defines neuropathic pain as “pain caused by a lesion or disease of the somatosensory nervous system”. We distinguish the difference between nociceptive and neuropathic pain in that neuropathic pain becomes pathological even in the absence of an obvious exogenous stimulus (such as capsaicin or methylglyoxal administration). Much work has been completed to elucidate the mechanisms of neuropathic pain after traumatic nerve injury, post-herpetic neuralgia, chemotherapeutic neuropathy, trigeminal neuralgia, or diabetes. Here we used three different models of neuropathic pain to investigate the mechanisms of PPARγ analgesia as well as the contribution of methylglyoxal to diabetic neuropathic pain. For reviews of neuropathic pain mechanism see the following references (Campbell and Meyer, 2006; Scholz and Woolf, 2007; Sandkühler, 2009; Jensen and Finnerup, 2014).
1.6.2.1 Traumatic (Spared) Nerve Injury

There are several models of traumatic nerve injury utilized in rodents. Most involve either ligation, by tying sutures around a nerve to induce neuroinflammation and associated sensitization, or ligation followed by transection, where a piece of the nerve is removed. There are two major nerves utilized in these types of studies: trigeminal and sciatic. Here we focus on manipulation of the sciatic nerve to induce neuropathic pain. Spared nerve injury (SNI) is a method by which the branches of the sciatic nerve trifurcation (tibial, common peroneal, sural) are differentially ligated and/or transected. Transection of one or more of these branches results in hypersensitivity and sensitization of the intact, or spared, nerve. In our studies, we transect the tibial and common peroneal branches of the sciatic nerve and spare the sural nerve. The procedure (Decosterd and Woolf, 2000) and innervation territories of these nerves in the spinal cord dorsal horn (Corder et al, 2010) are described previously and elaborated upon in section 3.3.2

1.6.2.2 Diabetic neuropathic pain

Models of both type 1 and type 2 diabetes exist in the pain research community. The most common model of diabetes and associated pain-like behavior is induced by streptozotocin (STZ), a glucose analog and pancreatic beta cell toxin. STZ induces beta cell destruction resulting in the loss of insulin production and hyperglycemia. STZ models a type 1 diabetic-like phenotype. Here we focus on models of type 2 diabetic pain for reasons described in section 4.2. We use both rat (Zucker Diabetic Fatty; ZDF) and mouse (Lepr<sup>db/db</sup>; db/db) genetic models of type 2 diabetes. These rodents develop a type 2 diabetic phenotype because of an inbred genetic mutation that produces a non-functional leptin receptor. At early stages of diabetes, ZDF and db/db exhibit behavioral hypersensitivity to noxious mechanical and heat stimuli. However, as diabetes
progresses the animals become hyposensitive. This allows for investigating the mechanisms of both painful and insensate neuropathy in diabetes. Here we focus on the period of hypersensitivity in order to study painful diabetic neuropathy (PDN), or the neuropathic pain associated with type 2 diabetes.

The Zucker Diabetic Fatty (ZDF) rat originated from a colony harboring a mutation in their leptin receptor causing a truncated, non-functional protein product. Inbreeding of ZDF rats resulted in a reliable model of genetically induced spontaneous type 2 diabetes. ZDFs develop type 2 diabetes by age 2-3 months (Pickavance et al, 1998; Shibata et al, 2000; Li et al, 2006; Brussee et al, 2008; Sugimoto et al, 2008). Control Zucker Lean (ZL) rats are heterozygous for the leptin receptor mutation and are not diabetic. Thermal hyperalgesia in ZDF rats is present from age 14 weeks (Li et al, 2006; Donahue et al, 2012) and can last up to age 28 weeks (Oltman et al, 2008). Mechanical hyperalgesia occurs as early as age 14 weeks (Zhuang et al, 1997; Li et al, 2006; Romanovsky et al, 2008; Sugimoto et al, 2008) while mechanical allodynia develops at age 25 weeks and can last up to age 29 weeks (Otto et al, 2011). Our lab was unable to see evidence of tactile allodynia in ZDF rats up to age 24 weeks (Donahue et al, 2012). ZDF rats develop motor and sensory nerve conduction velocity deficits by age 16 weeks (Brussee et al, 2008; Oltman et al, 2008). Hypoalgesia is a common symptom of diabetics in later stages of disease progression and this condition also has been shown in ZDF rats (Oltman et al, 2008); however, this proposal aims to characterize the hypersensitivity seen after diabetes develops but before progression to hypoalgesia.

1.6.2.2.1 Methylglyoxal (chronic administration)

Several studies suggest that chronic administration of methylglyoxal (MG) may be a suitable model of diabetic complications (Dhar et al, 2011; Dornadula et al, 2015; Illien-Junger et al, 2015). These groups showed that chronic MG administration in the drinking
water, via repeated systemic injection, or minipump induces mild hyperglycemia, mitochondrial dysfunction, and neuroinflammation. However, pain-like behavior in a chronic MG model has not been investigated. Here we show that acute administration of MG produces pain-like behavior. We attempted to induce chronic neuropathic pain by using minipumps to administer MG in naïve, wild-type mice. However, experimental complications limited our conclusions from this study (see Figure 6-5). Therefore a future aim of this work is to develop a chronic neuropathic pain model induced by long-term administration of MG in mice and/or rats (see 6.3.1).

1.7 Measuring preclinical “pain” in rodents

It is virtually impossible to measure pain in rodents when considering the true definition of pain, which includes the words unpleasant and emotional. We simply cannot ask the rodent subject whether they hurt, or whether a particular stimulus is unpleasant or induces negative emotion. We can only observe behavior and make inferences based upon these observations. Instead of ascribing the word pain to behavioral observations that may or may not be unpleasant or emotional, we can refer to these behaviors as pain-like. A majority of pain research has used stimulus-evoked measures of pain-like behavioral hypersensitivity. New assays, or the repurposing of previously described behavioral assays from the fields of psychology and addiction, are now available to go beyond assessment of the sensory-discriminative dimension of pain.

1.7.1 Stimulus-evoked measures

Sensory organs and receptors in the skin allow animals to discern both quality and quantity of a potentially painful stimulus. Stimulus modalities include mechanical, thermal, and chemical. Primary afferents that innervate the skin and sensory organs express various proteins that each contribute to sensing stimuli in different modalities.
Here we investigate two such receptors: TRPV1, the primary heat receptor that is activated by capsaicin; and TRPA1, primarily a cooling receptor that is activated by methylglyoxal. Both TRPV1 and TRPA1 can contribute to mechanosensation in naïve and pathological pain conditions.

Stimulation of primary afferent terminals in the periphery can result in activation of nociceptive neurons in the spinal cord dorsal horn. These spinal neurons act as relay neurons that transmit pain information to circuits within the spinal cord as well as to third and fourth order neurons in the brainstem, thalamus, and cortex. This first (and potentially second) synapse in the spinal cord can be modulated by inhibitory and/or excitatory interneurons in the spinal cord or by descending projections from structures within the brainstem. However, a growing body of evidence suggests that the sensory aspects of stimulus-evoked pain-like behaviors are predominantly spinally mediated reflexes, and therefore do not reflect the affective-motivational or cognitive-evaluative aspects of pain. Nonetheless, the methods of assessing stimulus-evoked pain-like behavior in rodents are often quite similar to those used in humans, and therefore may still have translatable value. However, we argue that simultaneous assessment of both stimulus-evoked and non-evoked measures of pain in rodents is necessary in studies aiming to evaluate the clinical utility of drugs that may potentially relieve pain.

Assessment of stimulus-evoked pain-like behavior is accomplished by measuring the threshold of stimulation at which the animals responds, or withdraws from the given stimulus. Conversely, the magnitude of the response, as is the case for chemical-induced nociception by capsaicin or methylglyoxal, can be measured. Here we use von Frey filaments and a modified Randall-Selitto pressure method to determine the force at which animals withdraw/respond to non-noxious or noxious stimulation, respectively. Similarly, we use a hotplate to subject animals to a heat stimulus and measure the latency, or the duration of time until the animal withdraws/responds. For mechanical or
heat stimulation, a decrease in the withdraw threshold or latency indicates hypersensitivity. To measure cold sensitivity we either evaluate the duration of withdraw response after stimulation with acetone (presumably evaporation results in a cooling sensation) or quantify the number of responses in animals subjected to a coldplate.

1.7.2 Stimulus-independent measures

1.7.2.1 Chemical-induced nociception

Activation of primary afferent nociceptors by endogenous and exogenous moieties produces pain-related behaviors. Compounds (e.g. capsaicin, formalin, carrageenan, methylglyoxal) are administered subcutaneously beneath the epidermis (intradermal). We refer to this as an intraplantar injection (i.pl.) because compounds are given into the ventral plantar surface of the hindpaw. Chemical activation of receptors on the free nerve endings of nociceptors results in spinally mediated reflex withdraw behaviors including licking, lifting, and/or flinching of the affected limb. We deem these spontaneous measures of nociception or nocireponsive behaviors because the effect is immediate, typically short-lived on the order of minutes, and does not require repeated stimulation (i.e. administration of the compound of interest) as is the case for stimulus-evoked mechanical or thermal hyperalgesia. Quantification of the pain-related behaviors allows us to test how various manipulations (e.g. a receptor antagonist) affect the specific peripheral nociceptive pathway and/or how modulation of the spinal neuron reflex system changes nociception.

1.7.2.2 Mechanical conflict avoidance

A novel Mechanical Conflict Avoidance System (MCS) was recently developed by our colleagues (Lau et al, 2012) to incorporate cognitive processing in the measurement of preclinical motivational-affective pain in rodents. MCS gives the subjects a choice to
remain in an environment containing an aversive light stimulus or to enter an environment that contains an aversive mechanical stimulus in the form of height-adjustable, blunted metal probes. By navigating through the mechanical stimulus environment the subjects are rewarded with access to a darkened environment without probes. If animals chose to leave the light chamber and cross the probes in the stimulus chamber, they then had the option to enter a darkened reward chamber. This method is described in detail in section 4.3.4 and used to test the hypothesis that type 2 diabetes is associated with affective pain in ZDF rats (section 4.4.1).

1.7.2.3 Conditioned Place Preference/Avoidance

Assessment of clinical and preclinical pain is not always performed using the same assays, which contributes to the lack of clinical efficacy of drugs that reverse reflexive pain in rodents (Vierck et al, 2008). Alternatively, Bennett suggests that the lack of success in clinical trials is not due to measuring the wrong outcome in animals (i.e. evoked sensitivity), but that clinicians are evaluating imprecise endpoints in patients (Bennett, 2012). Therefore, assays that go beyond evoked measures and that can be performed using similar experimental designs in both rodents and humans are needed.

Conditioned place preference (CPP) is used to measure the rewarding (Bardo and Bevins, 2000) and pain relieving (Sufka, 1994; King et al, 2009) effects of experimental drugs in rodents and humans. CPP has gained attention as an approach to unmask the affective dimension of pain in preclinical rat (King et al, 2011) and mouse (He et al, 2012) models of peripheral nerve injury. The CPP approach harnesses the fact that an animal presumably in pain will be motivated to seek out an environment previously associated with pain relief. Since pain is aversive, negative reinforcement of the aversive stimulus is rewarding. Animals are trained by the unconditioned stimulus (i.e. the behavioral effects of analgesic drug administration) in an environment with distinctive
sensory cues that serves as the conditioned stimulus. After conditioning animals are allowed to choose whether or not they prefer to spend more time in the drug-paired environment in the absence of any drug administration. Pairing the affective consequences of analgesic drug administration takes into consideration the motivational aspects of pain and allows for measurement of preclinical analgesia in the absence of an exogenous stimulus (Tzschentke, 2007). One caveat to CPP/CPA is that diseases that affect memory may confound results from this assay, as memory is needed to recognize the environment where pain relief was received. We validate the method of CPP in a model of nerve injury (see 1.8 ) and use this assay to determine the effect of methylglyoxal on motivational-affective pain.

Several reports indicate dissociation between decreased evoked hypersensitivity and negative reinforcement of pain (i.e. CPP) after analgesic drug administration. After injury, rodents exhibit CPP to inhibition of spinal or peripheral nerve transmission by local injection of lidocaine (King et al., 2009; King et al., 2011). This suggests that ongoing activity from peripheral neurons in the pain pathway contributes to aversive aspects of supposed pain after injury. However, pharmacological inhibition of TRPV1 (Okun et al., 2011), COX2, or TRPA1 (Okun et al., 2012) does not reverse ongoing pain (i.e. eliminate a preference for peripheral nerve block after injury) but alleviates evoked hypersensitivity. In the streptozotocin model of painful diabetic neuropathy, inhibition of TRPA1 reversed mechanical hypersensitivity, but not spontaneous pain as assessed using the CPP paradigm (Wei et al., 2013). The failure of TRPA1 and TRPV1 inhibitors in producing CPP preclinically is mimicked by the limited ability of TRPA1 or TRPV1 antagonists to reduce pain in humans (Brederson et al., 2013). These studies highlight the importance of measuring both sensory/discriminative and affective/motivational outcomes in preclinical models in order to better predict clinical utility of analgesic drugs.
1.8 Preface

My current results reflect six years of work investigating chronic neuropathic pain using preclinical rodent models. I began this work by validating models for nociceptive pain (e.g. capsaicin and methylglyoxal), traumatic nerve injury (e.g. spared nerve injury), and painful type 2 diabetes (e.g. db/db mice and Zucker Diabetic Fatty rats). I used various methods to characterize and evaluate pain-related behaviors in rodents, including validating the use of conditioned place preference testing to assess the motivational-affective component of pain in Chapter 2: . In Chapter 3: I elucidated the rapid antihyperalgesic effect of PPARγ agonists and determined that the inhibition of spinal sensitization by pioglitazone is mediated through a non-genomic, anisomycin-insensitive, astrocyte-related mechanism. Next in Chapter 4: and Section 6.4.2 I simultaneously characterized models of painful diabetic neuropathy (i.e. ZDF and db/db) and determined that pioglitazone, a drug approved by the FDA for treating type 2 diabetes, inhibits painful type 2 diabetes independent from its effect on decreasing blood glucose. I also validated a second method for measuring pain-like negative affect using a novel mechanical conflict avoidance, showing that diabetic ZDF rats exhibit motivational-affective pain-like behavior. Finally, in Chapter 5: I extended findings from the overgeneralized streptozotocin model of painful diabetes, providing strong evidence implicating TRPA1 in sustained diabetic hypersensitivity in a superior model, type 2 diabetic db/db mice. Using this model along with exogenous administration of the glucose metabolite methylglyoxal, I will show that MG is sufficient to produce behavioral hypersensitivity and affective pain (i.e. conditioned place aversion) through activation of peripheral TRPA1 and subsequent sensitization of spinal neurons through a TRPA1-AC1 mechanism. Furthermore, I investigate the inhibition of methylglyoxal-induced pain by the methylglyoxal scavenging peptide GERP10 and pioglitazone.
My results are the first to report evidence of affective pain in a model of type 2 diabetes as well as the reduction of painful type 2 diabetes GERP10 and pioglitazone. My long-term goal for this work is to inform the initiation of future clinical studies investigating the analgesic properties of pioglitazone and/or GERP10. In Chapter 6: I evaluate my results alongside the available literature to synthesize new insights into the mechanism(s) by which PPARγ mediates its antihyperalgesic effects in neuropathic pain conditions. I also discuss evidence suggesting that targeting TRPA1, AC1, or GLO1 may alleviate painful type 2 diabetes. Future studies discussed in the following content chapters and the overall discussion in Chapter 6: are proposed to extend our knowledge of the involvement of MG, GLO1, TRPA1, AC1, and PPARγ in mediating and inhibiting chronic neuropathic pain associated with type 2 diabetes.
CHAPTER 2: Gabapentin alleviates affective pain after traumatic nerve injury

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2.1 Abstract

Gabapentin reduces behavioral signs of stimulus-evoked allodynia and hyperalgesia in preclinical studies of traumatic nerve injury, but its effects on more clinically-relevant measures of stimulus-independent pain are unclear. To address this gap, we determined whether gabapentin would relieve affective pain after spared nerve injury (SNI). Twelve days after sham or SNI surgery, we administered gabapentin over three consecutive conditioning days and then evaluated conditioned place preference (CPP). Gabapentin produced CPP and reversed mechanical hypersensitivity in SNI but not sham rats at a dose (100 mg/kg) that did not change open field activity. These results show for the first time that gabapentin provides relief from affective pain without producing locomotor sedation, and adds to a limited clinical literature suggesting that its use can be extended to treat pain arising from traumatic nerve injury.
2.2 Introduction

Preclinical research targeting discovery of novel treatments for neuropathic pain primarily rely on mechanical or thermal stimulus-evoked behavioral outcomes. However, this approach fails to mimic the affective and spontaneous aspects of chronic pain that are most relevant to pharmacotherapy in humans, as indicated by the high failure rate of analgesic drug candidates in clinical trials. The use of conditioned place preference (CPP) to assess non-evoked pain, originally described two decades ago (Sufka, 1994), has re-emerged as a leading measure of affective neuropathic pain (King et al., 2009) and has the potential to address the disconnect between preclinical and clinical efficacy (King and Porreca, 2014). The use of CPP to measure preclinical pain relief is advantageous because the test is performed in the absence of an exogenous stimulus (Tzschtenke, 2007), incorporates the motivation to seek reward (Bardo and Bevins, 2000), and evaluates the affective (King et al., 2009) pain relieving effects of analgesic drug administration (King and Porreca, 2014).

Gabapentin (Neurontin®) is a primary treatment for neuropathic pain (Attal et al., 2010) in patients with trigeminal neuralgia (Lemos et al., 2011), post-herpetic neuralgia (Rowbotham et al., 1998), painful chemoneuropathy (Caraceni et al., 2004), and painful diabetic neuropathy (Backonja et al., 1998; Vincent et al., 2011). Reverse translation studies in rodents indicate that gabapentin attenuates affective pain produced by cisplatin (Park et al., 2013) or streptozotocin (Wagner et al., 2014), as well as the evoked hypersensitivity associated with traumatic nerve injury (Xie et al., 2014). However, no study has evaluated whether gabapentin reduces affective pain after traumatic nerve injury. To address this question, we performed gabapentin CPP in rats with spared nerve injury (SNI), a widely used preclinical model of traumatic nerve injury (Decosterd and Woolf, 2000).
2.3 Methods

2.3.1 Animals

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 200-250 g at the time of surgery and 300-350 g at the time of behavioral procedures were housed 2 per cage on a 12-hour light/dark cycle (7am lights on / 7pm lights off) in a temperature (68-72º F) and humidity controlled room with food and water provided ad libitum. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to in vivo techniques, in accordance with the International Association for the Study of Pain and the National Institutes of Health Office of Laboratory Animal Welfare Guide for the Care and Use of Laboratory Animals. All behavioral procedures were carried out between 8am-6pm and approved by the Institutional Animal Care and Use Committee (IACUC) at University of Kentucky. Behavioral measurements were performed by an observer blinded to experimental treatments.

2.3.2 Spared Nerve Injury (SNI) surgery

Sham and SNI surgeries were performed as previously described (Decosterd and Woolf, 2000). To generate surgical sham control subjects, all steps were performed except ligation and transection of the common peroneal and tibial nerves. The day of sham or SNI surgery is referred to as day 0.

2.3.3 Measurement of pain-like behavior and open field activity

Animals were acclimated in individual Plexiglas boxes (4" x 8" x 4") on top of a raised stainless steel mesh grid for 1 h. Mechanical hypersensitivity was assessed using von Frey filaments (Stoelting, Inc., Wood Dale, IL) using a modified up-down method (Dixon,
1980; Chaplan et al, 1994) as previously described (Morgenweck et al, 2013). The calculated 50% withdraw threshold is reported.

A photobeam activity system (PAS; 16 x 16 array; San Diego Instruments, San Diego, CA) was used to measure exploratory locomotion in a clear, square box surrounded by the photobeam array. Saline or gabapentin (100 mg/kg) was administered i.p. prior to placing the rat into the open field chamber. The total number of photobeam breaks was automatically quantified by the PAS software for 30 min in 5 min bins in the absence of any observer.

2.3.4 Conditioned Place Preference

The use of conditioned place preference (CPP) as a tool to measure the ongoing aversiveness (i.e. affective pain) after injury or preference for rewards has been well established (Sufka, 1994; Bardo and Bevins, 2000; King et al, 2009; Wei et al, 2013). Eight rat CPP boxes (Med Associates, St Albans, VT) were used to assess chamber preference before and after the drug conditioning phase. The experimental timeline and details of the CPP apparatus are illustrated in Figure 1. Rats were able to discriminate the drug- versus vehicle-paired chamber using visual (wall color), tactile (flooring), and olfactory (Lipsmackers Chapstick, Bonne Bell, Westlake, OH) cues. Preliminary experiments indicated no preference for vanilla (white chamber) or kiwi (black chamber) chapstick olfactory cues in sham or SNI rats. To reduce time spent in the gray chamber, lighting in the white and black chambers was adjusted to 25% of that in the grey chamber. Manual guillotine doors were used to isolate the white and black pairing chambers from the grey chamber during conditioning. Each individual CPP box was fully contained in a sound and light attenuating enclosure. Time of testing, animal handling method, and cleaning of the CPP boxes were held constant.
Preconditioning. The CPP procedure spanned six consecutive days. On Day 1, subjects were acclimated to the CPP boxes for 30 min, with open access to each of the three chambers. On Day 2 (preconditioning), animals were placed in the grey middle chamber, and then we determined time spent in the white or black pairing chambers for 15 min. Animals that spent <20% or >80% time in the black and/or white chamber (i.e. showing an apparatus bias or initial, unconditioned preference) during preconditioning were removed from the experiment (King et al, 2009). By these criteria, ten animals were removed from von Frey and CPP analyses.

Conditioning. On Days 3, 4 and 5 (conditioning), we used a biased assignment approach to drug pairing: saline was paired with the preferred chamber in the morning, and gabapentin was paired with the non-preferred chamber in the afternoon. Our biased approach was chosen for five reasons: 1) increases assay sensitivity; 2) allows for a within subjects design and statistical analysis (Cunningham et al, 2003; Tzschantke, 2007); 3) of all CPP studies in 2001, 30% used a biased approach and 42% analyzed results using a difference score (postconditioning minus preconditioning) (Cunningham et al, 2003); 4) a biased approach was very recently used to assess gabapentin CPP in the streptozotocin model of painful diabetic neuropathy (Wagner et al, 2014); 5) Cunningham et al demonstrated that if the CPP apparatus is not biased (time spent in the white chamber = time spend in black chamber when average across all subjects, as in the current study) , then the use of either a biased or unbiased chamber-assignment approach does not affect the ability to produce CPP (Cunningham et al, 2003).

Conditioning consisted of the following sequential steps: i.p. injection, return of the animal to its home cage for 5 min, and then placement within the white or black chamber for 30 min (injections were never paired with the grey, middle chamber). We used a 30 min conditioning time based on reports that gabapentin maximally reduced mechanical hypersensitivity at 30-60 min after injection (Park et al, 2013; Wagner et al, 2014). We
chose 3 d of gabapentin conditioning because 1 d was not sufficient to produce CPP in a mouse model of chemoneuropathy (Park et al., 2013).

**Postconditioning and analysis.** On Day 6 (postconditioning), animals were placed into the grey chamber and we evaluated time spent in either the white or black chamber. The difference score for each subject was calculated by subtracting the time spent in the saline- or gabapentin paired chamber before pairing (during preconditioning) from the time spent in each chamber after pairing (postconditioning), and then averaged within each group.

**Figure 2-1.** Experimental timeline and diagram of the conditioning place preference (CPP) apparatus. (Top) Baseline von Frey thresholds were measured on d -1 prior to Sham or Spared Nerve Injury (SNI) surgery on d 0. Mechanical hypersensitivity (von Frey) was measured on d 9 (pre CPP) and d 16 (post CPP). The Conditioned Place Preference (CPP) assay was performed on d 10 – 15 (open arrows) and consisted of acclimation, preconditioning, conditioning, and postconditioning. Open field activity was determined on d 17. (Bottom) The CPP apparatus consisted of: (left) a white chamber with grid flooring, vanilla chapstick, and 25% light intensity; (middle) a grey chamber with solid flooring, no olfactory cue, and 100% light intensity; (right) a black chamber with bar flooring, kiwi chapstick, and 25% light intensity.
2.3.5 Experimental Design

Evoked mechanical sensitivity was measured prior to sham or SNI surgery (day 0), 9 d after surgery (pre CPP), and after completion of the CPP procedure on d 15 (post CPP). The CPP assay was performed on days 10 – 15. Following post CPP measurement of von Frey withdraw thresholds to confirm the sustained presence of mechanical hypersensitivity, saline or gabapentin (100 mg/kg) was injected i.p. and von Frey thresholds were recorded 15, 30, and 60 min later. A 24 h timepoint was taken to determine whether the anti-hypersensitivity effects of gabapentin endured from one conditioning day to the next. Open field activity after i.p. saline or gabapentin administration was performed at the conclusion of von Frey and CPP experiments on d 17.

2.3.6 Drugs

Gabapentin (Spectrum Chemical, Gardena, CA) was dissolved in 0.9% saline immediately prior to injections and administered i.p. in a volume of 0.5-1.0 ml (final dose = 100 mg / kg body weight).

2.3.7 Statistical Analysis

A paired t-test was used to compare the effect of sham or SNI surgery on mechanical sensitivity prior to CPP, preconditioning versus postconditioning time spent in CPP chambers, and CPP difference scores. Gabapentin effect on behavior in the von Frey assay was compared for significant differences over time using repeated measures two-way ANOVA followed by Holm-Sidak multiple comparison correction. An alpha value of $\alpha = 0.05$ was used to determine statistical significance. All data were analyzed and graphed using Prism 6.0 (GraphPad, La Jolla, CA) and are presented as mean ± SEM.
2.4 Results

2.4.1 SNI produces evoked mechanical hypersensitivity

Spared nerve injury (SNI) evokes mechanical hypersensitivity that begins a few days after surgery and lasts for at least 6 months (Decosterd and Woolf, 2000). To compare evoked mechanical hypersensitivity and affective pain, we performed von Frey testing prior to surgery (baseline), before CPP (pre), after CPP (post), and for 60 min following i.p. gabapentin administration in sham and SNI rats. CPP conditioning occurred at day 12-14 during established mechanical hypersensitivity. As illustrated in Fig 2A, SNI produced hypersensitivity to von Frey mechanical stimulation at the pre CPP timepoint [p < 0.0001]. There was no change in mechanical thresholds in sham animals [p > 0.05].

2.4.2 Gabapentin reverses SNI-induced evoked mechanical hypersensitivity

After CPP testing, we assessed inhibition of SNI-induced mechanical hypersensitivity by measuring von Frey withdraw thresholds after systemic administration of gabapentin at the same dose used during CPP conditioning (100 mg/kg). Fig 2B illustrates that gabapentin significantly attenuated evoked mechanical hypersensitivity in rats with SNI at 30 [p < 0.05] and 60 [p < 0.05] min after i.p. injection [drug x time; F (3, 36) = 17.5; P < 0.0001]. Mechanical withdraw thresholds were slightly, but insignificantly, increased at 15 min. Sham animals did not exhibit evoked mechanical hypersensitivity (compared to baseline) [p > 0.05] and von Frey withdraw thresholds were unaltered by gabapentin [drug x time; F (3, 18) = 0.015; P > 0.05].
Figure 2-2. Gabapentin reverses evoked mechanical hypersensitivity associated with nerve injury.

(A) Mechanical thresholds are shown at baseline (before surgery) and d 9 (pre CPP) after sham or spared nerve injury (SNI) surgery prior to conditioned place preference testing. SNI (n=17) decreased mechanical thresholds relative to sham (n=8) controls. (B) On d 16 (post CPP), mechanical thresholds remain decreased in SNI but not sham animals. Gabapentin (100 mg/kg; i.p.) increased mechanical thresholds in SNI (n=11) but not sham (n=5) animals. Saline did not change mechanical thresholds in sham (n=3) or SNI (6) animals. (A), ★ “SNI” at the pre CPP timepoint significantly different from all other groups. (B), ★ “SNI – Gabapentin” significantly different from “SNI – Saline”.

2.4.3 Gabapentin produces CPP in rats with SNI but not sham surgery

To confirm that animals do not prefer one chamber over another, we assessed time spent in each. This was done prior to drug-pairing during conditioning. In sham rats, time spent in the white [355.1 ± 16.0 s] and black [363.2 ± 18.8 s] chambers was similar [p = 0.37]. In SNI rats, time spent in the white [336.2 ± 15.6 s] and black [346.1 ± 18.6 s] chambers was also similar [p = 0.34]. These data indicate that there is no initial bias for the CPP apparatus and that injury did not alter preconditioning preferences.

To determine whether gabapentin alleviates affective pain after traumatic nerve injury, we assessed CPP in sham and SNI rats. Fig 3A illustrates our biased conditioning approach: preconditioning time spent in the saline-paired chamber was greater than time
spent in the gabapentin-paired chamber in both sham [p < 0.05] and SNI [p < 0.0001] rats. Biased drug pairing remained counterbalanced, where half the animals received gabapentin in the white chamber and half in the black chamber. Conditioning to gabapentin produced an increase in time spent in the gabapentin-paired chamber in SNI [p = 0.0043] but not sham rats [p = 0.2]. When compared to saline difference scores, Fig 3B illustrates a significantly higher gabapentin difference score in SNI [p < 0.0001] but not sham [p = 0.70] rats. These results indicate that gabapentin produces CPP in SNI but not sham rats at 2 wks after injury. A previous study indicates that the antihyperalgesic effect of gabapentin varies over time after nerve injury (Hama and Borsook, 2005). Future studies could investigate the ability of gabapentin to produce CPP at later timepoints.

Figure 2-3. Gabapentin attenuates affective pain associated with traumatic nerve injury.
To determine affective pain relief we performed CPP with three days of conditioning (saline or gabapentin; 100 mg/kg; i.p.). (A) Time spent in the saline-paired chamber during preconditioning was greater than time spent in the gabapentin-paired chamber as a result of our biased drug-pairing approach. In sham rats (n=8), there was no change in preference during postconditioning (“Post”) when compared to preconditioning baselines (“Pre”). In SNI rats (n=17), gabapentin produced an increase in time spent in the gabapentin-paired chamber when compared to preconditioning baseline. (B) Saline and gabapentin difference scores were
significantly different in SNI but not sham rats. These results taken together indicate
gabapentin induces CPP thereby relieving affective pain in rats with traumatic nerve
injury. # Significantly different from preconditioning saline-paired in SNI. $ Significantly
different from preconditioning gabapentin-paired in SNI. ★ Significant
difference between indicated groups.

2.4.4 Gabapentin did not change locomotor activity in sham or SNI rats

Gabapentin produces adverse effects in humans including somnolence, dizziness,
peripheral edema, infection, and ataxia (Rowbotham et al, 1998). To address the
potential effect of gabapentin on exploratory or somatomotor activity, resulting in
confounding alterations in mechanical thresholds or CPP, we assessed open field
activity. Fig 4A indicates that gabapentin did not change locomotor activity in sham
[drug; F (1, 6) = 0.18; P > 0.05] or SNI [drug; F (1, 8) = 0.005; P > 0.05] rats.
Furthermore, there was no difference in activity between sham and SNI animals treated
with saline [injury; F (1, 8) = 0.02; P = 0.89] indicating that SNI did not change locomotor
function.

Figure 2-4. Gabapentin does not alter locomotor activity.
(A) The number of beam breaks in sham or SNI animals treated with saline or
gabapentin (100 mg/kg; i.p.) was not different in an open field photobeam activity
assay (n=5-6).
2.5 Discussion

Here we present the first data indicating that gabapentin relieves affective pain (i.e. produces CPP) associated with traumatic nerve injury in a preclinical model. Our current results are consistent with recent findings in other rodent models of neuropathic pain. For example, gabapentin produces CPP in mice following chronic cisplatin treatment (Park et al, 2013) or in the streptozotocin model of type I painful diabetic neuropathy (Wagner et al, 2014). Xie et al reported that gabapentin reversed mechanical hyperalgesia associated with spinal nerve ligation (SNL), without measuring its effect on affective pain using CPP (Xie et al, 2014). Repetition of our study in an alternative model of traumatic nerve injury such as SNL or using a dose response could provide additional support for our conclusion that gabapentin alleviates affective pain generalized to multiple types of neuropathic conditions.

The current study shares important experimental design characteristics with Park et al and Wagner et al (Park et al, 2013; Wagner et al, 2014), but in the setting of traumatic nerve injury. First, a systemic dose of 100 mg/kg was administered over three conditioning days. Second, gabapentin produced CPP rapidly, within 30 min of administration. Third, gabapentin did not produce CPP in control animals, ruling out the possibility that gabapentin is intrinsically rewarding. This is in contrast to rewarding analgesic drugs such as morphine, which produce CPP in naïve or uninjured subjects, thus complicating interpretation of effects on affective pain (Sufka, 1994; Tzschentke, 2007). Fourth, gabapentin reduced both evoked and affective measures of pain. This is striking in light of recent reports indicating that other analgesic drugs such as TRP antagonists (Brederson et al, 2013) inhibit evoked but not affective pain in preclinical models of inflammation (Okun et al, 2011), osteoarthritis (Okun et al, 2012), type I diabetes (Wei et al, 2013), and SNI (Wei et al, 2013).
We conclude that gabapentin alleviates affective pain after SNI in rodents, and suggest further studies to determine the clinical efficacy of gabapentin for the treatment of chronic pain associated with traumatic nerve injury. Indeed, a randomized, double-blind, placebo-controlled, cross-over, multicenter clinical trial involving patients with peripheral nerve injury due to trauma or surgery reported that, compared to placebo, gabapentin provided better pain relief and increased the number of subjects with a pain reduction of at least 30% (Gordh et al, 2008). In addition, our results highlight the importance of measuring the affective component of pain in preclinical studies to better predict clinical efficacy of pain-relieving drugs.

2.6 Supplemental Discussion

2.6.1 Gabapentin reduction of neuropathic pain in humans and rodents

Gabapentin (Neurontin®) is an anticonvulsant drug that is a primary treatment (Attal et al, 2010) for neuropathic pain in patients with diabetes (Vincent et al, 2011), trigeminal neuralgia (Lemos et al, 2011), cancer (Caraceni et al, 2004), traumatic nerve injury (Gordh et al, 2008), and post-herpetic neuralgia (Kukkar et al, 2013). Gabapentin also decreases evoked measures of pain-like hypersensitivity in rodent models of painful diabetic neuropathy (Field et al, 1999; Zhang et al, 2013), orofacial nerve injury (Nakai et al, 2014), spinal cord injury (Kitzman et al, 2007; Rabchevsky et al, 2011), hindlimb nerve injury (Luo et al, 2002; Yezierski et al, 2013), and chemoneuropathy (Park et al, 2013). Gabapentin likely produces analgesia by reducing neuronal excitability through inhibition of α2δ1 subunits presynaptic calcium channels (Brown et al, 1998; Kukkar et al, 2013). Relief from pain-like hypersensitivity is associated with decreased Fos expression in the spinal cord and PAG after nerve injury (Morgado et al, 2010), attenuation of fMRI BOLD signals in the cortical pain matrix in nerve injured mice (Takemura et al, 2011), and attenuated overexpression of Nav1.7 and pERK in
streptozotocin-treated rats (Zhang et al, 2013). Because gabapentin is currently recommended for the treatment of neuropathic pain patients (Attal et al, 2010), and it is unknown whether gabapentin attenuates preclinical spontaneous pain, we performed a reverse translation study to assess gabapentin induced CPP. Our finding that gabapentin decreases affective pain in rodents with traumatic nerve injury highlights the ability of the CPP approach to predict clinical efficacy. Future reverse translation studies could investigate whether gabapentin relieves affective pain in a preclinical model of painful type 2 diabetes, such as db/db or ZDF.

Evoked mechanical hypersensitivity in SNI, but not sham, animals begins to subside at 15 min, is significantly reduced at 30 min, and is maximally reversed at 60 min after systemic administration of gabapentin. This efficacy time course is similar to that of Park et al, where mechanical hypersensitivity alleviation and production of CPP occurs within 30 min of gabapentin administration in cisplatin-treated mice (Park et al, 2013).

Rapid attenuation of both evoked and spontaneous pain-like behavior after injury is likely due to gabapentin acting on α2δ calcium channel subunits to reduce neuronal excitability (Kukkar et al, 2013). Our results suggest that 30 min is enough time for gabapentin to affect α2δ channels at presynaptic terminals. The finding that gabapentin does not produce CPP or change mechanical thresholds in sham animals may be due to the finding that expression of α2δ subunits is increased after peripheral nerve injury (Luo et al, 2002; Nieto-Rostro et al, 2014). Furthermore, we found no effect of gabapentin on locomotor activity suggesting that adverse effects related to motor and exploratory activity are not present at the current dose. Effects on exploratory behavior would confound our conclusion that production of CPP indicates alleviation of spontaneous pain-like behavior after SNI. This is an important measure that is easily obtained by monitoring activity during CPP conditioning.
2.6.2 Methodological considerations in CPP

2.6.2.1 Production of CPP to systemic gabapentin after injury

To our knowledge, Park et al (2013) were the first to investigate CPP to gabapentin with the notable difference that the neuropathic pain-like model was generated by administration of the chemotherapeutic cisplatin. Additionally, the authors report that two but not one day of gabapentin pairing was necessary to produce a preference in cisplatin but not control mice. Gabapentin was administered systemically (i.p.) at the same dose used in the current study. They achieved CPP only after two days of conditioning with vehicle pairing in the morning and drug pairing in the afternoon. In another very recent study, gabapentin produced CPP in streptozotocin (STZ) diabetic but not naïve mice indicating, contrary to other reports, that STZ induction of type 1 diabetes produces a state of ongoing pain (Wagner et al, 2014). Production of CPP to gabapentin in STZ mice required three conditioning sessions and the authors used a biased approach (i.e. pairing gabapentin to the least-preferred chamber) because the CPP apparatus showed initial bias after the preconditioning test. We also used a biased approach while Park et al counterbalanced their pairings.

It should be noted that even though we used a biased approach, pairings were indeed counterbalanced because half of the rats received gabapentin in the white chamber while half were paired with the black, an approach also used by Wagner et al. Cunningham et al demonstrated that when there is no apparatus bias (i.e. on average, animals spend equal time in the black and white pairing chambers as in the current study), then the use of a biased or unbiased chamber-assignment approach does not affect the ability to produce CPP (Cunningham et al, 2003). Furthermore, the lack of gabapentin CPP in sham rats (current results) or vehicle-treated mice (Park et al, 2013) indicates that gabapentin is not intrinsically rewarding. This is important given the abuse
potential of analgesic drugs such as morphine that produce CPP in naïve, uninjured animals (Sufka, 1994; Tzschentke, 2007) and Figure 2-5.

**Figure 2-5. Morphine produces conditioned place preference in rats.** Difference scores (postconditioning minus preconditioning time spent in chamber) for animals that were conditioned with saline vehicle in both chambers (left) or vehicle in one chamber and morphine in the other (right). Systemic administration of morphine induced a significant place preference in rats.

2.6.2.2 Constant experimental conditions are critical to yield consistent CPP results

Neither SNI nor sham surgery altered baseline initial preference indicating that our CPP apparatus is unbiased, which is an important methodological consideration to eliminate experimental confounds. We were able to replicate very similar baseline values in SNI animals between Experiment 1 and Experiment 2 even though these studies were performed six months apart. Our CPP setup includes different tactile cues in the white and black pairing chambers; therefore, it is important to note that injury alone did not produce a preference to either tactile cue (grating versus bars) as evidenced by equal baseline preferences for the white and black chambers. Additionally, preference for a vanilla (white chamber) or kiwi (black chamber) chapstick olfactory cue was equal in
sham and SNI rats in both experiments (see Figure 2-6). We were extremely careful to handle the animals in the same manner within and between experiments and in the different injury groups. We used eight different CPP boxes and were able to achieve consistent average baselines between experiments. Although not directly tested, we should stress that experimental variables such as time of day of testing, handling method, the way animals are placed into the neutral grey chamber, cleaning boxes before and after each CPP session, etc. should be highly controlled to achieve consistent baselines as reported here. Carefully controlled variables will allow easier replication and increase the predictive value (i.e. sensitivity) of the CPP approach.

Figure 2-6. Baseline preferences with the addition of a chapstick olfactory cue. Counterbalanced addition of vanilla and kiwi chapstick to the pairing chambers in the conditioned place apparatus did not introduce a scent bias, as evidenced by equal time spent in each vanilla- or kiwi-scented chamber.

2.6.2.3 Minimization of time spent outside the pairing chambers

We cannot ignore the fact that, for whatever reason, conditioning the animals in both experiments resulted in an increase in time spent in the grey neutral chamber. It is possible that the stress of vehicle or gabapentin pairing (e.g. i.p. injections, handling,
cage manipulations) resulted in a slight aversion to the black and white chambers after conditioning, resulting in increased grey time in postconditioning. However, the negative reinforcement of pain by the gabapentin unconditioned stimulus was great enough to overcome any positive associations made to the grey chamber as evidenced by a significant difference score between saline and gabapentin and no differences between saline and grey or gabapentin and grey. If anything, part of the gabapentin reward is masked by the slight increase in grey chamber times indicating that we are underestimating the magnitude of the effects on motivation to seek pain relief. Another perspective is that since animals were never placed into the grey chamber after i.p. injection, this area was less aversive than the white or black chambers, which were paired with three i.p. injections each during conditioning. A third explanation is that after three days of conditioning in only the white and black chambers novelty of the grey chamber was reintroduced during postconditioning. A recent report indicates that injection of i.p. saline during habituation or preconditioning does not alter production of CPP and may even increase avoidance of the grey chamber after conditioning (Zhang et al., 2014). The use of a two chamber apparatus may also overcome this limitation, but also introduces additional methodological considerations beyond the scope of this discussion.

2.6.2.4 Expression of CPP after systemic drug administration

The technical challenge to expression of CPP is that the desired effect of the analgesic drug (i.e. unconditioned stimulus) needs to be rapid so that the animals associate analgesia with chamber contextual cues (i.e. conditioned stimulus). Furthermore, differential effects of drugs on evoked versus ongoing pain may contribute to the failure of injured animals from exhibiting CPP. A negative result (i.e. no expression of CPP to analgesic drug pairing) indicates that the experiment should be repeated in
the presence of a positive control (e.g. morphine production of CPP in naïve animals, clonidine in SNI animals, others).

2.6.3 Conclusions when experiments fail to produce CPP

Several recent studies highlight the differences of analgesic drugs on evoked and spontaneous pain as assessed by CPP testing. Wei et al (2013) found that the TRPA1 antagonist Chembridge-5861528 alleviates mechanical hypersensitivity but does not produce CPP in streptozotocin (STZ) treated rats, a model of diabetic neuropathy. However, the authors were able to produce CPP in SNI animals with intrathecal clonidine indicating that the CPP method was appropriate. The authors suggest that 15 min of conditioning may not be enough time for drug effects on spontaneous pain (Wei et al, 2013). In another STZ study, systemic administration of the gabapentin analog pregabalin alleviated evoked mechanical hypersensitivity but not spontaneous pain as evidenced by the lack of expression of CPP (Rutten et al, 2011). The authors suggested that CPP is not suitable for measurement of spontaneous pain in the STZ model. However, due to the lack of a positive control for the CPP assay, an alternative explanation is that CPP was not expressed due to methodological concerns, or that STZ does not invoke a state of ongoing aversiveness in rodents.

Our results suggest that CPP is indeed a predictive tool of the clinical efficacy of analgesic drugs such as gabapentin for the treatment of spontaneous neuropathic pain. However, enthusiasm may be diminished by the notion that normal ambulatory behavior produces a pain-like stimulus evoked by the tactile environment (Bennett, 2012). In other words, exploratory behavior in the CPP apparatus induces an evoked pain-like state. This concern is mitigated by the findings of Qu et al, who found that analgesic drugs produced CPP in axotomized, but not sham, animals. The authors conclude that “these observations in animals with complete denervation of the hind paw also provide an
important control, eliminating concerns for pain resulting from tactile stimulation potentially arising from ambulation within the testing apparatus” (Qu et al, 2011). In addition, it is unlikely that the low threshold, innocuous stimulation of normal ambulatory, exploratory movement in the CPP chamber is sufficient to produce ongoing affective pain.
CHAPTER 3: Pioglitazone rapidly reduces neuropathic pain through astrocyte and non-genomic PPARγ mechanisms

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3.1 Abstract

Repeated administration of peroxisome proliferator-activated receptor gamma (PPARγ) agonists reduces neuropathic pain-like behavior and associated changes in glial activation in the spinal cord dorsal horn. As PPARγ is a nuclear receptor, sustained changes in gene expression are widely believed to be the mechanism of pain reduction. However, we recently reported that a single intrathecal injection of pioglitazone, a PPARγ agonist, reduced hyperalgesia within 30 minutes, a time frame that is typically less than that required for genomic mechanisms. To determine the very rapid anti-hyperalgesic actions of PPARγ activation we administered pioglitazone to rats with spared nerve injury (SNI) and evaluated hyperalgesia. Pioglitazone inhibited hyperalgesia within 5 min of injection, consistent with a non-genomic mechanism.
Systemic or intrathecal administration of GW9662, a PPARγ antagonist, inhibited the anti-hyperalgesic actions of intraperitoneal or intrathecal pioglitazone, suggesting a spinal PPARγ-dependent mechanism. To further address the contribution of non-genomic mechanisms, we blocked new protein synthesis in the spinal cord with anisomycin. When co-administered intrathecally, anisomycin did not change pioglitazone anti-hyperalgesia at an early 7.5 min timepoint, further supporting a rapid non-genomic mechanism. At later timepoints anisomycin reduced pioglitazone anti-hyperalgesia, suggesting a delayed recruitment of genomic mechanisms. Pioglitazone reduction of SNI-induced increases in GFAP expression occurred more rapidly than expected, within 60 min. We are the first to show that activation of spinal PPARγ rapidly reduces neuropathic pain independent from canonical genomic activity. We conclude that acute pioglitazone inhibits neuropathic pain in part by reducing astrocyte activation, and via both genomic and non-genomic PPARγ mechanisms.

3.2 Introduction

Peroxisome proliferator-activated receptor gamma (PPARγ) is activated by thiazolidinedione (TZD) drugs such as pioglitazone (Saltiel and Olefsky, 1996) that cross the blood brain barrier (Maeshiba et al, 1997). TZDs reduce molecular and behavioral sequelae of many neurological diseases [see (Feinstein, 2003; Kapadia et al, 2008; Maeda and Kishioka, 2009; Yonutas and Sullivan, 2013)]. It is widely assumed that genomic PPARγ activity mediates reduction of inflammatory (Oliveira et al, 2007; Morgenweck et al, 2010) and neuropathic (Maeda et al, 2008; Morgenweck et al, 2013) pain after repeated TZD dosing. However, agonists for PPARγ and other nuclear receptors (e.g. estrogen, glucocorticoid, PPARα) can modulate neuronal excitability (Filardo et al, 2002; Levin, 2008; Joels et al, 2012) and pain (LoVerme et al, 2006; Churi et al, 2008; Fehrenbacher et al, 2009; Zhang et al, 2012) within minutes. Thus, the anti-
hyperalgesic effects of nuclear receptor agonists may occur through multiple mechanisms including: 1) genomic, receptor-dependent transcription/translation, 2) non-genomic, receptor-dependent activation of membrane-bound receptors (Luconi et al., 2010; Rowan et al., 2010; Joels et al., 2012), 3) receptor-independent “off-target” effects at GPCRs (Maggiolini and Picard, 2010; Nakamoto et al., 2012; Nakamoto et al., 2013).

Traumatic nerve injury produces changes in the spinal cord such as astrocyte activation (Zhang and De Koninck, 2006; Vega-Avelaira et al., 2007), increased post-synaptic responses to glutamate (Wei et al., 2008), and long-term potentiation (Gruber-Schoffnegger et al., 2013) resulting in central sensitization (Scholz and Woolf, 2007). During chronic pain states, activated spinal astrocytes release pro-nociceptive mediators (Watkins et al., 2001; Ohtori et al., 2004) such as TNFα that facilitate pain sensitization [for review see (Grace et al., 2014)]; therefore, pharmacotherapeutic approaches that decrease astrocyte activation may reduce chronic pain. TZDs agonize PPARγ expressed in spinal cord (Moreno et al., 2004) and brain (Cristiano et al., 2001) astrocytes to reduce activation (Bernardo and Minghetti, 2008) and GFAP upregulation (Heneka et al., 2005). Dosing over several weeks produced anti-hyperalgesia that was associated with reductions in spinal GFAP (Maeda et al., 2008; Jia et al., 2013; Morgenweck et al., 2013), injury-induced pro-inflammatory cytokine expression (Jia et al., 2010), and in vitro astrocyte TNFα release (Storer et al., 2005; Gurley et al., 2008). A single injection of PPARγ agonist reduces neuropathic pain (Churi et al., 2008); however, an important gap is whether there are acute effects on spinal astrocytes.

Here, we characterized the effects of PPARγ activation in neuropathic (spared nerve injury; SNI) and acute nociceptive (capsaicin) conditions after acute drug administration. We hypothesized that rapid inhibition of pain by PPARγ agonists is mediated by receptor-dependent, spinal mechanisms and is independent of translation (i.e. non-genomic). We used systemic and spinal agonist/antagonist administration to determine
the site of PPARγ anti-hyperalgesia. We investigated pain-like hypersensitivity as early as 5 min after intrathecal injection of pioglitazone in the presence or absence of anisomycin, an inhibitor of protein synthesis (Grollman and Walsh, 1967; Frey et al, 1988; Schafe and LeDoux, 2000). Finally, we tested whether a single pioglitazone injection, as opposed to repeated administration (Morgenweck et al, 2013), would reduce astrocyte activation in the form of GFAP overexpression after SNI.

3.3 Materials & Methods

3.3.1 Animals

Male Sprague-Dawley rats (CD-IGS, Charles River Laboratories, Inc., Wilmington, MA) weighing 300-450g at the time of behavioral procedures were housed 2 per cage on a 12-hour light/dark cycle (7am lights on / 7pm lights off) in a temperature (68-72 °F) and humidity controlled room with food and water provided ad libitum. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to in vivo techniques, in accordance with the International Association for the Study of Pain and the National Institutes of Health Office of Laboratory Animal Welfare Guide for the Care and Use of Laboratory Animals. All behavioral procedures were performed between 8am-6pm (lights on) and approved by an Institutional Animal Care and Use Committee (IACUC) protocol. Behavioral measurements and immunohistochemistry quantification were performed by an observer blinded to experimental treatments (e.g. injury and/or drug).

3.3.2 Spared Nerve Injury (SNI) surgery

Surgical anesthesia was achieved with isoflurane (5% induction and 1.5% maintenance diluted in oxygen). As previously described (Decosterd and Woolf, 2000; Taylor et al, 2007), the skin was incised on the left hindlimb over the sciatic nerve
trifurcation. The overlying muscles were retracted to expose the common peroneal, tibial, and sural nerves. The common peroneal and tibial nerves were ligated with 6-0 silk (Ethicon, Somerville, NJ) and transected 1mm proximal and 1mm distal to the ligation. The ligation knot and adjacent nerve were removed. Sural nerve perturbations were avoided. The muscle and skin were closed with loosely tied 5-0 absorbable sutures (Ethicon) and 9mm stainless steel wound clips, respectively. During sham surgeries, all steps were performed except ligation and transection of the common peroneal and tibial nerves. SNI or sham surgery day is referred to as day 0.

### 3.3.3 Pain-like behavior.

Animals were acclimated in individual Plexiglas boxes (4” x 8” x 4”) on top of a raised stainless steel mesh grid (mechanical and cold) or Plexiglas floor (Hargreaves) for 1 h. Fluctuations in noise, vibrations, temperature, and other distractors in the behavioral testing room were avoided to optimize reliable measurements between cohorts of animals tested on different days.

Mechanical hypersensitivity was assessed using von Frey filaments (Stoelting, Inc., Wooddale, IL). The lateral aspect of the hindpaw plantar surface (sural receptive field) was stimulated with an incremental series of 8 monofilaments of logarithmic stiffness using a modified up-down method (Dixon, 1980; Chaplan et al, 1994). Each filament was applied to the sural receptive field three times at proximal, intermediate, and distal locations with respect to the heel. Testing began by applying an intermediate von Frey monofilament (number 4.31, exerts 2.0 g of force) perpendicular to the glabrous skin, causing a slight bending. In the case of a positive response (withdraw of the paw) a filament exerting less force was applied. In the case of a negative response, a filament exerting greater force was applied. The calculated 50% withdraw threshold is reported.
Cold hypersensitivity was assessed after application of a drop of acetone to the sural receptive field. We used a 3 mL syringe attached to an 8 cm length of PE-10 tubing flared to a diameter of 3.5 mm at the distal end. Surface tension maintained the volume of the drop to 10\textsuperscript{-12} μl. The amount of time the animal lifted, shook, or licked the affected hindpaw was recorded with a cutoff of 30 s after each acetone application. The average of three trials per subject at each timepoint is reported.

Heat hypersensitivity was assessed by recording paw withdraw latencies using the Hargreaves method and apparatus (Hargreaves et al, 1988). An adjustable infrared heat source (8 V, 50 W lamp, Ugo Basile, Italy) was positioned under the Plexiglas floor directly beneath the uninjured hindpaw. Prior to each behavioral testing session, intensity was adjusted so that the average latency to paw withdraw was 9 ± 2 s. If an animal did not respond within 20 s, the radiant beam was shut off to avoid tissue damage. The average of three trials per subject at each timepoint is reported.

3.3.4 Motor coordination.

Motor coordination was assessed by placing the animals on an accelerating rotarod (Stoelting, Wood Dale, IL). Beginning at 2 revolutions per minute (rpm), the rotarod machine was programmed to accelerate 0.5 rpm every 5 s until reaching 60 rpm. Animals were acclimated to the rotarod and subjected to one training session per d for 2 consecutive d prior to drug administration. During training and testing, the rats were placed on the rotating bar at 2 rpm, the acceleration program was started, and the time spent on the rotarod prior to fall was recorded. During the first training session, the animals were repeatedly placed on the rotarod until they performed 3 consecutive trials ≥ 150 s or reached a predetermined cutoff of 20 trials, whichever occurred first. By the second day of training, all rats successfully completed 3 consecutive trials ≥ 150 s prior to reaching 20 trials. Baseline measurement of time spent on the rotarod was recorded 7
d after SNI followed by reassessment at 1, 2, and 3 h after i.p. or i.t. drug administration. Three trials per timepoint were averaged.

3.3.5 *Intracerebroventricular cannulation.*

Surgery was performed one week before injury to allow for 5 μl of drug or vehicle injection directly into the ventricle. Surgical anesthesia was achieved with isoflurane (5% for induction, 1.5% for maintenance in oxygen). Rats were placed in a stereotaxic apparatus fitted with blunt ear bars (Stoelting, Kiel, WI). After an incision to expose the cranium, the dorsal surface of the skull was leveled by zeroing the dorsoventral coordinate at lambda and bregma. A 26Ga stainless steel guide cannula (Plastics One, Roanoke, VA) was lowered to the right lateral brain ventricle using the following stereotaxic coordinates: 0.7 mm posterior to bregma, 1.5 mm lateral from midline and 3.3-4.0 mm below the skull surface (Paxinos and Watson, 1997). Correct placement was indicated by the observation of the movement of 1μl sterile saline from a piece of PE-10 tubing attached to the cannula into the ventricle (Taylor et al, 1994). The cannula was fixed to the skull with 3 small screws and dental cement and after suturing the incision a 30 Ga stylet (Plastics One) was secured within the guide cannula.

3.3.6 *Drugs*

Pioglitazone potassium salt (10028, Cayman Chemical, Ann Arbor, MI) was dissolved in a 0.9 % saline slurry. GW9662 (70785, Cayman Chemical) was dissolved in a mixture of ethanol, ethoxylated castor oil, and saline (2:2:6) for i.t. administration and 50% DMSO in saline for i.p. administration. Neither vehicle solution altered pain-like behavior (see Fig 2). Anisomycin (A9789, Sigma-Aldrich, St. Louis, MO) was first dissolved in a small volume of 1 M HCl and then an equal volume of 0.9% saline was added. Next, the solution was titrated with NaOH to obtain a physiological pH of 7.0-7.4.
Finally, the anisomycin solution was further diluted in saline to a final concentration of 20 mg/ml. Capsaicin (M2028, Sigma-Aldrich) was first dissolved in 100% ethanol (5% final v/v) followed by addition of a Tween 80 (P1754, Sigma-Aldrich) in saline (8% v/v) solution to a final capsaicin concentration of 50 mg/ml (w/v). Capsaicin (50 μl) was injected into the subdermal space between the 2nd and 3rd digits on the ventral plantar surface using a 30 Ga ½” needle.

3.3.7 Drug Injections

Rats were anesthetized with isoflurane. Vehicle or drug solution (10-20 μl) was injected into the subarachnoid space using a 27 Ga 1” needle inserted into a stretch of PE-20 tubing and attached to a Hamilton micro syringe (Mestre et al, 1994). We confirmed needle placement by both visualization of cerebrospinal fluid aspiration within the tubing and/or a reflexive tail/hindpaw flick. For systemic pioglitazone studies, i.p. (2 or 10 mg/kg) or i.t. GW9662 (300 μg) was administered 15 min prior to pioglitazone. For intrathecal pioglitazone studies, GW9662 (300 μg) or anisomycin (0-200 μg) was co-administered with pioglitazone to avoid multiple i.t. injections.

3.3.8 Capsaicin-induced nociception.

Intrathecal vehicle (saline), pioglitazone (0-300 μg), or anisomycin (0-200 μg) were injected 20 min before intraplantar (i.pl.) capsaicin (50 μg in 50 μl). Behavior was recorded for 0-2 min following i.pl. capsaicin. The number of flinches and the time spent licking or lifting (s) the affected hindpaw were combined and reported as the number of nociceptive responses, where 1 s of licking/lifting is equal to one flinch response (Seabrook et al, 2002; Lu et al, 2009; Andersson et al, 2013). Sham injections consisted of capsaicin vehicle as a control. Rats were perfused 60 min following i.pl. capsaicin for Fos immunohistochemistry.
3.3.9 Immunohistochemical quantification of Fos and GFAP in the dorsal horn.

Rats were anesthetized with pentobarbital (Fatal Plus, Med-Vet International, Mettawa, IL) and perfused through the left ventricle with 250 ml of room temperature 0.1 M phosphate buffered saline (PBS) with heparin (10,000 USP units/L) followed by 250 ml of ice-cold fixative (10% phosphate buffered formalin). The lumbar spinal cord was removed and post-fixed overnight in 10% phosphate buffered formalin and then cryoprotected in 30% sucrose in 0.1 M PBS for several days. Transverse sections (30 μm) from L4-L5 were cut on a freezing microtome and collected in 0.1 M PBS. The sections were washed three times in 0.1 M PBS and then pretreated with blocking solution (3% normal goat serum and 0.3% Triton X-100 in 0.1 M PBS) for 1 h. Sections were incubated overnight at room temperature in blocking solution containing either rabbit anti-Fos (1:500, SC-52, Santa Cruz Biotechnology, Santa Cruz, CA) for capsaicin studies or anti-GFAP (1:1000, ab7779, Abcam, Cambridge, MA) for SNI studies. The slices were washed three times in 0.1 M PBS, incubated in goat anti-rabbit (1:800, Alexa 488 or 568, Molecular Probes, Grand Island, NY) for 90 min, washed in 0.1 M PBS then 0.01 M PB, mounted onto Superfrost Plus slides, air dried, and cover-slipped using Prolong Gold with DAPI mounting medium (Molecular Probes, Grand Island, NY).

All images were captured and analyzed on a Nikon Eclipse TE2000-E microscope using 4x or 10x objectives using NIS-Elements Advanced Research software. In capsaicin studies we quantified the number of Fos+ cell profiles in lamina I-V of the dorsal horn. In SNI studies the dorsal horn was separated into lamina I-II, lamina III-IV, and lamina V as well as medial, central, and lateral regions of interest (ROI). Medial-lateral ROI correspond to afferent input from the tibial (medial), common peroneal (central), and sural (lateral) receptive fields as previously described (Corder et al, 2010). Each of the six ROI were quantified and analyzed separately. For GFAP, pixel intensity values were summed, normalized to ROI area, and background subtracted. Background
intensity was determined by selecting a ROI in the devoid of GFAP+ cells. This method accounts for changes in both the number and the fluorescence intensity of GFAP+ cells. Slices were analyzed by an observer blinded to treatment. Quantification of 4-6 slices per subject was averaged for each ROI and n=3-4 rats per group are reported.

3.3.10 Western blotting of lumbar spinal GFAP.

Western blot analyses were performed on lumbar spinal cord quadrants that were sonicated on ice in 50 mM Tris buffer containing 100 mM 6-amino-n-caproic acid, 1 mM EDTA, 5 mM benzamidine, 0.2 mM phenylmethyl sulfonyl fluoride (in 100% ethanol), and protease inhibitors. After extraction, proteins were subjected to NuPAGE Bis–Tris (4-12%) gel electrophoresis under reducing conditions (Invitrogen, Carlsbad, CA) and then transferred to nitrocellulose membranes electrophoretically (Invitrogen, Carlsbad, CA). Nonspecific binding sites on the membrane were blocked with Odyssey Blocking Buffer (50%; LI-COR Biosciences, Lincoln, NE) in TBS containing 0.1% Tween-20, 0.05% Tris-Chloride, and 0.03% 5 M NaCl for 1 h at 22-24°C. Membranes were subsequently incubated with primary antibodies in Odyssey Blocking buffer containing 0.1% Tween-20 overnight at 4°C. The membranes were then washed with PBS containing 0.1% Tween-20, and probed with appropriate IRDye secondary antibodies (LI-COR Biosciences) in Odyssey Blocking buffer containing 0.1% Tween-20 for 1 h at 22-24°C, protected from light. Following washing with PBS containing 0.1% Tween-20, membranes were scanned on an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). Primary antibodies and dilution ratios used were rabbit GFAP (1:1000, z0334, Dako, Carpinteria, CA), and mouse β actin (1:100,000, A5316, Sigma-Aldrich, St. Louis, MO). Secondary antibodies used were goat anti-mouse IRDye 680RD (1:15,000, LI-COR Biosciences), goat anti-rabbit IRDye 800CW (1:15,000, LI-COR Biosciences). Bands were quantified using Image Studio (LI-COR Biosciences).
3.3.11 Statistical analysis.

For analysis of behavioral data and westerns, multiple groups were compared for significant differences over time using repeated measures two-way ANOVA or between groups using a standard two-way ANOVA followed by Holm-Sidak multiple comparison correction. Area-under-the-curve (AUC) was calculated using the trapezoidal method and analyzed using a one-way ANOVA with Holm-Sidak multiple comparison correction or an unpaired, two-tailed t-test. Ipsilateral versus contralateral (paired) or pioglitazone versus saline (unpaired) comparisons of GFAP immunohistochemistry ROIs (6 total analyses) or GFAP western quadrants were analyzed using a two-tailed t-test. An alpha value of $\alpha=0.05$ was used to determine statistical significance. All data were analyzed and graphed using Prism 6.0 (GraphPad, La Jolla, CA) and are presented as mean ± SEM.

3.4 Results

3.4.1 Systemic pioglitazone reduces mechanical and cold hypersensitivity

To determine whether PPARγ activation reduces hyperalgesia, we evaluated behavioral indices of neuropathic pain after a single i.p. injection of pioglitazone. Spared nerve injury (SNI) decreased mechanical [time; F (1, 37) = 1453; P < 0.0001] and cold [time; F (1, 37) = 86; P < 0.0001] sensitivities at 14 d after injury. As illustrated in Fig 1A-B, systemic pioglitazone attenuated mechanical [dose x time; F (21, 259) = 5.756; P < 0.0001] and cold hypersensitivity [F (21, 259) = 3.485; P < 0.0001]. Area under the curve (AUC) analyses in Fig 1C-D indicate that pioglitazone dose-dependently attenuated mechanical [dose; F (3, 37) = 6.747; P = 0.001] and cold hypersensitivity [F (3, 37) = 3.575; P = 0.023].
3.4.2 *PPARγ in the spinal cord mediates the anti-hyperalgesic actions of systemic pioglitazone*

Churi *et al.* reported that a single intrathecal injection of the PPARγ agonist rosiglitazone produced anti-hyperalgesia within one hour (Churi *et al.*, 2008). However, the authors did not test for PPARγ-dependency. To address this gap and test the hypothesis that PPARγ mediates the acute anti-hyperalgesic effects of i.p. pioglitazone, we pretreated SNI rats with GW9662, an irreversible PPARγ antagonist (Leesnitzer *et al.*, 2002; Chen *et al.*, 2004). As illustrated in Fig 2A-D, systemic GW9662 (i.p.) prevented pioglitazone reductions in mechanical [AUC; $F(2,25) = 13.14; P = 0.0001$] and cold [AUC; $F(2,27) = 3.992; P = 0.03$] hypersensitivity.

Next we sought to determine whether the anti-hyperalgesic effects of systemic pioglitazone are mediated through activation of spinal PPARγ. As illustrated in Fig 2E-H, intrathecal (i.t.) GW9662 attenuated pioglitazone reduction of mechanical [AUC; $F(3,23) = 18.55; P < 0.0001$] and cold [AUC; $F(3,23) =12.85; P < 0.0001$] hypersensitivity. Both systemic and spinal administration of GW9226 attenuated the reduction of mechanical and cold hypersensitivity by administration of systemic pioglitazone.
Figure 3-1. A single systemic injection of pioglitazone (Pio) dose-dependently reduced behavioral signs of neuropathic pain. Sparred nerve injury (SNI, arrow) resulted in pain-like hypersensitivity. Intraperitoneal (i.p.) pioglitazone dose-dependently reversed (A) mechanical and (B) cold hypersensitivity 14d after SNI. Pain-related behavioral responses to plantar application of von Frey and acetone stimuli were measured before (0d) and after (14d) SNI and at 15, 30, 60, 90, 120, 180, and 240 min after intraperitoneal (i.p.) injection of pioglitazone (0, 10, 30, 100 mg/kg) in saline vehicle. Area under the curve (AUC) is shown for (C) von Frey and (D) acetone pain-related behaviors. ★ “i.p. Pio 100 mg/kg” significantly different from “i.p. Saline”. † “i.p. Pio 30 mg/kg” and “i.p. Pio 100 mg/kg” significantly different from “i.p. Saline”. “n” are shown in parentheses.
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Figure 3-2. Anti-hyperalgesic actions of systemic pioglitazone are mediated by spinal PPARγ.
Systemic GW9662 (GW), a PPARγ antagonist, blocked pioglitazone (Pio) reduction of (A-B) mechanical and (C-D) cold hypersensitivity 14d after SNI. (E-F) Spinal GW completely abolished pioglitazone reduction of mechanical hyperalgesia and (G-H) partially blocked pioglitazone alleviation of cold hypersensitivity. GW was administered i.p. or i.t. 15 min prior to i.p. pioglitazone. ★ significantly different from “i.p. GW 10 mg/kg” or “i.p. Pio 100 mg/kg + i.t. GW 300μg”. † significantly different from “i.p. GW 2 mg/kg” and “i.p. GW 10 mg/kg”. # significantly different from “i.p. Saline + i.t. Vehicle”. ’n’ are shown in parentheses.

3.4.3 PPARγ in the spinal cord mediates the anti-hyperalgesic actions of intrathecal pioglitazone

To support the PPARγ antagonist experiment above in identifying the spinal cord as a key site of PPARγ anti-hyperalgesic action, we administered pioglitazone and/or GW9662 by the intrathecal or intracerebroventricular route. Fig 3A demonstrates that i.t. pioglitazone attenuated mechanical hyperalgesia [dose x time; F (18, 162) = 2.951; P = 0.0001] as rapid as 30 min after injection in a dose-dependent manner. To account for possible supraspinal activation of PPARγ after i.t. pioglitazone via the cerebrospinal fluid circulation, we injected the same doses via the intracerebroventricular (i.c.v.) route. We found no anti-hyperalgesic effect of pioglitazone at these doses [F (3, 21) = 1.959; P = 0.15]. Fig 3B illustrates that co-administration of the PPARγ antagonist GW9662 completely blocked the anti-hyperalgesic actions of i.t. pioglitazone [drug x time; F (18, 198) = 16.26; P < 0.0001].
Figure 3-3. Spinal PPARγ mediates pioglitazone anti-hyperalgesia. (A) Intrathecal (i.t.) pioglitazone (Pio) dose-dependently reduced mechanical hyperalgesia at doses that have no effect when administered by the intracerebroventricular (i.c.v.) route. (B) Intrathecal co-administration of the PPARγ antagonist GW9662 (GW) completely reversed pioglitazone anti-hyperalgesia. † high and medium ‡ high, medium, low dose Pio significantly different from “i.t. Saline”. ★ significantly different from “i.t. Saline + i.t. Vehicle”. ‘n’ are shown in parentheses.

3.4.4 PPARγ activation does not produce motor deficits or analgesia

We determined whether the anti-hyperalgesic actions of pioglitazone might be confounded by off-target effects on motor systems or transient reflexive pain (Taylor, 2001). Fig 4A-B illustrates that a relatively high dose of pioglitazone did not change rotarod performance after i.p. \( p = 0.81 \) or i.t. \( p = 0.35 \) injection. Because von Frey
mechanical withdraw thresholds are maximal in uninjured rats (e.g. 15 g), we assessed response latencies to noxious heat to determine if pioglitazone altered acute nociception in sham rats. Intrathecal pioglitazone did not change heat response latencies \([p = 0.5]\) suggesting that the effects of PPAR\(\gamma\) activation are not analgesic. Together with our previous reports (Churi et al, 2008; Morgenweck et al, 2010; Morgenweck et al, 2013), we conclude that reduction of mechanical hyperalgesia by PPAR\(\gamma\) agonists is not secondary to adverse effects on motor coordination or normal sensory thresholds.
Figure 3-4. Pioglitazone did not produce ataxia or changes in transient nociception.

Motor coordination was assessed on an accelerating rotarod before and at 1, 2, and 3 h after high dose pioglitazone (Pio) administration by (A) systemic (i.p. 100 mg/kg) or (B) spinal (i.t. 1000 μg) routes. Pioglitazone did not change time spent on the accelerating rotarod. (C) The analgesic effects of pioglitazone were tested using the
Hargreaves assay before and after intrathecal saline or pioglitazone administration in sham rats. Pioglitazone did not change paw withdraw latencies to noxious heat. n = 8-10 per group.

3.4.5 PPARγ agonists rapidly reduce nociceptive and neuropathic pain-like behavior

The above studies illustrate that i.t. administration of pioglitazone reduces tactile hypersensitivity within 30 min of administration in SNI rats. Consistent with this finding, intrathecal pioglitazone, compared to saline, reduced nociceptive behavior when given 20 min prior to intraplantar capsaicin (nociceptive responses; 16.3 ± 2.3 vs. 62.2 ± 16.1; n = 5-6) [p = 0.013]. To test the hypothesis that PPARγ anti-hyperalgesic mechanisms occur rapidly after traumatic nerve injury, we extended our temporal analysis of pain-like behavior in the SNI model to earlier time points. Fig 5A-B demonstrates that the PPARγ agonists rosiglitazone [F (1, 9) = 5.942; P = 0.0375] and pioglitazone [F (1, 8) = 28.24; P = 0.0007] attenuated SNI-associated mechanical hypersensitivity within 5 min of injection. We repeated the study with 7.5 min as the first behavioral timepoint to allow full recovery from isoflurane anesthesia, as evidenced by the slight increase in von Frey thresholds in the saline control group in Fig 5B. Fig 5C illustrates a robust anti-hyperalgesic effect of pioglitazone [F (1, 14) =26.62; P = 0.0001] and at the 7.5 minute timepoint [p < 0.0001] in the absence of a residual anesthetic effect [p = 0.9998]. Our findings that rosiglitazone and pioglitazone produce anti-hyperalgesia and anti-nociception within 5-20 min of administration suggest that non-genomic PPARγ mechanisms mediate rapid reduction of pain-like behavior.
Figure 3-5. PPARγ agonists rapidly reduce mechanical hyperalgesia.
Intrathecal injection of the PPARγ ligands (A) rosiglitazone (Rosi) or (B) pioglitazone (Pio) rapidly (within 5 min) reduced mechanical hypersensitivity 14d after SNI. Intrathecal injections were performed under isoflurane anesthesia, which resulted in a small anti-hyperalgesic effect at 5 min. Therefore, we repeated the experiment with 7.5 min the earliest timepoint tested. (C) Pioglitazone rapidly attenuated mechanical hypersensitivity that lasted from 7.5 to 90 min with no anesthesia effect.
in the saline group. ★ significantly different from “i.t. Saline”. ‘n’ are shown in parentheses.

3.4.6 In vivo blockade of protein translation in the dorsal horn by intrathecal anisomycin

To examine whether a translation-independent (i.e. non-genomic) mechanism mediates the rapid anti-hyperalgesic effects of PPARγ, we administered intrathecal anisomycin to block protein synthesis in the dorsal horn in vivo at a dose that did not produce confounding alterations in nociception (Kim et al., 1998). To achieve this, we evaluated the effect of multiple doses of anisomycin, based on previously used doses of 25 to 125 µg (Schafe and LeDoux, 2000; Parsons et al., 2006; Asiedu et al., 2011; Bonin and De Koninck, 2014), on capsaicin-induced protein (i.e. Fos) expression. Capsaicin activates TRPV1-positive primary afferent nociceptors (Brederson et al., 2013) resulting in spontaneous, dose-dependent pain in humans (Simone et al., 1989). Intraplantar injection of capsaicin in rodents elicits spontaneous nociceptive behavior (Simone et al., 1987) and induces Fos expression in the superficial dorsal horn within 30 to 90 min (Hossaini et al., 2010; Beaudry et al., 2011), providing a suitable tool for evaluating effects of anisomycin on new protein synthesis.

As depicted in Fig 6A, we administered intrathecal pioglitazone and/or anisomycin twenty minutes prior to evaluation of capsaicin-evoked nociceptive behavior. Sixty minutes after capsaicin injection, we euthanized animals for quantification of Fos expression in the L4-L5 dorsal horn. As illustrated in Fig 6B, combinations of capsaicin, pioglitazone, and anisomycin administration significantly altered Fos expression in laminae I-V [group; F (7, 17) = 9.38; P < 0.0001]. Compared to vehicle injection, intraplantar capsaicin increased Fos expression [p = 0.007], an effect that was dose-dependently inhibited by pretreatment with 100-200 µg doses of anisomycin [p < 0.05] but not 50 µg [p = 0.064]. Anisomycin (200 µg) also reduced Fos expression when co-
administered with pioglitazone \( [p = 0.0006] \). Pioglitazone did not alter capsaicin-evoked Fos \( [p = 0.74] \) when administered alone.

We offer two explanations for the lack of effect of pioglitazone on spinal Fos expression even though nociceptive behavior was reduced after intraplantar capsaicin injection. First, Fos and behavior do not always positively correlate: drug-induced reduction of pain-like behavior can occur in the absence of (Harris, 1998; Coggeshall, 2005; Gao and Ji, 2009) or with increases in (Orii et al., 2002) Fos expression. Second, the target of pioglitazone, PPARγ, directly activates c-fos transcription (Rogue et al., 2010). If pioglitazone directly increases Fos expression in inhibitory interneurons, which can account for a significant population of total cells expressing Fos (Todd et al., 1994), then this would confound the ability of our Fos method to reflect noxious stimulus-evoked spinal neuron activation.

3.4.7 Anisomycin does not alter the anti-nociceptive effects of intrathecal pioglitazone

Having validated that anisomycin reduces protein translation (i.e. Fos) in the dorsal horn, we used this method to explore the anti-nociceptive and translation-independent actions of PPARγ activation in the capsaicin model. As illustrated in Fig 6C, combinations of capsaicin, pioglitazone, and anisomycin administration significantly altered nociceptive behavior \([\text{group}; F (7, 35) = 5.476; P = 0.0003]\). Intraplantar capsaicin markedly increased nociceptive behavior when compared to vehicle injection \([p = 0.0055]\). Anisomycin alone (50-200 μg) did not alter capsaicin-evoked nociceptive behavior \([p > 0.05]\). Pioglitazone reduced nociceptive behavior when administered alone \([p = 0.0029]\) or in combination with the 200 μg dose of anisomycin \([p = 0.0029]\). These results indicate that anisomycin blocks genomic activity (i.e. Fos expression) in the dorsal horn without altering nociceptive behavior or pioglitazone anti-nociception, providing proof of principle for this method.
Figure 3-6. Anisomycin reduces translation of capsaicin-evoked Fos without blocking the rapid anti-nociceptive actions of pioglitazone.

(A) Experimental timeline to test the hypothesis that capsaicin evoked Fos translation is inhibited by intrathecal anisomycin pretreatment. (B) Vehicle (0μg Cap), pioglitazone (Pio), and/or anisomycin (Ani) were co-administered i.t. 20 min prior to i.pl. capsaicin (50μg). Capsaicin-induced spontaneous licking, lifting, and flinching nociceptive responses from 0-2 min after injection were almost completely abolished by pioglitazone (300μg) alone or in combination with anisomycin (200μg) pretreatment. Anisomycin (50-200μg) alone did not alter capsaicin nociception. (C) Capsaicin-evoked Fos expression was dose-dependently inhibited by anisomycin and unaltered by pioglitazone. (D) Sham injection of vehicle produced moderate Fos expression when compared to (E) i.t. vehicle injections followed by i.pl. capsaicin. (F) 50μg, (G) 100μg, (H) 150μg, and (I) 200μg anisomycin dose-dependently reduced Fos. (J) Pioglitazone alone had no effect on Fos expression while (K) anisomycin completely abolished Fos when co-administered with pioglitazone. ★ significantly different from capsaicin only (black bars). n = 3-9 for behavioral and n = 3-4 for Fos analyses.
3.4.8 *Spinal anisomycin does not change the rapid anti-hyperalgesic effects of pioglitazone*

Next we extended spinal anisomycin blockade of translation in the dorsal horn to the SNI model of neuropathic pain. We began with a dose of anisomycin that, when administered into the brain, inhibits conditioned taste aversion (Rosenblum *et al.*, 1993), auditory fear conditioning (Schafe and LeDoux, 2000; Parsons *et al.*, 2006; Parsons *et al.*, 2006), and incorporation of radioactive methionine into nascent proteins (Rosenblum *et al.*, 1993; Parsons *et al.*, 2006). We found that the 200 µg dose of anisomycin abolished capsaicin-evoked Fos, but also produced a confounding attenuation of SNI-induced mechanical hypersensitivity. By lowering the anisomycin dose to 100 µg, we obtained a significant reduction in Fos without altering SNI-induced hypersensitivity. As illustrated in Fig 7A, combinations of intrathecal vehicle, pioglitazone, or anisomycin changed mechanical sensitivity in SNI rats when analyzed across the entire 240 min time course [drug x time; F (24, 168) = 3.416; P < 0.0001]. Further analysis of the 7.5 - 120 min timepoints revealed that pioglitazone attenuated mechanical hypersensitivity when injected alone [vs. Vehicle + Saline; F (1, 9) = 29.41; P = 0.004] or with 100 µg anisomycin [vs. Vehicle + Saline; F (1, 11) = 5.098; P = 0.045]. At this dose, anisomycin alone did not inhibit mechanical hypersensitivity [p = 0.54]. To compare the effect of anisomycin on pioglitazone anti-hyperalgesia at early versus delayed timepoints, we analyzed the data at each of four timepoints (Fig 7B): before intrathecal injections (pre-drug), during early pioglitazone anti-hyperalgesia (7.5 min), during late pioglitazone anti-hyperalgesia (60 min), and after anti-hyperalgesia resolved (180 min). Compared to intrathecal vehicle, pioglitazone [p = 0.013] and pioglitazone plus anisomycin [p = 0.026] attenuated hypersensitivity at 7.5 min when compared to intrathecal vehicle only [drug; F (3, 20) = 5.173; P = 0.008]. By contrast, the delayed anti-hyperalgesic actions of
Pioglitazone at 60 min \( [p = 0.003 \text{ vs. Vehicle + Saline}] \) were significantly reduced by anisomycin \( [p = 0.021 \text{ vs. Vehicle + Pio}] \).

**Figure 3-7. Early but not late pioglitazone anti-hyperalgesia is independent of translation.**

To test whether immediate PPARγ anti-hyperalgesia is dependent on canonical genomic activity, we co-administered pioglitazone with an anisomycin dose that blocks translation but not nociception (100μg; see discussion for details). (A) Timecourse of mechanical thresholds after i.t. injection of pioglitazone (Pio) or anisomycin (Ani). (B) Analysis of mechanical thresholds at 7.5, 60 and 180 min timepoints. Anisomycin did not change the anti-hyperalgesic effects of pioglitazone at the early 7.5 min period, but reduced pioglitazone anti-hyperalgesia at 60 min. These results suggest that pioglitazone produces its anti-hyperalgesic effects by both translation-independent (7.5 min) and translational-dependent (60 min) mechanisms. * significantly different. ‘n’ are shown in parentheses.
3.4.9 *Acute pioglitazone reduces expression of GFAP after nerve injury*

Neuropathic pain is associated with astrocyte activation after nerve injury (Zhang and De Koninck, 2006). We previously reported that repeated administration of pioglitazone reduced not only established neuropathic pain, but also astrocyte activation in the dorsal horn of SNI rats (Morgenweck *et al*, 2013). To determine whether a single administration of pioglitazone also reduces astrocyte activation after SNI, we evaluated the protein expression of GFAP in the lumbar dorsal horn using both immunohistochemistry and western blot as soon as 60 min.

Similar to previous reports (Zhang and De Koninck, 2006), Fig 8A-B illustrates that SNI produced a unilateral increase in GFAP expression in the ipsilateral dorsal horn 14d after injury [ipsi vs. contra; F (1, 12) = 8.55; P = 0.0127]. This occurred in both injured (tibial and common peroneal) and uninjured (sural) innervation territories [t-test; p < 0.05]. Fig 8C-D illustrates that pioglitazone reduced GFAP expression in the contralateral and ipsilateral dorsal horn within 60 min of injection [drug; F (1, 8) = 74.93; P < 0.0001].
Figure 3-8. Acute PPARγ activation reduces GFAP expression.

(A) Spared nerve injury produced an increase in GFAP expression on the injured (ipsilateral) side when compared to the uninjured (contralateral) side of the lumbar dorsal horn. (B) SNI-induced a GFAP increase in injured (tibial and common peroneal) and uninjured (sural) innervation territories. (C-D) Pioglitazone (100 mg/kg i.p.; 1 h prior to perfusion) reduced GFAP expression on both the contralateral and ipsilateral dorsal horn. Each region of interest was analyzed separately using a two-tailed t-test. # significantly different from “contralateral” in the “Saline” group (paired). ★ significantly different from “Saline” (unpaired). n = 3 per group.

To confirm that the acute reduction of GFAP expression elicited by pioglitazone (Fig 8) did not result from structural changes that mask the GFAP antibody epitope in fixed tissue, we performed denaturing western blots. At 14 d after sham or SNI surgery, we administered i.p. pioglitazone, measured behavioral anti-hyperalgesia, and harvested L4-5 spinal cord quadrants 90 min later. As illustrated in Fig 9A, pioglitazone but not saline reduced mechanical hyperalgesia in SNI animals [drug; F (1, 10) = 11.17; P = 0.0075] at 60 [p = 0.023] and 90 [p < 0.0001] min after administration. Western blot analysis of the four lumbar quadrants is reported in Fig 9B (dorsal) and Fig 9C (ventral). Neither injury [p = 0.58] nor drug [p = 0.33] treatment changed GFAP expression in the
contralateral dorsal horn. By contrast, we found a significant injury x drug interaction in the ipsilateral dorsal horn [F (1, 20) = 11.39; P = 0.003]. Post-hoc tests revealed that SNI increased GFAP expression when compared to sham animals treated with saline [p = 0.0014]. This increase was reduced by pioglitazone in the ipsilateral [p = 0.0026] but not contralateral [p = 0.54] dorsal horn of SNI animals (Fig 9B). In the contralateral ventral horn, neither injury [p = 0.14] nor pioglitazone [p = 0.40] changed GFAP expression. Injury [p = 0.41] or pioglitazone [p = 0.84] did not change GFAP expression in the ipsilateral ventral horn.

Figure 3-9. SNI is required for pioglitazone reduction of pain and astrocyte activation.

To determine whether pioglitazone is toxic to astrocytes and to rule out conformational changes in the GFAP antibody epitope during immunohistochemical analysis we performed a denaturing western blot. Spinal cord quadrants (L4-5) were harvested 90 min after i.p. pioglitazone administration at d14 after sham or spared nerve injury (SNI) surgery. (A) Pioglitazone attenuated mechanical hypersensitivity in SNI, but not sham, animals. (B) Dorsal and (C) Ventral integrated densities normalized to Sham + Saline are shown. There was no effect of injury or drug treatment in the ventral horn or in the contralateral dorsal horn segments. GFAP expression was increased in the ipsilateral dorsal horn of SNI + Saline animals when compared to all other groups. Pioglitazone significantly reduced ipsilateral dorsal horn GFAP expression in SNI, but not sham, animals. This suggests that the anti-hyperalgesic effects of pioglitazone are associated with decreased astrocyte activation after nerve injury. ★ significantly different from "SNI + Saline". n=6-7 per group.
3.5 Discussion

3.5.1 Anisomycin to assess translation-independent activity of nuclear receptors in the spinal cord

Here we used in vivo administration of anisomycin to dissect genomic versus non-genomic mechanisms of a nuclear receptor in the spinal cord. Previous studies using anisomycin suggested that protein translation is necessary for late phase LTP in the brain (Krug et al., 1984; Frey et al., 1988), hyperalgesic priming in the peripheral nervous system (Asiedu et al., 2011), ongoing pain transmission in the spinal cord (Kim et al., 1998), and reduction of established hyperalgesia during pain memory reconsolidation in the spinal cord (Bonin and De Koninck, 2014). As these studies did not confirm inhibition of translation, we found it important to demonstrate that intrathecal anisomycin inhibits spinal protein expression. We found that anisomycin, at a dose of 100 µg, decreased expression of capsaicin-evoked Fos without changing SNI-induced hyperalgesia. This provides proof of principle for using this approach to determine the contribution of non-genomic PPARγ mechanisms to the rapid and delayed phases of pioglitazone anti-hyperalgesia.

3.5.2 Non-genomic PPARγ activity mediates the early anti-hyperalgesic effect of pioglitazone

Based on our finding that the anti-hyperalgesic effect of pioglitazone occurred very rapidly (7.5 min) and this was maintained in the presence of 100 µg anisomycin, we suggest the involvement of a non-genomic PPARγ mechanism. Non-genomic mechanisms in the dorsal horn might also explain the rapid anti-hyperalgesic effects observed after interruption of other nuclear receptors including estrogen and PPARα receptors. For example, in vivo administration of 17β-estradiol rapidly (within 15 min) enhanced bradykinin-induced hyperalgesia (Rowan et al., 2010) and inhibited opioid
receptor-like 1 anti-nociception (Small et al., 2013): these were unaffected by pretreatment with anisomycin or conjugation of 17β-estradiol to membrane-impermeable BSA (Rowan et al., 2010; Small et al., 2013) suggesting that membrane estrogen receptors contribute to rapid pain modulation. Also, the inhibition of neuropathic pain (within 30 min) by the PPARα agonists PEA and GW7647 (Costa et al., 2008) was abrogated in PPARα knockout mice or by pharmacological blockade of calcium-activated potassium channels (Lo Verme et al., 2005; LoVerme et al., 2006; Costa et al., 2008) suggesting both rapid and PPARα-dependent analgesic actions. We speculate that, in a similar manner, the rapid anti-hyperalgesic effect of pioglitazone is mediated by membrane PPARγ that is linked to ion channel activity, neuronal excitability, and/or central sensitization mechanisms.

3.5.3 Genomic PPARγ activity mediates the late anti-hyperalgesic effects of pioglitazone

We found that anisomycin significantly reduced the anti-hyperalgesic effects of pioglitazone at later time points (60 minutes), supporting a contribution of classical genomic mechanisms involving transcription and translation. This is consistent with several reports indicating that repeated pioglitazone administration produced sustained anti-hyperalgesic actions persisting for days to weeks after cessation of drug administration (Takahashi et al., 2011; Morgenweck et al., 2013), beyond the time necessary for drug clearance (Maeshiba et al., 1997). Similarly, our current results and previous studies (Park et al., 2007; Churi et al., 2008; Morgenweck et al., 2010) indicate that a single TZD injection produces anti-hyperalgesia lasting for several hours, much longer than the half-life of pioglitazone (Maeshiba et al., 1997). In summary, our results suggest pioglitazone anti-hyperalgesia transitions from a rapid, anisomycin–resistant
mechanism to a delayed, anisomycin-sensitive mechanism within approximately 60 minutes.

3.5.4 The anti-hyperalgesic effect of pioglitazone is mediated by spinal PPARγ

PPARγ agonists reduce tactile hypersensitivity after nerve injury, but the site(s) of action and selectivity of these agents remain elusive. Here, systemic or spinal pioglitazone decreased nerve injury-induced tactile hypersensitivity, and this effect was lost when given in the presence of intrathecal GW9662. We conclude that PPARγ in the dorsal horn of the spinal cord contributes to the anti-hyperalgesic effects of pioglitazone. This is consistent with previous studies showing that: 1) PPARγ is expressed in the lumbar spinal cord (Churi et al., 2008; Maeda et al., 2008); 2) hyperalgesia after SNI was exacerbated by intrathecal administration of the PPARγ antagonist BADGE (Fehrenbacher et al., 2009); and 3) intrathecal GW9662 inhibited the anti-hyperalgesic effect of both repeated systemic or single intrathecal administration of rosiglitazone or 15d-PGJ2 (Churi et al., 2008; Morgenweck et al., 2013). It is unlikely that brain PPARγ mediates anti-hyperalgesia after intrathecal pioglitazone, because i.c.v. administration of comparable doses had no effect; this is consistent with previous results showing that low i.c.v. doses of rosiglitazone or 15d-PGJ2 (Churi et al., 2008) did not change tactile hypersensitivity.

Our studies do not rule out “off-target” effects that might contribute to rapid pioglitazone anti-hyperalgesia. Gras et al. (2009) reported that rosiglitazone-stimulated calcium mobilization was lost after GPR40 siRNA knockdown, suggesting that TZDs (i.e. PPARγ agonists) may activate GPR40 (Gras et al., 2009). Activation of GPR40, which is expressed in brain (Ma et al., 2007; Ma et al., 2008) and spinal cord neurons (Nakamoto et al., 2012), by endogenous (DHA) and exogenous (GW9508) ligands reduced
inflammatory pain (Nakamoto et al, 2012; Nakamoto et al, 2013). Thus GPR40 is an intriguing target for future studies.

3.5.5 Pioglitazone acutely inhibits astrocyte activation

We previously reported that repeated pioglitazone administration spanning several weeks reduced GFAP in the dorsal horn (Morgenweck et al, 2013). Here we observed the same result in a timeframe of just 60 minutes following a single dose of pioglitazone, leading us to speculate that astrocytes contribute to rapid anti-hyperalgesia mechanisms. This might be PPARγ-dependent, since markers of astrocyte activation are reduced by other PPARγ agonists (Storer et al, 2005; Gurley et al, 2008).

Our results are not the first to describe that an acute intervention (i.e. pioglitazone) can alter astrocyte function. For example, increases in GFAP expression occur as soon as 30 min after electrical stimulation of primary afferents (Wang et al, 2010) or intraplantar injection of CFA (Wang et al, 2010) or snake venom toxin sPLA2-Asp49 (Chacur et al, 2004). Second, the astrocyte toxins l-α-aminoadipate and fluorocitrate reduced neuropathic pain and GFAP expression within 30 to 60 min of administration (Zhuang et al, 2006; Wei et al, 2008). Third, fluorocitrate prevented the early phase of TNFα-induced long term potentiation (LTP) in the ex vivo spinal cord (Gruber-Schoffnegger et al, 2013). Therefore, based on the current finding that pioglitazone reduced GFAP in the dorsal horn, we propose that pioglitazone inhibits the astrocytic release of neuron-sensitizing molecules that contribute to LTP and, ultimately, the maintenance of chronic neuropathic pain. Indeed, pioglitazone reduced the release of TNFα from astrocytes in culture (Storer et al, 2005).

Our results do not rule out a supraspinal component to the anti-hyperalgesic actions of systemically-administered pioglitazone. This hypothesis is suggested by the findings that: 1) in models of CNS injury, pioglitazone reduced mitochondrial dysfunction (Hunter
and promoted glucose metabolism in astrocytes (Dello Russo et al., 2003; Pancani et al., 2011); 2) pioglitazone reduced Aβ-induced astrocyte activation in the hippocampus (Heneka et al., 2005); 3) PPARγ is expressed in cortical astrocytes (Cristiano et al., 2001; Moreno et al., 2004); 4) astrocytes in the anterior cingulate cortex facilitate pain sensitization and inhibition (Chen et al., 2012; Ikeda et al., 2013; Grace et al., 2014; Yamashita et al., 2014). This provides for the possibility that systemic pioglitazone administration produces anti-hyperalgesia through cortical, in addition to spinal, astrocytic mechanisms.

Investigations of nerve injury-induced activation of astrocytes in areas of the dorsal horn innervated by injured versus uninjured afferents are sparse (Zhang and De Koninck, 2006; Beggs and Salter, 2007; Corder et al., 2010). Here, our immunohistochemical quantification method (Corder et al., 2010) revealed that nerve injury increased GFAP expression in regions of the ipsilateral dorsal horn innervated by both injured tibial afferents (medial) and uninjured sural afferents (lateral). This is consistent with qualitative studies showing that astrocyte activation extends beyond injured territories (Beggs and Salter, 2007), and suggests that astrocytes induce hyperalgesia by sensitizing intact nociceptive pathways.

Validation of immunohistochemical results with a complementary approach is essential because IHC assays can be misinterpreted due to drug or injury-induced cytotoxic changes in the conformation and/or the formalin sensitivity of the GFAP epitope (i.e. epitope masking) (Bell et al., 1987; Eng et al., 2000). We chose an approach involving a denaturing western blot, and the results indicate that pioglitazone does not change GFAP expression in sham animals, nor on the contralateral side in SNI animals, nor in the ventral horn in sham or SNI animals. This not only suggests that astrocyte viability is preserved after pioglitazone injection, but also that epitope masking does not
confound our conclusion that a single injection of pioglitazone exerts anti-hyperalgesic actions in part by reducing astrocyte activation.

3.5.6 Conclusions

Our results indicate that pioglitazone acts at spinal PPARγ to inhibit astrocyte activation and to produce fast-acting, dose-dependent, and translation-independent inhibition of pain-like hypersensitivity after traumatic nerve injury. The specific mechanisms of rapid pain reduction by PPARγ and the involvement of astrocytes warrant further investigation. Our studies do not rule out actions of PPARγ agonists in the peripheral nervous system (Maeda et al, 2008; Takahashi et al, 2011) that would reduce spinal sensitization, but do illustrate an important behavioral and neurobiological role for non-genomic inhibition of pain in the spinal cord by a nuclear receptor.
CHAPTER 4: Pioglitazone inhibits the development of hyperalgesia and sensitization of spinal nocireponsive neurons in type 2 diabetes

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4.1 Abstract

Thiazolidinedione drugs (TZDs) such as pioglitazone are FDA-approved for the treatment of insulin resistance in type 2 diabetes. However, whether TZDs reduce painful diabetic neuropathy (PDN) remains unknown. Therefore we tested the hypothesis that chronic administration of pioglitazone would reduce PDN in Zucker Diabetic Fatty (ZDF\textsuperscript{fa/fa}) rats. Compared to Zucker Lean (ZL\textsuperscript{fa/+}) controls, ZDF developed: (1) elevated blood glucose, HbA1c, methylglyoxal and insulin; (2) mechanical and thermal hyperalgesia at the hindpaw; (3) increased avoidance of noxious mechanical probes in a mechanical conflict avoidance behavioral assay, the first report of a measure of affective-motivational pain-like behavior in ZDF; and (4) exaggerated lumbar dorsal horn immunohistochemical expression of pressure-evoked phosphorylated extracellular signal-regulated kinase (pERK). Seven weeks of pioglitazone (30 mg · kg\textsuperscript{-1} · d\textsuperscript{-1} in food)
reduced blood glucose, HbA1c, hyperalgesia, and pERK in ZDF. This is the first report to reveal hyperalgesia and spinal sensitization in the same ZDF animals, both evoked by a noxious mechanical stimulus that reflects pressure pain frequently associated with clinical PDN. As pioglitazone provides the combined benefit of reducing hyperglycemia, hyperalgesia, and central sensitization, we suggest that TZDs represent an attractive pharmacotherapy in patients with type 2 diabetes-associated pain.

4.2 Introduction

Approximately one-third of patients with diabetes experience pain (Davies et al., 2006; Abbott et al., 2011; Lee-Kubli et al., 2014), commonly referred to as painful diabetic neuropathy (PDN) (Vincent et al., 2011). A majority of preclinical PDN studies focus on the streptozotocin (STZ) model of type 1 diabetes (Calcutt et al., 2009). However, 90% of diabetic patients are type 2 (World Health Organization), the prevalence of PDN is greater in patients with type 2 (Van Acker et al., 2009; Abbott et al., 2011), and pain mechanisms in type 1 versus type 2 likely differ (Schmidt et al., 2003; Schmidt et al., 2004; Kamiya et al., 2005; Schuelert et al., 2015). Therefore we chose to study a genetic model of type 2 PDN, the Zucker Diabetic Fatty (ZDF^{fa/FA}) rat (Clark et al., 1983).

Hyperglycemia develops within 6-10 wks of age (Clark et al., 1983; Li et al., 2006; Brussee et al., 2008; Sugimoto et al., 2008) in ZDF but not Zucker Lean (ZL^{fa/+}) controls, and is followed by behavioral correlates of PDN, including hypersensitivity to mechanical (Roane and Porter, 1986; Zhuang et al., 1997; Brussee et al., 2008; Sugimoto et al., 2008; Otto et al., 2011; Vera et al., 2012; Galloway and Chattopadhyay, 2013) and thermal (Roane and Porter, 1986; Li et al., 2006; Rong and Ma, 2011; Galloway and Chattopadhyay, 2013) somatosensory stimulation. However, conflicting studies report either hypoalgesia (Sugimoto et al., 2008; Shevalye et al., 2012) or hyperalgesia (Roane and Porter, 1986; Zhuang et al., 1997) in similarly aged ZDF. This raises concerns
regarding examination of sensory thresholds at just a single age without regard to the developmental stage of diabetes (Roane and Porter, 1986; Zhuang et al, 1997; Li et al, 2006; Brussee et al, 2008; Shevalye et al, 2012), or omission of ZL controls when multiple ages are assessed (Otto et al, 2011). These deficiencies in the literature led us to rigorously evaluate multiple pain-like behaviors in ZDF and ZL at various ages in a well-controlled study.

We hypothesized that spinal cord plasticity contributes to pain in type 2 diabetes because: (1) hyperalgesic db/db mice exhibit dorsal horn increases in both phosphorylated extracellular signal-regulated kinase (pERK) (Dauch et al, 2012; Xu et al, 2014), a marker of nocireponsive neuron activation (Gao and Ji, 2009) in the spinal dorsal horn (Ji et al, 1999; Zhuang et al, 2005; Morgenweck et al, 2013), and the astrocyte activation marker GFAP (Liao et al, 2011; Dauch et al, 2012; Ren et al, 2012; Xu et al, 2014); (2) intrathecal injection of either the ERK phosphorylation inhibitor U0216 (Xu et al, 2014) or the astrocyte toxin L-α-aminoadipate (Liao et al, 2011) reverses hyperalgesia; and (3) augmented NMDA and AMPA receptor expression and function in the spinal cord (Li et al, 1999) may contribute to hyperalgesia in ob/ob mice (Latham et al, 2009). A recent report indicated that spinal neurons in ZDF exhibit central sensitization in response to non-noxious hindpaw stimulation (Schuelert et al, 2015). However, concurrent evaluation of pain-like behavior was not performed. We go beyond these studies by evaluating spinal plasticity in PDN using a clinically relevant noxious pressure stimulus to evoke not only pERK in the spinal dorsal horn but also behavioral hyperalgesia in the same subjects.

Thiazolidinedione drugs (TZDs) such as pioglitazone (Actos®) are approved by the U.S. Food and Drug Administration for the treatment of type 2 diabetes. TZDs also reduce the molecular and behavioral sequelae of neurological disease (Feinstein, 2003; Kapadia et al, 2008), as well as neuropathic pain associated with brain (Sauerbeck et al,
2011; Yonutas and Sullivan, 2013), spinal cord (Park et al, 2007; McTigue, 2008) or nerve (Churi et al, 2008; Maeda et al, 2008; Takahashi et al, 2011; Morgenweck et al, 2013; Griggs et al, 2015) injury. For example, we recently reported that pioglitazone reduced hypersensitivity to non-noxious mechanical stimulation in rats with traumatic nerve injury (Morgenweck et al, 2013). However, it still remains unclear whether TZDs reduce the neuropathic pain associated with type 2 diabetes (Taylor, 2015). In addition, behavioral signs of PDN in rodents are more frequently associated with hypersensitivity to noxious, rather than non-noxious stimulation. To address these gaps, we tested the hypothesis that chronic administration of Actos® would prevent the development of noxious pressure-evoked hyperalgesia and spinal central sensitization in ZDF rats.

4.3 Materials and Methods

4.3.1 Subjects

Experiments were carried out in accordance with the Institutional Animal Care and Use Committee at the University of Kentucky (Approved Protocol # 2009-0429). All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to in vivo techniques, in accordance with the International Association for the Study of Pain (Zimmermann, 1983) and the National Institutes of Health Office of Laboratory Animal Welfare Guide for the Care and Use of Laboratory Animals.

Male ZL and ZDF rats (http://www.criver.com/products-services/basic-research/find-a-model/zucker-diabetic-fatty-(zdf)-rat, Charles River, Wilmington, MA) aged 4-19 weeks were used for all experiments. “Obese” ZDF rats are homozygous for the loss-of-function “fatty” mutation in the leptin receptor (fa/fa) that results in the development of type 2 diabetes (Clark et al, 1983). “Lean” ZL rats are heterozygous (fa/+), do not develop a diabetic phenotype, and are genetic controls for ZDF. All rats were housed in a
temperature- and humidity-controlled room on a 12 hour light – 12 hour dark cycle with lights on from 7:00am to 7:00pm. Prior to any behavioral or dietary manipulations, all rats were provided water and Formulab 5008 (TestDiets, Purina Mills, Richmond, IN) chow ad libitum. Formulab 5008 (formerly Purina 5008) food yields reliable diabetic symptoms including hyperglycemia, hyperlipidemia, impaired glucose tolerance and insulin insensitivity as reported by Charles River.

4.3.2 Measurement of Blood Glucose & HbA1c

Blood was collected at sacrifice for the measurement of MG-AGE and insulin at 19 wks of age in the Characterization of PDN study, and for the measurement HbA1c both before (12 wks) and after (19 wks) drug treatment in the Pioglitazone Administration study. Blood glucose was measured at weekly intervals in both studies. The experimental designs for these studies are described below.

Rats were lightly restrained in a towel and the distal tail wiped with an alcohol swab. A small nick was made at the distal tip of the tail using a #11 scalpel blade. Initial bleeding was wiped clean with gauze and subsequent drops of blood were either loaded into a room temperature HbA1c cartridge and analyzed using a DCA Vantage Analyzer (Siemens, Munich, Germany), or placed on a glucose test strip in triplicate and inserted into a glucose monitor (TrueTrack, Walgreens, Deerfield, IL). To avoid perturbations in pain-like behavior elicited by fasting or exogenous glucose administration in a tolerance test (Dobretsov et al, 2001; Dobretsov et al, 2003), non-fasted blood glucose was measured. A random blood glucose level greater than 200 mg/dL (11.1 mmol/L) was defined as hyperglycemia (Sacks, 2011). We measured blood glucose at the same time each week to minimize circadian-induced fluctuations.
4.3.3 Pain-Like Behavior: Stimulus-Evoked

Fluctuations in noise, vibrations, temperature, and other distractors in the behavioral testing room were minimized to optimize reliable measurements between cohorts of animals tested during different behavioral sessions. To further reduce variability and acclimate animals to the different testing apparatuses, two weeks of training were performed prior to behavioral measures reported at 8 weeks of age.

Heat hyperalgesia was assessed by placing the animals on a heated surface (52.5 ± 1 °C) within an acrylic enclosure (Hotplate; Columbus Instruments, Columbus, OH). The time until a hindpaw withdraw response (e.g. jumping, licking, flicking) occurred was recorded. The animal was immediately removed after the withdraw response or at a cutoff of 30 s to avoid tissue injury. Three trials, with an inter-trial interval of at least 5 min, were averaged for each time point.

Mechanical hyperalgesia was measured using the Randall Selitto method (Randall et al, 1957) with slight modification to accommodate an electronic device with a force transducer (IITC Life Science Inc., Woodland Hills, CA). The thoracic body and head were lightly restrained in a towel with both hindpaws exposed. To start, one hindpaw was placed into the calipers with the blunted-point of the force transducer placed on the plantar surface between the 2nd and 3rd digits. The force exerted on the surface of the hindpaw by the blunted-point was gradually and uniformly increased until a withdraw reflex occurred. Animals were not required to vocalize. The gram force required to elicit a withdraw response was recorded. Because patients report increased responsiveness to repeated mechanical stimuli (Otto et al, 2003), we used multiple trials per time point to assess pressure hyperalgesia. The force until paw withdraw was measured three times for each hindpaw and then averaged together, as there was no significant difference in left versus right withdraw thresholds. The combined average of hindpaw withdraw thresholds is reported at each timepoint.
Cold hyperalgesia was assessed by placing the animal on a cooled surface (4°C ± 0.5°C) within an acrylic enclosure (Coldplate; IITC Life Science Inc., Woodland Hills, CA) for five minutes. The combined number of nociceptive responses (e.g. jumping, licking, flicking) for both hindpaws during the 5 min period is reported (Jasmin et al, 1998).

4.3.4 Pain-Like Behavior: Affective-Motivational

To assess the affective-motivational component of pain, we utilized a novel Mechanical Conflict Avoidance System (MCS) (Lau et al, 2012). MCS gives animals the option to remain in a start chamber containing an aversive light stimulus or choose to cross a chamber containing an aversive mechanical stimulus in the form of height-adjustable, blunted probes. If animals chose to leave the light chamber and cross the probes in the stimulus chamber, they then had the option to enter a darkened reward chamber. MCS behavioral testing began at 17 wks of age and consisted of three stages: familiarization, training, and testing. The latency to exit the light chamber was used as a measure of avoidance of the mechanical stimulus, and therefore a measure of affective-motivational pain-like behavior.

Familiarization – 1 day. Animals were placed in the light chamber with the light off; access to the stimulus chamber was restricted by a closed guillotine door. After 15 s of darkness, the light was turned on. After 20 s of light exposure, the guillotine door was raised and the animal was free to explore the entire apparatus, including the dark chamber, for 5 min. The animal was then returned to the homecage.

Training – 4 days. Animals were trained to move from the light chamber, through the stimulus chamber at a probe height of 0 mm (i.e. no probes), and into the dark chamber. Animals were placed in the light chamber with the light off; access to the stimulus chamber was restricted by a closed guillotine door. After 15 s of darkness, the light was turned on. After 20 s of light exposure, the guillotine door was raised and a timer started
to measure the latency to exit the light chamber. If the animal did not exit the light chamber within 30 s, the guillotine door was closed and the animal was returned to its homecage. Upon entering the dark chamber, a second guillotine door was closed to restrict the animal to the dark chamber. Animals remained in the dark chamber for 45 s to reinforce the crossing of the stimulus chamber and then were returned to the homecage. Each animal underwent four training trials on each of the four training days.

**Testing – 3 days.** Assessment of the latency to exit in the presence of varying degrees of mechanical stimulus (1, 3, 4 mm) was initiated at 18 wks of age. Each testing day began with 1 trial in the absence of a stimulus (0 mm) followed by 3 trials with the probes raised to a predetermined height: 1 mm (d 1), 3 mm (d 2), 4 mm (d 3). The training procedure described above was also used for testing in either the absence (trial 1) or presence (trials 2-4) of mechanical probes in the stimulus chamber. Only one probe height was tested per day. Assessment at the 0 mm probe height was used to confirm that animals were still trained to cross the stimulus chamber.

Animals remained in their home cage for at least 10 min between training and testing trials. All four paws were required to leave the light chamber to determine the latency to exit. The average of 4 trials for each training day or 3 trials for each testing probe height is reported for each animal.

4.3.5 *pERK Quantification via Immunohistochemistry*

Upon general anesthesia with isoflurane (5% induction, 1.5% maintenance), the plantar surface between the 2nd and 3rd digits of the left hindpaw was stimulated with 110 g of constant force for 30 s per minute over a 5 min period. These stimulus parameters were chosen to mimic those used to elicit withdraw responses to noxious pressure as described above for mechanical hyperalgesia testing. Ten minutes after initiating pressure stimulation, animals were perfused through the left ventricle with 250 ml of
room temperature 0.1 M phosphate buffered saline (PBS) with heparin (10,000 USP units/L) followed by 250 ml of ice-cold fixative (10% phosphate buffered formalin). The lumbar spinal cord was removed and post-fixed overnight in 10% phosphate buffered formalin and then cryoprotected in 30% sucrose in 0.1 M PBS for several days. Transverse sections (30 μm) from L4-L5 were cut on a freezing microtome and collected in 0.1 M PBS. The sections were washed three times in 0.1 M PBS and then pretreated with blocking solution (3% normal goat serum and 0.3% Triton X-100 in 0.1 M PBS) for 1 h. Sections were then incubated in blocking solution containing the primary antibodies rabbit anti-pERK (1:250, #4370, Cell Signaling Technology, Danvers, MA) and Alexa Fluor 488 conjugated mouse anti-NeuN (1:200, MAB377X, EMD Millipore, Billerica, MA) overnight at room temperature on a slow rocker in the dark. The sections were washed three times in 0.1M PBS, and incubated in goat anti-rabbit secondary antibody (1:800, Alexa 568, Molecular Probes, Grand Island, NY) for 90 min, washed in 0.1M PBS, 0.01M PBS, then 0.01M PB, and non-sequentially mounted onto Superfrost Plus slides, air dried, and cover-slipped with Prolong Gold with DAPI mounting medium (Molecular Probes). 4-6 high quality sections were randomly selected for quantification.

All images were captured on a Nikon Eclipse TE2000-E microscope using a 10x objective and analyzed using NIS-Elements Advanced Research software. We focused our quantification of the number of pERK immunopositive cell profiles within lamina I-II, where the majority of C-fibers and nociceptive peripheral afferents terminate within the dorsal horn (Basbaum et al, 2009; Corder et al, 2010). Colabeling of pERK with NeuN is expressed as the percentage of the total number of pERK profiles in laminae I-II also expressing NeuN. Each spinal cord slice was analyzed by an observer blinded to treatment. The average of n=5-6 animals per group is reported.
4.3.6 **Quantification of Methylglyoxal-Derived Advanced Glycation End-Products (MG-AGEs)**

MG-AGEs were quantified using a competitive ELISA according to the manufacturer’s instructions (STA-811, Cell BioLabs, San Diego, CA). This ELISA uses a primary antibody that recognizes the hydroimidazolone (H1) moiety created by the modification of protein residues by methylglyoxal (Ahmed, 2003). Whole blood taken from the left ventricle prior to transcardial perfusions was collected in serum separator tubes (SST™, BD Biosciences, Franklin Lakes, NJ) and allowed to clot for 30 min. Clotted blood in SSTs was centrifuged at 5000 x g for 10 min at 4°C and the serum was transferred to fresh microcentrifuge tubes and stored at -80°C until MG-H1 ELISA analysis. Serum samples were diluted 1:2 in 0.1 M PBS to obtain concentrations in the span of the standard curve.

4.3.7 **Insulin Quantification via ELISA**

Non-fasted insulin levels were quantified by ELISA according to the manufacturer's recommendations (EMD Millipore; EZRMI-13K; Darmstadt, Germany) from serum obtained as described above in serum separator tubes (SST™, BD Biosciences) at time of sacrifice.

4.3.8 **Pioglitazone Incorporation into Food**

Actos® (pioglitazone hydrochloride; Takeda Pharmaceuticals U.S.A., Inc., Deerfield, IL) was obtained from the University of Kentucky pharmacy in 30 mg tablets of 25% purity, and was incorporated into Formulab 5008 rat chow (TestDiets, Purina Mills, Richmond, IN). Average body weight and food consumption (Saitoh et al, 2007) were used to determine the concentration of Actos in chow required to achieve a dosing of 30 mg pioglitazone / kg body weight / day. For ZDFs the concentration of Actos in chow
was 0.16% (catalog no. 5W01) and for ZLs the concentration was 0.207% (catalog no. 5W02). Food was provided *ab libitum* and consumption and body weights were monitored in order to calculate actual dosing.

4.3.9 *Experimental Design: Characterization of PDN*

To determine the time course of development of pain-like behavior, we monitored ZL and ZDF rats weekly from 4 to 18 weeks of age with n=12 per group. Weekly measurement of pain-like behaviors occurred on Monday thru Friday in the following order: coldplate, pressure, hotplate. Assessment of pain-like behaviors occurred prior to determination of blood glucose and mass at 2-4pm on Fridays. Animals were not fasted in order to minimize the potential effects of fasting on pain-like behavior. At least one day separated coldplate and hotplate determinations to avoid cross-modality sensitization. After behavioral and metabolic outcomes were assessed at 18 wks, the left hind paws of control and diabetic rats were stimulated, using the same pressure device used to determine behavioral hyperalgesia, in order to evoke pERK. Next, cardiac blood was taken prior to transcardial perfusion and fixation, and then tissues were harvested for subsequent assays.

4.3.10 *Experimental Design: Pioglitazone Administration*

Rats were divided into the following treatment groups using a 2x2 experimental design: ZL Vehicle (n=10), ZL Pioglitazone (n=10), ZDF Vehicle (n=10), ZDF Pioglitazone (n=9). Vehicle food was defined as unaltered Formulab 5008 chow.

Pain-like behaviors, blood glucose, and weight were measured weekly from 10 to 19 wks of age. HbA1c was measured at 12 and 19 wks. Vehicle or pioglitazone food treatment began after assessment of behavioral and metabolic outcomes at 12 wks and all further testing was performed by an observer blinded to drug treatment group. Food
consumption was measured 3 times per week by calculating the mass difference between food added to the cage and food remaining in the cage then dividing by 2, as rats were housed in pairs, and the number of days that had lapsed between food additions. After outcome measurements were obtained during week 19, animals were pressure-stimulated to evoke pERK, perfused and fixed with buffered formalin for immunohistochemical analyses of spinal cords.

One rat from the ZDF Pioglitazone group was removed from all analyses prior to beginning pioglitazone administration due to an abnormally high baseline blood glucose level of 514 mg/dL (more than double all other animals). Because the phenotype for coldplate responses in ZDF was modest, we did not assess the effect of pioglitazone on cold hypersensitivity.

4.3.11 Statistical Analysis

Mass, glucose, hotplate, pressure, coldplate, and MCS were compared using a repeated measures two-way ANOVA followed by Holm-Sidak multiple comparison correction. To compare ZL vs. ZDF, the outcome measures insulin, MG-AGE, HbA1c, and area-under-the-curve (AUC; calculated via the trapezoidal method) were analyzed using an unpaired, two-tailed t-test. To compare ZL vs. ZDF and vehicle vs. pioglitazone, the outcomes HbA1c and AUC were analyzed via a two-way ANOVA followed by Holm-Sidak multiple comparison correction. Ipsilateral vs. contralateral or pioglitazone vs. vehicle comparisons of pERK immunohistochemistry in ZL vs. ZDF were analyzed by two-way ANOVA followed by Holm-Sidak multiple comparison correction. A value of $\alpha=0.05$ was used to determine statistical significance. All data were analyzed and graphed using Prism 6.0 (GraphPad, La Jolla, CA) and are presented as mean ± SEM.
4.4 Results

4.4.1 The ZDF Rat is a Model of Progressive Painful Diabetic Neuropathy

When compared to control heterozygotes or wild-types, only ZDF rats homozygous for the fatty (fa/fa) leptin receptor mutation develop cardinal symptoms of glucose-intolerant diabetes (Clark et al., 1983) including hyperphagia (Saitoh et al., 2007; Otto et al., 2011), hyperinsulinemia (Munoz et al., 2001; Sugimoto et al., 2008; Li et al., 2014), obesity (Otto et al., 2011), hyperglycemia (Piercy et al., 1999; Otto et al., 2011; Vera et al., 2012), elevated HbA1c (Sugimoto et al., 2008; Li et al., 2014), and increased MG-AGE (Shevalye et al., 2012). In the first experiment we characterized the progression of hyperglycemia and pain-like behavior in diabetic ZDF and control ZL rats. As illustrated in Figure 1A-C, ZDF had elevated mass [strain x time; F (14, 308) = 8.249; P < 0.0001] from 4 to 18 wks [p < 0.01], elevated blood glucose [strain x time; F (14, 308) = 46.31; P < 0.0001] from 8 to 18 wks [p < 0.0001], and elevated serum insulin [p = 0.0009] at 19 wks.

Advanced glycation end-products (AGEs) such as methylglyoxal (MG) derived hydroimidazolone (MG-H1) are associated with diabetic pain in patients (Sveen et al., 2013). MG-AGEs result from the accumulation of MG (Illien-Junger et al., 2015), a metabolite of glucose found in blood that is elevated in type 2 diabetes (Kender et al., 2014) and exacerbated in patients and mice with PDN (Bierhaus et al., 2012). Therefore we measured MG-AGE levels and hemoglobin glycation in the form of HbA1c, an AGE considered by clinicians to be the ‘gold standard’ diagnostic for diabetes (Sacks, 2011). Figure 1D indicates that 19 wk old ZDFs had elevated blood levels of MG-AGE [p < 0.05]. In a separate group of subjects, Figure 1E indicates that 12 wk-old ZDFs had elevated blood levels of HbA1c [p < 0.0001]. Thus, consistent with the literature, we found that ZDFs exhibited: (1) obesity after weaning that persisted until the conclusion of the study; (2) hyperglycemia beginning at 6 wks of age that progressively increased
throughout the study; (3) hyperinsulinemia, elevated MG-AGE, and elevated HbA1c levels at 12 wks.

Figure 4-1. ZDF rats develop a type 2 diabetes phenotype. (A) Body mass (n=12) and (B) blood glucose levels (n=12), serum (C) insulin (n=6) at 19 wks, (D) methylglyoxal-derived advanced glycation end-products (MG-AGE; n=3) at 19 weeks and (E) HbA1c at 12 weeks of age (n=10) in Zucker Diabetic Fatty (ZDF) and Zucker Lean (ZL) controls. * p < 0.05, ZDF vs. ZL.
To rigorously evaluate the ZDF rat as a behavioral model of PDN, we measured not only evoked responses to noxious stimuli over an extended time course, but also signs of affective pain. First, to assess stimulus-evoked hyperalgesia, we measured responses to noxious mechanical and thermal stimuli. Figure 2A-B illustrates that ZDFs were hypersensitive to a noxious hotplate [strain; F (1, 22) = 14.2; P = 0.0011] at 15, 17, and 18 weeks of age [p < 0.05], exhibiting an overall decrease in heat response threshold from 14 to 18 wks [AUC; p < 0.05]. Figure 2C-D illustrates that ZDFs were hyperresponsive to noxious mechanical pressure [strain x time; F (10, 220) = 6.045; P < 0.0001] from 15 to 18 wks [p < 0.001], exhibiting an overall decrease in pressure threshold from 14 to 18 wks [AUC; p < 0.0001]. Figure 2E-F illustrates that ZDFs exhibited more hindpaw responses in a coldplate test [strain x time; F (10, 220) = 2.372; P = 0.0110] at 18 wks [p < 0.01], with an overall increase from 14 to 18 wks [AUC; p < 0.001].
Figure 4-2. ZDF rats develop hyperalgesia.
Paw withdraw responses to (A-B) heat, (C-D) pressure, and (E-F) cold stimuli in ZL and ZDF rats. Area under the curve (AUC) analyses for 14-18 wks are shown. n=12 per group. * p < 0.05, ZDF vs. ZL.

Preclinical research targeting the mechanisms of PDN primarily relies on stimulus-evoked behavioral outcomes (Obrosova, 2009). However, PDN symptoms include not just pressure and thermal hyperalgesia but also aching, paresthesia/dysesthesia, and affective pain (Liberman et al, 2014). To our knowledge there are no reports of affective
pain in a rodent model of type 2 diabetes (Lee-Kubli et al, 2014). To address this gap and determine whether ZDFs possess the affective-motivational component of PDN that is most relevant to diabetic patients, we utilized a mechanical conflict-avoidance system (MCS) (Morrow and Harte, 2010; Donahue et al, 2012; Lau et al, 2012) as shown in Figure 3A. Figure 3B illustrates that during the four training trials when no pins were present (0 mm probe height), latency to exit the light chamber was similar between ZL and ZDF rats (p > 0.05), indicating no differences in light aversion or learned motivation/ability to find the dark chamber. Despite contrasting results in an open field test, where we (unpublished findings; R.B. Griggs et al, 2013) and others (Jiménez-Aranda et al, 2013) found diminished locomotor activity in ZDF, exploration in a novel open field environment is not equitable to behavioral training to seek the dark chamber in MCS. Raising the probe height produced an increase in the latency to exit the light chamber [strain x probe height; F (6, 102) = 4.262; P = 0.0007] in ZL at 4 mm (vs. 1 mm; p = 0.0051) and in ZDF at both 3 mm (p < 0.0001) and 4 mm (p < 0.0001) heights, indicating aversion to the mechanical stimulus in both control and diabetic rats. Importantly, the latency to exit the light chamber was higher in ZDF compared to ZL at both 3 mm (p = 0.0493) and 4 mm (p < 0.0001) heights, indicating that diabetes potentiates the avoidance of a noxious mechanical stimulus.
Figure 4-3. Diabetes increases the avoidance of mechanical probes.

(A) Diagram of the mechanical conflict avoidance system (MCS) used as a measure of affective-motivational pain in ZL and ZDF rats. MCS behavioral testing began at 17 wks of age and consisted of three stages: familiarization (1 d), training (4 d), and testing (3 d).

Familiarization: Animals were initially placed in the light chamber and allowed free access to explore the entire MCS apparatus.

Training: Animals were trained to move from the light chamber, through the stimulus chamber with mechanical probes set to a height of 0 mm (i.e. no probes), and into the dark chamber. Each animal underwent four training trials on each of the four consecutive training days.

Testing: Animals were placed in the light chamber and allowed to cross the stimulus chamber at probe heights of 1 mm, 3 mm, and 4 mm. Each animal was tested for 3 trials at each probe height with only one probe height tested on each of the three testing days.

(B) The latency to exit the light chamber (latency) in ZL and ZDF rats is shown for the 4 d of training (left) and 3 d of testing (right). The latency was similar in the absence of mechanical probes (probe height = 0 mm) during training and at a 1 mm probe height during testing. Raising the testing probe height increased the latency in ZL at 3 mm and ZDF at 3 and 4 mm. The latency was greater in ZDFs compared to ZLs at the 3 and 4 mm probe height. n=9-10. * p < 0.05. † p<0.05 vs. ZL at 1mm. ‡ p<0.05 vs. ZDF at 1 mm.

Central sensitization is defined as the increased responsiveness of nociceptive neurons in the central nervous system to their normal or subthreshold afferent input (Woolf, 2011). As an example, expression of phosphorylated extracellular signal-regulated kinase (pERK) in the dorsal horn is exacerbated by light touch stimulation after peripheral nerve injury (Morgenweck et al, 2013) or inflammation (Gao and Ji, 2010) but not uninjured controls, and is therefore an important marker of spinal central sensitization (Gao and Ji, 2009). To test the hypothesis that diabetes results in central sensitization, we evaluated noxious pressure-evoked phosphorylation of ERK (pERK) in
dorsal horn neurons. We chose pressure as the evoking stimulus because it is a hallmark pain modality in PDN patients (Otto et al., 2003). Figure 4A shows that unilateral stimulation of the tibial receptive field produced unilateral pERK within the superficial laminae of the dorsal horn. pERK was restricted to the side ipsilateral to stimulation and was predominantly located within the medial extent of the dorsal horn, respecting previously-described somatotopic boundaries of tibial nerve anatomy (Swett and Woolf, 1985; Corder et al., 2010). Figure 4B shows representative images indicating that pERK predominantly colabeled with NeuN, an established immunohistochemical marker for neurons (Kim et al., 2009). The percentage of pERK cell-profiles that colabeled with NeuN was (mean ± SEM): ZL contralateral (81.5 ± 6.5), ZL ipsilateral (88.3 ± 3.6), ZDF contralateral (80.5 ± 3.1), ZDF ipsilateral (92.1 ± 1.3). The degree of colocalization was similar between ZL and ZDF at either contralateral [p = 0.93] or ipsilateral [p = 0.89] dorsal horns. Figure 4C illustrates that pressure increased pERK in ZL [ipsilateral vs. contralateral; p < 0.0001] and ZDF rats [p < 0.0001]. Diabetes exacerbated this increase [ZDF vs. ZL; ipsilateral only; p = 0.001], and there was an interaction between Strain and Stimulation [F (1, 20) = 8.051; P = 0.0102].

Figure 4-4. Diabetes exacerbates noxious pressure-induced activation of dorsal horn neurons.
Pressure-evoked phosphorylation of ERK and colabeling with NeuN in the L4/5 dorsal horn at 18 wks of age in ZL and ZDF rats. Representative images of pERK
(A) in the stimulated (ipsilateral) or unstimulated (contralateral) side and (B) colabeling with the neuronal marker NeuN in the medial portion of the ipsilateral lumbar dorsal horn after pressure stimulation. (C) Quantification of cell-like profiles labeled with pERK in laminae I-II. n=6. † p<0.05 vs. ZL ipsilateral. ‡ p<0.05 vs. ZDF ipsilateral. * p<0.05. Scale bars = 100 µm.

4.4.2 Chronic Administration of Oral Pioglitazone Reduces Pathological Signs of Diabetes

Numerous reports indicate that oral administration of pioglitazone or rosiglitazone reduces hyperglycemia in db/db mice (Takahashi et al, 2015), ZDF rats (Piercy et al, 1999), and humans (Patel et al, 1999; Aronoff et al, 2000; Xu et al, 2015) as well as HbA1c levels in ZDF (Watanabe et al, 2015) and diet-induced type 2 diabetes (Ibrahim et al, 2015). To confirm that pioglitazone alleviated signs of type 2 diabetes in ZDF, we measured blood glucose and HbA1c. The average dose of pioglitazone (mg · kg⁻¹ · d⁻¹) received by ZL (35.53 ± 1.94) and ZDF (32.19 ± 2.52) was similar over the 7 wk treatment period, and was comparable to previous studies in db/db mice (4 wks of 30 mg · kg⁻¹ · d⁻¹) (Takahashi et al, 2015) and ZDF rats (6 wks of 10 mg · kg⁻¹ · d⁻¹) (Watanabe et al, 2015). As illustrated in Figure 5A, pioglitazone decreased blood glucose in ZDF [F (1, 18) = 16.51; P = 0.0007] but not in ZLS [F (1, 18) = 3.51; P=0.077] from 13 to 19 wks of age. Figure 5B illustrates that blood levels of HbA1c were elevated in ZDF rats at 19 wks of age [p < 0.0001], even greater than observed at 12 wks (Fig 1E). Pioglitazone normalized HbA1c levels in ZDF to the level of ZL [p < 0.0001].
Figure 4-5. Pioglitazone reduces pathological signs of type 2 diabetes.
Effect of vehicle or pioglitazone (administered in food, yellow bar) on blood levels of (A) glucose (n=9-10) from 11 to 19 wks and (B) HbA1c (n=9-10) at 19 wks of age in ZL and ZDF rats. ‡ p<0.05 vs. both ZL groups. † p<0.05, ZDF Pioglitazone vs. ZDF Vehicle. *

4.4.3 Chronic Oral Pioglitazone Inhibits the Development of PDN and Reduces Evoked pERK

Hyperalgesia develops in ZDF at approximately 14 wks of age (Fig 2). To test the hypothesis that pioglitazone prevents the development of hyperalgesia, we initiated its administration at 12 wks of age. As illustrated in Figure 6A-B, ZDF exhibited lower heat response thresholds as compared to ZL [strain x time; F (8, 144) = 2.039; P = 0.0458] from 13 to 19 wks. Pioglitazone temporarily normalized heat thresholds in ZDF [F (1, 17) = 22.09; P = 0.0002] from 13 to 15 wks [p < 0.01], and then heat hypersensitivity returned at 16 wks. Figure 6C-D illustrates that ZDF exhibited a decrease in pressure response thresholds compared to ZL [strain x time; F (8, 144) = 2.178; P = 0.0324] from 11 to 19 wks [p < 0.05]. Pioglitazone normalized pressure thresholds in ZDF [drug; F (1, 17) = 104.9; P < 0.0001] throughout the duration of treatment, from 13 to 19 wks [p < 0.05]. Further studies are needed to investigate the differential effect of pioglitazone on heat and noxious mechanical sensitivity. In addition, we speculate that pioglitazone
would reduce cold hypersensitivity as we recently reported that pioglitazone reduced the development of \cite{63} and reversed \cite{30} hyperresponsivity to acetone in the spared nerve injury model of neuropathic pain.

Because we began pioglitazone administration prior to the development of hyperalgesia, further intervention studies are needed to determine if the mechanism of pioglitazone antihyperalgesia is independent from its reduction of hyperglycemia. This may indeed be the case, since we found that a single intraperitoneal injection of pioglitazone, given after the development of hyperalgesia in ZDF, reversed heat hypersensitivity without changing blood glucose levels (unpublished data, R.R. Donahue et al, 2014).

Figure 4-6. Pioglitazone attenuates pain-like behavior.
Effect of vehicle or pioglitazone in food (yellow bar) on the development of (A-B) heat and (C-D) mechanical hyperalgesia (n=9-10). Pioglitazone inhibits the development of heat and mechanical hyperalgesia in ZDF. ‡ p<0.05 ZDF Vehicle vs. all other groups. * p<0.05.

To determine whether chronic pioglitazone treatment reduces spinal nocireceptive neuron activation, we quantified pERK after noxious mechanical stimulation of the hindpaw. Figure 7A-B illustrates that noxious pressure evoked greater pERK in vehicle-treated ZDF as compared to ZL [p = 0.0006]. Pioglitazone but not vehicle normalized pERK in ZDF [p = 0.011] but not ZL [p = 0.96]. There was an interaction between Strain and Drug [F (1, 19) = 6.048; P = 0.0237].

Figure 4-7. Pioglitazone attenuates dorsal horn neuron activation.
Noxious pressure-evoked pERK in the lumbar superficial dorsal horn of ZL and ZDF rats at 19 wks of age following a 7 wk treatment with vehicle or pioglitazone administered in chow. (A) Representative images of pERK immunostaining. (B) Pioglitazone reduced pERK in ZDFs to the level of ZLS. n=5-6. * p<0.05. Scale bar = 100 µm.

4.5 Discussion

4.5.1 ZDFs Develop Multiple Types of Pain-Like Behavior

Consistent with previous reports, ZDFs developed mechanical (Roane and Porter, 1986; Zhuang et al, 1997; Li et al, 2006; Brussee et al, 2008; Sugimoto et al, 2008; Otto
et al, 2011; Rong and Ma, 2011; Vera et al, 2012; Galloway and Chattopadhyay, 2013), heat (Roane and Porter, 1986; Li et al, 2006; Rong and Ma, 2011; Galloway and Chattopadhyay, 2013), and cold hyperalgesia (Rong and Ma, 2011) at 14 to 18 wks of age (Otto et al, 2011; Vera et al, 2012; Galloway and Chattopadhyay, 2013), results which we replicated in two separate cohorts. In contrast, a comparatively small number of ZDF studies report either mechanical hypoalgesia (Shevalye et al, 2012), heat hypoalgesia (Sugimoto et al, 2008; Shevalye et al, 2012; Vera et al, 2012), no difference in heat (Brussee et al, 2008) or pressure (Piercy et al, 1999) response thresholds, and/or pressure hyperalgesia prior to hyperglycemia (Romanovsky et al, 2008). Discrepancies in heat responses could be due to differences in the stimulus type: we used a hotplate while other studies used an infrared beam directed at the tail (Sugimoto et al, 2008) or hindpaw (Brussee et al, 2008; Shevalye et al, 2012; Vera et al, 2012).

A recent review of preclinical models of PDN highlights the need to establish indices of spontaneous/affective pain (Lee-Kubli et al, 2014). One study used conditioned place preference (CPP), an assay that has re-emerged (Sufka, 1994) as a leading preclinical measure of tonic pain in rats (King et al, 2009) and mice (He et al, 2012), to reveal affective pain in type 1 diabetic STZ mice in the absence of an evoking stimulus (Wagner et al, 2014). Here, we discovered that type 2 diabetes exacerbates the avoidance of a noxious mechanical stimulus. Unlike CPP, where presumed relief of non-evoked affective pain reinforces a chamber preference, MCS utilizes the avoidance of an evoked stimulus as a measure of the motivational component of pain. Our results demonstrate for the first time the existence of a motivational-affective component of PDN in a preclinical model of type 2 diabetes.
4.5.2 ZDFs Develop Central Sensitization in Spinal Dorsal Horn Neurons

The dorsal horn is a key site of nociceptive integration (Basbaum et al, 2009) including central sensitization after tissue or nerve injury (Woolf, 2011). A recent electrophysiological study in ZDF spinal cord slices indicated that non-noxious mechanical stimulation increased afterdischarges and spontaneous activity of dorsal horn neurons (Schuelert et al, 2015). However, the authors did not investigate pain-like behavior and they analyzed dorsal horn neurons at 32-34 wks, after ZDF aged 26 wks are reported to be hypoalgesic (Lee-Kubli et al, 2014). Thus, at this advanced diabetic stage, it is unclear whether central sensitization contributed to painful diabetic neuropathy. In our study using younger ZDF, we resolved this uncertainty by evaluating both behavioral hypersensitivity and stimulus-evoked pERK in the same subjects.

We are the first to demonstrate both pain-like behavior and elevated spinal neuron activation in ZDF. Both of these results were observed in response to a noxious mechanical stimulus, and this fits with the clinical manifestations of PDN: up to 71% of patients (Otto et al, 2003) report hyperalgesia in response to application of a similar, static, noxious pressure stimulus (Wienemann et al, 2012) used in the current study on ZDF. By contrast, Schuelert et al reported that spinal neurons were not sensitized to noxious mechanical stimuli (Schuelert et al, 2015). Albeit, our results provide strong evidence in support of the hypothesis proposed by Schuelert et al that central sensitization contributes to hyperalgesia in type 2 diabetes (Schuelert et al, 2015). If true, then therapies aimed at reducing central sensitization could alleviate PDN. Indeed, spinal administration of MEK inhibitors, which prevent phosphorylation of ERK, attenuated hyperalgesia in db/db (Xu et al, 2014) and STZ (Ciruela et al, 2003) models.
4.5.3 Pioglitazone Reduces Pathological Signs of PDN in ZDF by Actions at PPARγ

Several lines of evidence lead us to speculate that activation of spinal peroxisome proliferator-activated receptor gamma (PPARγ) mediated the antihyperalgesic actions of pioglitazone reported here in ZDF. First, spinal sites are rich in PPARγ expression (Moreno et al., 2004; Churi et al., 2008; Maeda et al., 2008) and pioglitazone crosses the blood-brain barrier (Maeshiba et al., 1997). Second, intrathecal administration of PPARγ antagonists inhibits the antihyperalgesic effect of both repeated systemic or single intrathecal administration of PPARγ agonists (Churi et al., 2008; Morgenweck et al., 2013; Griggs et al., 2015). Alternatively, non-PPARγ mechanisms can be proposed for the antihyperalgesic effects of pioglitazone in PDN. Pioglitazone inhibits HMGB1-RAGE signaling in spinal neurons (Wang et al., 2014) and reduces plasma RAGE (Ibrahim et al., 2015), both of which are implicated in the pathogenesis of neuropathic pain in models of traumatic nerve injury (Feldman et al., 2012) and PDN (Ren et al., 2012; Hidmark et al., 2014). Whether pioglitazone reduces the contribution of spinal gliosis and/or HMGB1-RAGE signaling to PDN remains an important direction of future studies.

Our current results suggest that pioglitazone decreases central sensitization to reduce PDN in type 2 diabetes. In support of this idea, chronic oral pioglitazone (at the same 30 mg · kg⁻¹ · d⁻¹ dose used in the current study) reduced non-noxious stimulus-induced mechanical allodynia and spinal pERK in a traumatic nerve injury model of neuropathic pain (Morgenweck et al., 2013). Our results extend this finding by showing that pioglitazone reduces spinal pERK following a different stimulus (noxious pressure) and in a different neuropathic pain model that is associated with disease (type 2 diabetes).

Our studies aimed to evaluate whether ZDF exhibited hyperalgesia alongside spinal sensitization and whether this could be reduced by a TZD, and therefore the focus was not on peripheral mechanisms. However, several studies suggest that pioglitazone can
inhibit peripheral mechanisms of chronic pain: 1) PPARγ is expressed in sciatic nerve (Yamagishi et al, 2008); 2) TZDs decreased hyperalgesia, proinflammatory cytokine expression, and macrophage infiltration in sciatic nerve after nerve injury (Maeda et al, 2008; Takahashi et al, 2011); 3) Pioglitazone reduced macrophage infiltration and pERK in the sciatic nerve of STZ rats (Yamagishi et al, 2008); 4) Pioglitazone reduced pERK in sciatic nerve of db/db mice (Dasu et al, 2009); and 5) Rosiglitazone and resolvin D1 coadministration to the site of hindpaw incision in db/db mice promotes a M2 macrophage phenotype and reduces mechanical hypersensitivity (Saito et al, 2015). Future studies in preclinical models of type 2 PDN could investigate the effect of local TZD administration to the peripheral nerve (Takahashi et al, 2011; Hasegawa-Moriyama et al, 2012) on: macrophage polarization (Hasegawa-Moriyama et al, 2012); TNFα, which is elevated in patients with PDN (Uceyler et al, 2007; Purwata, 2011); nerve conduction velocity, which is decreased in type 2 diabetes (Brussee et al, 2008); and pain-like behaviors.

4.5.4 Pioglitazone Reduces PDN Independent of its Anti-Hyperglycemic Action

Although we found that hyperglycemia preceded pain-like behavior as it does in type 2 diabetic patients (Abbott et al, 2011), we propose that pioglitazone reduces PDN independent of its simultaneous reduction of blood glucose. This is based on several lines of evidence. First, pioglitazone and other PPARγ agonists reduce neuropathic pain-like behavior in normoglycemic animals after various routes of administration including: repeated oral (Maeda et al, 2008; Morgenweck et al, 2013) or intraperitoneal (Maeda et al, 2008; Takahashi et al, 2011; Hasegawa-Moriyama et al, 2012; Morgenweck et al, 2013); local injection to the injured sciatic nerve (Takahashi et al, 2011) or incised paw (Hasegawa-Moriyama et al, 2012); or a single spinal (Park et al, 2007; Churi et al, 2008; Morgenweck et al, 2010; Griggs et al, 2015) or brain (Morgenweck et al, 2010) injection.
Second, numerous drugs can reduce PDN without reducing hyperglycemia in patients with PDN, including tapentadol (Schwartz et al, 2011) or the FDA-approved PDN medications duloxetine and pregabalin (Mendell and Sahenk, 2003; Smith and Argoff, 2011). Furthermore, the antioxidant taurine (Li et al, 2006), aldose reductase inhibitors (Calcutt et al, 2004; Ramos et al, 2007; Obrosova, 2009), insulin growth factor (Zhuang et al, 1996; Zhuang et al, 1997), or poly-ADP-ribose inhibitors (Obrosova et al, 2005; Obrosova et al, 2008; Drel et al, 2010) reduce hyperalgesia in animal models of PDN without inhibiting hyperglycemia. Consistent with these findings, pioglitazone normalized peripheral nerve conduction velocities without affecting hyperglycemia in the STZ model (Yamagishi et al, 2008). Third, mechanical hyperalgesia that is not associated with hyperglycemia has been reported in ZDFs, which suggests that hyperalgesia can be independent from blood glucose levels (Romanovsky et al, 2008). Finally, it is important to note that normalization of blood glucose alone cannot reverse the neurotoxic effects resulting from long-term hyperglycemia (Tomlinson and Gardiner, 2008) that contribute to PDN (Vincent et al, 2011).

The results from this pretreatment study are clinically important as they suggest that pre-diabetic patients could benefit from adjuvant treatment with pioglitazone or an alternative PPARγ agonist in addition to recommended lifestyle changes such as diet and exercise. This is supported by preclinical studies indicating that TZD treatment prior to or coinciding with peripheral nerve injury are quite effective at reducing hyperalgesia (Maeda et al, 2008; Takahashi et al, 2011; Morgenweck et al, 2013). Further study is needed to definitively dissociate the antihyperglycemic and antihyperalgesic effects of targeting PPARγ with pioglitazone. For example, alternative agonists such as 15d-PGJ2 that alleviate neuropathic pain (Churi et al, 2008) without alteration of glucose or HbA1c could be tested in ZDF. Also, we could test a "control" drug that normalizes glucose
metabolism without affecting hyperalgesia or investigate whether administration of glucose to pioglitazone-treated ZDF could reinstate hyperalgesia.

4.5.5 Conclusions and Future Directions

The current results are the first to demonstrate in a preclinical model of type 2 diabetes that pioglitazone prevents the development of not only pain-like behavior but also noxious stimulation-evoked central sensitization – within the same subjects. We go beyond the use of non-noxious von Frey hairs to evaluate spinal sensitization in ZDF (Schuelert et al, 2015) or nerve injury (Morgenweck et al, 2013) by evoking spinal pERK and hyperalgesia using a clinically relevant pressure stimulus (Otto et al, 2003). Our results extend the potential efficacy of pioglitazone from resolving neuropathic pain after traumatic nerve injury to the growing problem of PDN.

Alternative strategies using other TZDs or related therapies may bypass the human safety risks of pioglitazone (Lewis et al, 2011). First, the non-TZD PPARγ agonists FK614 (Minoura et al, 2007), MBX-102 (Chandalia et al, 2009; Gregoire et al, 2009), or MDG548 (Lecca et al, 2015) reduce insulin insensitivity, hyperglycemia, and inflammation and are neuroprotective (Lecca et al, 2015). Second, TZDs (Geldenhuys et al, 2010; Yonutas and Sullivan, 2013) or the novel ligand TT01001 (Takahashi et al, 2015) reduce diabetic mitochondrial dysfunction by targeting the mitochondrial protein mitoNEET, which is thought to alleviate other neurological diseases (Feinstein et al, 2005; Yonutas and Sullivan, 2013). Finally, a multiple drug strategy, such as coadministration of pioglitazone with canagliflozin (Watanabe et al, 2015) or geraniol (Ibrahim et al, 2015) might reduce diabetes without the adverse effects of adipocyte differentiation, fat deposition, and weight gain associated with pioglitazone-only treatment. PDN patients may also benefit from other diabetic drugs such as metformin, which reduces peripheral neuropathic pain (Mao-Ying et al, 2014) and PDN in STZ rats
(Byrne et al., 2015; Ma et al., 2015), as well as decreases hyperglycemia, HbA1c, and MG in patients with type 2 diabetes (Kender et al., 2014). Future clinical studies could investigate whether the above treatments or other PPARγ-directed therapies alleviate both hyperglycemia and neuropathic pain, as simultaneous inhibition is necessary to maximize quality of life in type 2 diabetic patients.

4.6 Supplemental Data & Discussion

4.6.1 Pioglitazone produces weight gain and hyperphagia.

In Figure 4-8 we illustrate that chronic oral administration of pioglitazone produced an increase in food consumption that presumably led to exacerbated weight gain in both ZL and ZDF. Hyperphagia and obesity did not change the average dose received by ZL and ZDF during the period of administration (see 0). Weight gain and edema are consistent with the known side effects of TZDs. Figure 4-9 illustrates that the same dose of pioglitazone (30 mg/kg/d) modestly increased weight gain in nerve injured Sprague-Dawley rats. It appears that the increase in weight gain is exacerbated by type 2
diabetes, as 7 wks of pioglitazone treatment resulted in SNI animals weighing roughly 450 g while ZDF were almost 700 g. These results indicate that weight gain should be monitored during long-term pioglitazone treatment and that future studies could investigate ways to reduce TZD-associated weight gain.

![Figure 4-9. Pioglitazone effect on weight gain in Sprague-Dawley rats after traumatic nerve injury.](image)

*Data courtesy of Renee Donahue.*

Pioglitazone administration in food began 7 d prior to spared nerve injury (SNI). (A) Pioglitazone modestly increased weight gain at 6-7 weeks after beginning administration at the same dose (30 mg/kg/d) used to treat PDN in ZDF. (B) However, pioglitazone did not increase the weight when analyzed using area-under-the-curve calculation for 0 to 42 d after SNI. * p<0.05 Pio 30 vs. Vehicle. † p<0.05 Pio 30 and Pio 3 vs. Vehicle.

4.6.2 *Exploratory locomotor behavior in ZDF decreases as PDN develops*

In order to measure the effect of diabetes on sedentary behavior we measured locomotor activity using an open field photo beam array. Rearing number, rearing duration, distance travelled, total beam breaks, and active/resting duration were quantified in control ZL and diabetic ZDF rats at 6, 12, and 18 weeks of age. Figure 4-10 shows that there are no significant differences in any of the activity measures at 6 or 12 wks but by 18 wks diabetic animals showed decreased activity levels in all measures
tested. These results are consistent with decreased locomotor activity in ZDF rats (Jiménez-Aranda et al., 2013) and db/db mice (Sharma et al., 2010) at similar stages of type 2 diabetes. Five weeks of rosiglitazone treatment (20 mg/kg/d orally) at a similar dose to the 30 mg/kg/d of pioglitazone in the current studies did not attenuate hypolocomotive behavior in db/db mice. Rosiglitazone did however attenuate hyperglycemia (Sharma et al., 2012). These results indicate that alleviating type 2 diabetes in rodents is not sufficient to reduce the hypolocomotion that may reflect affective components of animal behavior.

4.6.3 Mechanisms of PDN

Other mechanisms in addition to neuronal central sensitization might drive PDN in type 2 diabetes. First, increased expression of NGF (Cheng et al., 2009), pp38 (Cheng et al., 2010; Galloway and Chattopadhyay, 2013), Nav1.6 (Ren et al., 2012), Nav1.7 (Galloway and Chattopadhyay, 2013), TNFα (Cheng et al., 2010; Galloway and Chattopadhyay, 2013), and IL-1β (Galloway and Chattopadhyay, 2013) in the lumbar DRG of db/db mice and ZDF rats with PDN suggests peripheral pain sensitization. Further supporting expressional studies, administration of anti-NGF (Cheng et al., 2009) or a p38 kinase antagonist (Cheng et al., 2010) reduced mechanical allodynia in 8 wk old db/db mice. Second, PDN is associated with an increase in spinal expression of markers of astrocyte (GFAP) (Liao et al., 2011; Dauch et al., 2012; Ren et al., 2012; Xu et al., 2014) but not microglia (Ox42) (Liao et al., 2011; Xu et al., 2014) gliosis. Indeed, the astrocyte toxin LAA but not the microglial inhibitor minocycline reversed mechanical hyperalgesia in db/db mice (Liao et al., 2011). However, our preliminary results in ZDF contrast with previous reports indicating upregulation of glial markers in the spinal cord in PDN. Figure 4-11 illustrates that the expression of microglial and astrocyte activation markers is similar in ZL and ZDF at 18 wks of age. Given that this is the first study to investigate
glial activation in the spinal cord in ZDF, it is possible that species differences between db/db mice and ZDF rats contribute to the lack of upregulation in Iba1 or GFAP in the current study. In the future, assessment of functional markers for glial activation could be performed to investigate the interplay between neurons and glia in peripheral, spinal, and brain areas and to evaluate their contribution to PDN.
Figure 4-10. Locomotor activity decreases with age in ZDF.
General exploratory behavior in an open field activity box was measured in 5 min bins for 45 min at 6, 12, and 18 wks of age in ZL and ZDF. The number of rearing events, rearing duration, total beam breaks, distance travelled, active duration, and resting duration are shown. * p<0.05 ZDF vs. ZL.

Figure 4-11. Immunohistochemical quantification of microglial and astrocyte markers.
(A) Representative images of the ipsilateral dorsal horn in ZL and ZDF rats aged 18 wks from the first experiment characterizing the development of PDN. Quantification of (B) the microglial marker Iba1 and (C) the astrocyte marker GFAP revealed no significant differences in the expression of gliosis markers in the spinal cord. Integrated density was determined by thresholding the images using the default algorithm within NIS Elements to reduce background and include positively stained cells. Integrated density of the region of interest (ROI) is equal to the product of ROI area and mean intensity value. The mean intensity value represents the sum of the intensity values for all pixels above the threshold in the ROI divided by the number of pixels above threshold within the ROI. This method controls for differences in fluorescence background levels between slices and subjects.

We offer several explanations for our result that pioglitazone did not significantly reduce blood insulin levels in diabetic ZDFs: (1) Hyperinsulinemia in ZDFs occurs at an
earlier age (Gregoire et al., 2009; Ibrahim et al., 2015; Watanabe et al., 2015) and therefore insulin production in diabetic ZDFs may already be diminished in the vehicle group; (2) Pioglitazone treatment produces a modest increase in insulin production as evidenced by our control ZDFs; or (3) Pioglitazone did not reduce insulin in similarly aged diabetic ZDFs (Watanabe et al., 2015); (4) The duration of pioglitazone treatment was not long enough to reduce the overcompensation of pancreatic beta cell insulin production, although insulin sensitivity was enhanced as evidenced by the reduction in hyperglycemia. However, this finding does rule out an alternative mechanism of pioglitazone analgesia providing support to the conclusion that antihyperalgesia is independent of the metabolic benefits of pioglitazone.

Pioglitazone may decrease PDN by reducing diabetes-associated inflammation. In humans, TNF-α plasma levels and macrophage TNF-α expression are positively correlated with diabetic neuropathy-associated pain (Purwata, 2011). TZDs reduce proinflammatory factors such as: (1) blood TNF-α in a long-term model of diabetes (Qiang et al., 1998) or after methylglyoxal treatment (Hsu et al., 2013); (2) adipose TNF-α in ZDFs (Minoura et al., 2007); (3) in vitro production of TNF-α in activated macrophages (Jiang et al., 1998); (4) proinflammatory cytokine release in TLR-activated human monocytes and db/db mice (Dasu et al., 2009); (5) pioglitazone reduces diabetes-associated elevations in urinary (Ye et al., 2010) and monocyte (Dasu et al., 2009) expression of the chemokine MCP-1/CCL2; (6) glucose induction of TNF-α and MCP-1 release from rat microglia (Quan et al., 2011) may be diminished by pioglitazone reduction of hyperglycemia; and (7) molecules implicated in the generation of pain such as proinflammatory cytokines, Nav1.7, and pp38 are elevated in the DRG from diabetic ZDF (Galloway and Chattopadhyay, 2013). We speculate that chronic pioglitazone treatment decreases the neuroinflammatory state produced by type 2 diabetes, thereby reducing PDN.
Separating improvements in hyperglycemia and insulin resistance from analgesic effects of PDN treatments is non-trivial. Several lines of evidence make it unclear as to whether the correction of hyperglycemia (by treating the underlying insulin resistance in type 2 diabetes or by insulin infusion in type 1) results in decreased pain. (1) Localized hyperglycemia in the sciatic nerve or DRG induces pain-like behavior and was reversed by an aldose reductase inhibitor. This suggests that production of reactive oxygen species (ROS), the endpoint of the glucose-polyol pathway, is responsible for pain-like hypersensitivity (Dobretsov et al., 2003) rather than the direct neurotoxic effect of glucose (Tomlinson and Gardiner, 2008). (2) Taurine, an antioxidant, improves nerve conduction velocity and attenuates mechanical and thermal hyperalgesia without affecting hyperglycemia (Li et al., 2006). (3) Markers for oxidative or nitrosative stress, which can lead to pain, are reduced by Poly(ADP)-ribose polymerase inhibition without eliminating hyperglycemia (Obrosova et al., 2005). (4) Hyperglycemia persists when pain-like behavior is reduced in STZ (Ramos et al., 2007) and ZDF (Piercy et al., 1999) rats after treatment with IGF (Zhuang et al., 1996), IGF-II (Zhuang et al., 1997), or aldose reductase inhibitors (Ramos et al., 2007). (5) Pain behavior can persist even after correction of hyperglycemia (Lee and McCarty, 1992). Contrasting with the above, studies indicate that acute exposure to glucose can produce hyperalgesia (Dobretsov et al., 2001; Dobretsov et al., 2003) and glycemic control correlates with pain relief in diabetic patients (Oyibo et al., 2002). Nevertheless, treatments such as TZDs and metformin that reduce both hyperglycemia and pain-like behavior in preclinical models need further clinical investigation.
CHAPTER 5: Methylglyoxal produces pain in type 2 diabetes via sensitization of TRPA1 and AC1

Manuscript in preparation.

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5.1 Abstract

Type 2 diabetic pain remains difficult to treat, in part because the mechanisms that lead to painful diabetic neuropathy (PDN) are poorly understood. Metabolic dysregulation in diabetes produces an increase in the glucose metabolite methylglyoxal (MG) and blood levels of MG correlate with PDN in patients. The current literature led to our hypothesis that elevated MG contributes to pain in type 2 diabetes by sensitizing peripheral and/or spinal nociceptive neurons via activation of TRPA1 and AC1.

We confirmed that methylglyoxal is increased in the db/db mouse model of type 2 PDN. Intraplantar injection of MG produced multiple types of pain-like behaviors, including spontaneous nociceptive responses, mechanical and heat hypersensitivity, and conditioned place aversion, a measure of affective pain in preclinical studies. MG also triggered spinal nociceptive transmission, as it evoked phosphorylation of ERK and increased intracellular calcium in superficial dorsal horn neurons. Pharmacological and genetic disruption of TRPA1 attenuated pERK and pain-like behavioral responses to MG administration as well as reversed hyperalgesia in db/db mice. Knockout of AC1
abolished MG-induced hyperalgesia but not nociceptive responses while the AC1 inhibitor NB001 attenuated hyperalgesia in db/db mice. Finally, the novel MG scavenging peptide GERP10 attenuated hyperalgesia in db/db mice without altering hyperglycemia or elevated HbA1c. We conclude that elevated MG in type 2 diabetes contributes to PDN through a TRPA1-AC1 dependent pathway.

5.2 Introduction

Painful diabetic neuropathy (PDN) is associated with accumulation of methylglyoxal (MG), a major metabolite of glycolysis, in the blood in both patients and rodent models (Bierhaus et al, 2012). For example, in healthy control subjects blood MG levels are 150-300 nM (McLellan et al, 1992; Bierhaus et al, 2012) and ~600 nM in diabetic patients, though levels are greatly exacerbated in diabetic patients reporting pain (~900 nM). This suggests a potential role for MG in the production of pain associated with diabetes.

Previous studies indicate that acute administration of MG produces spontaneous nociception (Andersson et al, 2013) and evoked heat hypersensitivity in mice (Bierhaus et al, 2012). Data from knockout mice suggest activation of transient receptor potential, ankyrin subtype 1 (TRPA1) as a mechanism of these effects in primary afferents. However, additional measures of evoked and affective pain-like behavior beyond MG-induced nociception and the use of additional pharmacological and genetic tools in preclinical models are needed to assess the contribution of MG and TRPA1 to ongoing PDN in type 2 diabetes.

A majority of PDN studies focus on the streptozotocin (STZ) model of type 1 diabetes (Calcutt et al, 2009). Indeed, using the STZ model or administration of exogenous MG, Bierhaus et al showed that MG scavenging compounds (e.g. ALT-711, aminoguanidine) or an arginine-rich peptide (i.e. GERP10) reduced MG-associated hypersensitivity (Bierhaus et al, 2012). Here we focus on db/db mice (Kodama et al, 1994), a model of
type 2 diabetes that is ideal because: (1) type 2 diabetes represents the majority (90%) of diabetic patients (World Health Organization) and has a greater incidence of PDN compared to type 1 (Van Acker et al, 2009; Abbott et al, 2011); (2) pain mechanisms in type 1 versus type 2 likely differ (Schmidt et al, 2003; Schmidt et al, 2004; Kamiya et al, 2005; Schuelert et al, 2015); (3) it is unknown whether administration of the MG scavenger GERP10 will alleviate type 2 PDN as it does in STZ (Bierhaus et al, 2012); and (4) STZ may activate TRPA1 to produce peripheral neuropathy and pain prior to the development of diabetic hyperglycemia (Andersson et al, 2015) thereby confounding conclusions from previous STZ studies investigating the role of TRPA1 in development of PDN (Wei et al, 2009; Wei et al, 2010; Koivisto et al, 2012).

Several lines of evidence suggest that sensitization of nociceptive neurons in the spinal dorsal horn of primary afferents may contribute to painful type 2 diabetes (aka PDN) through a TRPA1 mechanism. First, increased phosphorylation of extracellular signal-regulated kinase (pERK), a marker for nocireponsive spinal neuron activation (Ji et al, 1999; Gao and Ji, 2009), is associated with pain-like hypersensitivity in type 2 diabetic rats (see Chapter 4: ). Second, inhibition of spinal ERK phosphorylation in db/db mice reversed pain-like hypersensitivity (Xu et al, 2014). Third, MG-evoked nociception and calcium mobilization in DRG neurons was abolished in TRPA1 knockout mice (Eberhardt et al, 2012; Andersson et al, 2013). Fourth, MG-evoked activation of SH-SY5Y cells was prevented by NMDA receptor inhibition (de Arriba et al, 2006). Along these lines, we hypothesized that: (1) MG activation of peripheral TRPA1-positive nociceptors induces spinal phosphorylation of ERK (pERK) that is dependent on peripheral and/or spinal TRPA1; and (2) elevated MG in type 2 diabetes produces painful spinal sensitization as evidenced by exacerbated glutamate- and/or MG-evoked calcium responses in spinal slices and dissociated DRG neurons from wild-type and db/db mice.
Calcium activated adenylyl cyclase 1 (AC1) (Xia and Storm, 1997) is a key modulator of pain plasticity in the central nervous system (Zhuo, 2012). For example, AC1 mediates spinal LTP and ERK activation induced by capsaicin or glutamate in mice (Wei et al, 2006) as well as NMDA-induced pain reinstatement after injury (Corder et al, 2013). As glutamate and NMDA receptors play a role in both normal and sensitized pain transmission, and activation of TRPA1 leads to a rise in intracellular calcium (Eberhardt et al, 2012; Ohkawara et al, 2012), we tested the hypothesis that AC1 mediates pain-like responses after MG administration and in db/db mice with PDN.

5.3 Materials and Methods

5.3.1 Subjects

Experiments were carried out in accordance with the Institutional Animal Care and Use Committee at the University of Kentucky (Approved Protocol # 2009-0486). All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to in vivo techniques, in accordance with the International Association for the Study of Pain (Zimmermann, 1983) and the National Institutes of Health Office of Laboratory Animal Welfare Guide for the Care and Use of Laboratory Animals.

Animals were housed in a temperature and humidity controlled room on a 12 hour light – 12 hour dark cycle with lights on from 07:00 to 19:00. Animals were provided water and chow ad libitum. All rats and mice used were male. Male Sprague-Dawley rats (CD-IGS, Charles River Laboratories, Inc., Wilmington, MA) weighing 300-450g at the time of behavioral procedures were used. TRPA1 knockout (-/-) and wild-type littermates (+/+ ) mice were graciously provided by Dr. Gregory Frolenkov (Department of Physiology, University of Kentucky, Lexington, KY), courtesy of Drs. Kelvin Kwan and David Corey (Harvard University, Cambridge, MA), and were used to setup a TRPA1
knockout colony. AC1/- and AC1+/+ mice were graciously provided by Dr. Daniel Storm (Washington University, Seattle, WA, USA) and were used to setup an AC1 knockout colony. Both congenic knockout mouse lines were maintained using a heterozygote breeding strategy onto a C57BL/6J (000664, JAX Mice, The Jackson Laboratory, Bar Harbor, ME) background. Genotypes were confirmed by PCR. Control (C57BLKS/J; BKS) and diabetic (Lepr<sup>db</sup>; db/db) mice (The Jackson Laboratory, Bar Harbor, ME; Stock No. 000642) were aged 6-14 wks. Mice aged 8-12 wks were used for all non-db/db studies with C57BL/6J serving as congenic controls.

5.3.2 Drugs & Injections

Methylglyoxal (M0252, Sigma-Aldrich, St. Louis, MO) was diluted in 0.9% sterile saline for intraplantar injections (0-1000 µg) or aCSF (0-10 mM) for calcium imaging studies. NB001 (SML0060, Sigma-Aldrich) was diluted in water for intrathecal injections. GEAP10 and GERP10 were graciously provided by Drs. Thomas Fleming and Peter Nawroth (University Hospital Heidelberg, Heidelberg, Germany) and diluted in 0.9% saline for intraperitoneal injections. For intrathecal (5-10 µL), intraperitoneal (100 µL), and intraplantar (5-25 µL) injections un-anaesthetized mice were lightly restrained and a 30Ga½" needle attached to a Hamilton microsyringe was used to administer drug solutions. Intrathecal injections were performed by puncturing the dura between the L5/L6 vertebrae and confirmed by presence of reflexive tail or hindpaw flick as previously described (Fairbanks, 2003).

Stock solutions of methylglyoxal contain <1% of impurities such as formalin, which is known to be an agonist for TRPA1 (McNamara <i>et al</i>, 2007). However, concern that contaminates in the Sigma-Aldrich solution used in this study could confound our conclusions is mitigated by the finding that electrophysiological results using MG from
Sigma-Aldrich and synthesized pure MG were identical in a previous CNS study (Distler et al., 2012).

5.3.3 Pain-like Behavior

Fluctuations in noise, vibrations, temperature, and other distractors in the behavioral testing room were minimized to optimize reliable measurements between cohorts of animals tested on different days. Animals were acclimated to the different testing apparatuses prior to commencing behavioral studies and for 30-60 min on the day(s) of testing.

Nociceptive responses were measured by placing the animals within an acrylic enclosure immediately following intraplantar injection of saline or methylglyoxal. A mirror was positioned below the enclosure to facilitate quantification of spontaneous licking/lifting (s) or flinching (#) of the injected hindpaw for five minutes. The seconds of licking/lifting and the number of flinches were added together to obtain a combined number of nociceptive responses, where one second of licking/lifting or one flinch were equated to one nociceptive response.

Heat hypersensitivity was assessed by placing the animals on a heated surface (52.5 ± 1 °C) within an acrylic enclosure (Hotplate; Columbus Instruments, Columbus, OH). The time until a hindpaw withdraw response (e.g. jumping, licking, flicking) occurred was recorded. The animal was immediately removed after the withdraw response or at a cutoff of 30 s to avoid tissue injury. Three trials were averaged for each timepoint.

Mechanical hypersensitivity was assessed by placing animals within a rectangular acrylic box (15x4x4 cm; 3 white opaque walls and 1 clear wall) and stimulating the ventral-medial hindpaw plantar surface with an incremental series of 8 von Frey monofilaments (Stoelting, Inc., Wooddale, IL) of logarithmic stiffness using a modified up-down method (Dixon, 1980; Chaplan et al., 1994). Testing began by applying an
intermediate von Frey monofilament (number 4.31, exerts 2.0 g of force) perpendicular to the glabrous skin, causing a slight bending. In the case of a positive response (withdraw of the paw) a filament exerting less force was applied. In the case of a negative response, a filament exerting greater force was applied. The calculated 50% withdraw threshold is reported.

Conditioned place aversion (CPA) testing was completed using a three-chambered acrylic enclosure with manual doors and the time spent in each chamber was quantified using a 4 x 16 photobeam array (Place Preference, San Diego Instruments, San Diego, CA; http://www.sandiegoinstruments.com/place-preference/). Printed paper was used to cover the outside of the clear box so that the middle chamber was grey and the end chambers had either vertical or horizontal black and white stripes that were ¾” wide. Mice were acclimated to the CPA box on d1-2 and then preconditioning preferences were assessed on d3. During conditioning on d4, intraplantar saline injection-pairing in the morning was followed by methylglyoxal (300µg) injection-pairing to the opposite chamber in the afternoon. On d5, the same conditioning procedure was repeated with methylglyoxal injected into the hindpaw that received saline on d4. Drug pairings and the hindpaw initially receiving methylglyoxal were counterbalanced. On d6, postconditioning preferences were assessed in the absence of any injections.

5.3.4 pERK quantification via immunohistochemistry

Animals were injected intraplantar with saline or methylglyoxal, nociceptive responses were quantified, and then mice were anesthetized with isoflurane (5% induction, 2% maintenance). Ten minutes after intraplantar injection, animals were perfused through the left ventricle with room temperature 0.1 M phosphate buffered saline (PBS) with heparin (10,000 USP units/L) followed by ice-cold fixative (10% phosphate buffered formalin). The lumbar spinal cord was removed and post-fixed
overnight in 10% phosphate buffered formalin and then cryoprotected in 30% sucrose in 0.1 M PBS for several days. Transverse sections (30 μm) from L3-L4 were cut on a freezing microtome and collected in 0.1 M PBS. The sections were washed three times in 0.1 M PBS and then pretreated with blocking solution (3% normal goat serum and 0.3% Triton X-100 in 0.1 M PBS) for 1 h. Sections were then incubated in blocking solution containing the primary antibody rabbit anti-pERK (1:250, #4370, Cell Signaling Technology, Danvers, MA) overnight at room temperature on a slow rocker. The sections were washed three times in 0.1M PBS, and incubated goat anti-rabbit secondary antibody (1:800, Alexa 568, Molecular Probes, Grand Island, NY) for 90 min, washed in 0.1M PBS, 0.01M PBS, then 0.01M PB, and non-sequentially mounted onto Superfrost Plus slides, air dried, and cover-slipped with Prolong Gold with DAPI mounting medium (Molecular Probes).

All images were captured on a Nikon Eclipse TE2000-E microscope using a 10x objective and analyzed using NIS-Elements Advanced Research software. We focused our quantification of the number of pERK immunopositive cell profiles within lamina I-II, where the majority of C-fibers and nociceptive peripheral afferents terminate within the dorsal horn (Basbaum et al, 2009; Corder et al, 2010). Each spinal cord slice was analyzed by an observer blinded to treatment. 4-6 high quality sections were randomly selected for quantification.

5.3.5 Calcium imaging

Preparation of adult mouse spinal cord slices. Mice were anesthetized with 5% isoflurane and quickly perfused transcardially with 10 ml of ice-cold sucrose-containing artificial cerebrospinal fluid (aCSF) (sucrose-aCSF) that contained (in mM): NaCl 95, KCl 1.8, KH₂PO₄ 1.2, CaCl₂ 0.5, MgSO₄ 7, NaHCO₃ 26, glucose 15, sucrose 50, kynurenic acid 1, oxygenated with 95% O₂, 5% CO₂; pH 7.4. The lumbar spinal cord was rapidly
isolated by laminectomy from the cervical enlargement to the cauda equina, placed in oxygenated ice-cold sucrose-aCSF, cleaned of dura mater and ventral roots, and super-glued vertically to a block of 4% agar (Fisher Scientific, Pittsburgh, PA) on the stage of a Campden 5000mz vibratome (Lafayette, IN). Transverse slices (450 µm) from lumbar segments L3/L4 were cut in ice-cold sucrose-aCSF.

**Fluorometric Ca²⁺ measurements.** Slices were incubated for 30 min with Fura-2 AM (10 μM), pluronic acid (0.1%) in oxygenated aCSF containing (in mM): NaCl 127, KCl 1.8, KH₂PO₄ 1.2, CaCl₂ 2.4, MgSO₄ 1.3, NaHCO₃ 26, glucose 15 at 37°C, followed by a 20 min de-esterification period in normal aCSF. Prior to recording, slices were kept at RT in a chamber containing approximately 150 ml of oxygenated aCSF. Slices were perfused at 1-2 ml/min with normal aCSF in an RC-25 recording chamber (Warner Instruments, Hamden, CT) mounted on a Nikon FN-1 upright microscope fitted with a 79000 ET FURA2 Hybrid filter set (Nikon Instruments, Melville, NY) and a Photometrics CoolSNAP HQ2 camera (Tucson, AZ). Relative intracellular Ca²⁺ levels were determined by measuring the change in ratio of fluorescence emission at 510 nm in response to excitation at 340 and 380 nm (200 ms exposure). Paired images were collected at 1-1.5 seconds/frame. Relative changes in Ca²⁺ levels were evaluated using Nikon Elements software by creating a region of interest over the cell body and calculating the peak change in ratio. The peak magnitude of Ca²⁺ response to 90 s exposure to MG was normalized to the Ca²⁺ response to 1 mM glutamate (10 s). Only cells that displayed a consistent response to 1 mM glutamate at the beginning and end of the experiment (showing a less than 40% decrease in glutamate-evoked Ca²⁺ transients) were included in this study.
5.3.6 Quantification of Methylglyoxal-derived advanced glycation end-products (MG-AGEs)

MG-AGEs were quantified using a competitive ELISA according to the manufacturer’s instructions (STA-811, Cell BioLabs, San Diego, CA). This ELISA uses a primary antibody that recognizes the hydromidazolone (H1) moiety created by the modification of protein residues by methylglyoxal (Ahmed, 2003). Whole blood taken from the left ventricle prior to transcardial perfusions was collected in serum separator tubes (SST™, BD, Franklin Lakes, NJ) and allowed to clot for 30 min. Clotted blood in SSTs was centrifuged at 5000 x g for 10 min at 4ºC and the serum was transferred to fresh microcentrifuge tubes and stored at -80ºC until MG-H1 ELISA analysis. Serum samples were diluted in 0.1M PBS to obtain values in the span of the standard curve.

5.3.7 Measurement of blood glucose & HbA1c

Mice were lightly restrained in a towel and the distal tail wiped with an alcohol swab. A small nick was made at the distal tip of the tail using a #11 scalpel blade. Initial bleeding was wiped clean with gauze and subsequent drops of blood were either loaded into a room temperature HbA1c cartridge and analyzed using a DCA Vantage Analyzer (Siemens, Munich, Germany), or placed on a glucose test strip in duplicate and inserted into a glucose monitor (TrueTrack, Walgreens, Deerfield, IL). To avoid perturbations in pain-like behavior elicited by fasting or exogenous glucose administration (Dobretsov et al, 2001; Dobretsov et al, 2003), non-fasted blood glucose was measured. A random blood glucose level greater than 200 mg/dL (11.1 mmol/L) was defined as hyperglycemia (Capes et al, 2000; Sacks, 2011).
5.3.8 Data analysis and statistics

Nociceptive behavior and calcium responses in wild-type rats and mice were analyzed using a one-way ANOVA followed by Holm-Sidak multiple comparison correction. Nociceptive behavior, pERK immunohistochemistry, blood glucose, and HbA1c in wild-type, TRPA1-/-, AC1-/-, and db/db mice were analyzed using a two-way ANOVA followed by Holm-Sidak multiple comparison correction. Nociceptive behavior, mechanical hypersensitivity, heat hypersensitivity, conditioned place aversion, and blood glucose in wild-type, TRPA1-/-, AC1-/-, and db/db mice were analyzed using a repeated measures two-way ANOVA followed by a Holm-Sidak multiple comparison correction. An alpha value of α=0.05 was used to determine statistical significance. All data were analyzed and graphed using Prism 6.0 (GraphPad, La Jolla, CA) and are presented as mean ± SEM.

5.4 Results

5.4.1 Methylglyoxal produces pain-like behavior

First we determined whether exogenous intraplantar administration of methylglyoxal into the hindpaw is sufficient to produce pain-like behaviors. As illustrated in Figure 1A-C, methylglyoxal produced dose-dependent nociceptive responses in rats [F(2,9) = 6.87; P = 0.015] and mice [F(7,41) = 88.2; p < 0.0001], with 1000 μg of MG producing nociceptive responses that lasted for one hour [drug x time; F(15,90) = 3.4; p = 0.0002]. As illustrated in Figure 1D-E, methylglyoxal produced hypersensitivity to mechanical [drug x time; F(5,85) = 3.54; p = 0.0059] and heat [dose x time; F(15,100) = 5.0; p < 0.0001] stimuli.

To determine an aspect of affective pain we tested whether methylglyoxal produced conditioned place aversion. Baseline preconditioning time spent in the saline-paired (mean ± SEM; 334.7 s ± 38.9) or methylglyoxal-paired (354.2 s ± 42.35) chambers was
similar \( p = 0.75 \), indicating our injection pairings were counterbalanced. Postconditioning time spent in the methylglyoxal-paired chamber (273.9 s ± 38.6) was less than time spent in the saline-paired chamber (489.6 s ± 49.9) \( p = 0.0026 \), indicating avoidance of methylglyoxal administration. As illustrated in Figure 1E, the difference scores for saline and methylglyoxal were significantly different \( p = 0.021 \), indicating conditioned place aversion to injection of methylglyoxal.

**Figure 5-1.** Exogenous methylglyoxal produces spontaneous, evoked, and affective pain-like behavior.
Nociceptive behavioral responses in (A) rats (n=3-5) and (B-C) mice (n=3-14) as well as evoked (D) mechanical (n=9-10) and (E) heat (n=4-12) thresholds after intraplantar (i.pl.) injection of methylglyoxal (MG). (E) Difference scores (post minus pre conditioning) for conditioned place aversion testing using two days of i.pl. MG (300 μg) injections in mice (n=8). ★ p<0.05 vs. Saline. # p<0.05, MG 10 μg vs. Saline. † p<0.05, MG 30 μg vs. Saline. ‡ p<0.05 MG 100 μg vs. Saline.

5.4.2 Methylglyoxal activates spinal neurons to produce PDN

Previous studies indicate methylglyoxal activates dorsal root ganglia neurons via TRPA1 to produce nociception (Eberhardt et al, 2012; Andersson et al, 2013). However, whether elevated methylglyoxal in type 2 diabetes sensitizes spinal neurons remains unknown. Therefore we tested the hypothesis methylglyoxal activates nocireponsive neurons in the spinal cord dorsal horn. Figure 2A illustrates that intrathecal injection of methylglyoxal produces hindpaw mechanical hypersensitivity at 30-120 min [p < 0.05] with a significant Drug by Time interaction [F(6,36) = 4.712; p = 0.0012].

Figure 5-2. Methylglyoxal activates nociceptive neurons in the spinal cord dorsal horn to produce pain-like behavior.
Data courtesy of Renee Donahue. I formulated the hypothesis, designed the experiment, and analyzed the data.
(A) Mechanical sensitivity before and after intrathecal (i.t.) injection of MG (n=4). ★ p<0.05 vs. Saline.
5.4.3 TRPA1 mediates spinal sensitization and pain in type 2 diabetes

Phosphorylated extracellular signal-regulated kinase (pERK) in the spinal cord dorsal horn (Ji et al., 1999; Zhuang et al., 2005) is a marker of nocireponsive neuron activation (Gao and Ji, 2009) and stimulus-evoked central sensitization (Morgenweck et al., 2013)(Griggs et al., 2015, under review at Journal of Pain), which is defined as the increased responsiveness of nociceptive neurons in the central nervous system to their normal or subthreshold afferent input (Woolf, 2011). To evaluate methylglyoxal-induced spinal sensitization we measured the number of cell profiles displaying pERK in the superficial dorsal horn after MG hindpaw administration. To determine the contribution of peripheral and/or spinal TRPA1 we administered MG in wild-type and TRPA1 knockout (-/-) mice, or after administration of intrathecal vehicle or HC030031, a TRPA1 inhibitor, to wild-type mice.

Figure 3A-B shows that MG injection increased pERK in wild type [p < 0.0001], but not TRPA1-/- [p = 0.95] mice [drug x strain; F(1,11) = 48.41; p < 0.0001] as well as in wild-type mice with intrathecal (i.t.) vehicle [p < 0.0001] but not HC [p = 0.088] administration [drug x drug; F(1,12) = 67.02; p < 0.0001]. As illustrated in Figure 3C, methylglyoxal produced nociception in wild-type [vs. saline; p < 0.0001] but not TRPA1-/- [p = 0.77] mice [F(1,14) = 44.66; p < 0.0001] as well as in wild-type mice with i.t. vehicle [p < 0.0001] administration [drug x drug; F(1,19) = 29.57; p < 0.0001]. Importantly, although MG produced modest nociceptive behavior in HC-treated mice [vs. HC Saline; p = 0.0026], nociceptive response was significantly less than that in vehicle-MG mice [p < 0.0001]. Figure 3D illustrates that, compared to saline, methylglyoxal decreased withdraw latency to heat in wild-type but not TRPA1-/- mice [strain x time; F(5,45) = 10.67; p < 0.0001]. Compared to TRPA1-/-, methylglyoxal produced heat hypersensitivity in wild-types at 30 min [p < 0.0001]. As illustrated in Figure 3E, intrathecal administration
of HC030031 reversed heat hypersensitivity in diabetic db/db but not control BKS mice
[F(5,30) = 7.088; p = 0.0002] from 15-45 min [p < 0.05].
Figure 5-3. TRPA1 mediates methylglyoxal-induced spinal nociceptive neuron activation and hyperalgesia in painful diabetic neuropathy.

To determine whether activation of spinal neurons is dependent upon TRPA1 we injected methylglyoxal (MG; i.pl.) in wild-type mice alongside intrathecal (i.t.) HC030031 (HC) or in TRPA1 knockouts and measured phosphorylated ERK (pERK), a marker of nociceptive neuron activation, in the L4-L5 spinal cord. (A) Representative dorsal horn images ipsilateral to injection of saline or MG. Quantification of (B) pERK+ cells, (C) nociceptive responses, or (D) hotplate behavior after i.pl. saline or MG injection in animals treated with with i.t. vehicle or HC (10 μg), or in wild-type (+/+ ) or TRPA1 knockout (-/- ) mice. pERK, nociceptive responses, and heat hyperalgesia were abolished after pharmacological or genetic inhibition of TRPA1. (E) Heat thresholds in control BKS and diabetic db/db mice after i.t. injection of the TRPA1 antagonist (10 μg) at 10 wks of age. ★ p<0.05 vs. all other groups. # p<0.05 vs. db/db Vehicle.

5.4.4 AC1 facilitates methylglyoxal-induced hypersensitivity

Our finding that activation of TRPA1 by methylglyoxal resulted in subsequent heat hypersensitivity is puzzling, considering TRPA1 primarily responds to cold (Story et al., 2003), mechanical (Kwan et al., 2009), or chemical (Bautista et al., 2005; McNamara et al., 2007; Trevisani et al., 2007; Eberhardt et al., 2012) stimuli. However, previous studies suggest a functional link between TRPA1 and TRPV1 containing primary afferents (Fischer et al., 2014; Spahn et al., 2014; Weng et al., 2015), a phenomena that might be mediated by AC1 (Spahn et al., 2014). Therefore we tested the hypothesis that AC1 mediates pain-related behaviors in wild-type (WT) and AC1 knockout (AC1-/-; KO) mice after methylglyoxal administration, as well as in BKS and db/db mice with pretreatment with the AC1 inhibitor NB001.

Figure 4A illustrates that, compared to saline, methylglyoxal produced nociceptive responses [drug; F(1,14) = 193.6; p < 0.0001] in wild-type [vs. saline; p < 0.0001] and AC1-/- [p < 0.0001] mice. Saline or methylglyoxal-induced responses were similar in wild-type and AC1-/- mice [p = 0.99]. As illustrated in Figure 4B, compared to saline injection methylglyoxal produced heat hypersensitivity in wild-type mice [drug in WT; F(5,30) = 8.72; p < 0.0001] at 30-60 min [p < 0.001] but not in AC1-/- [drug in KO;
F(5,30) = 1.12; p = 0.37. Compared to AC1-/−, methylglyoxal produced heat hyperalgesia in wild-types at 30 min [p = 0.03]. As illustrated in Figure 4C, intraplantar methylglyoxal injection had no effect on mechanical thresholds in AC1-/− at any timepoint measured [F(5,35) = 0.575; p = 0.72]. As illustrated in Figure 4D, intrathecal injection of the AC1 inhibitor NB001 had no effect on heat thresholds in control BKS mice [F(1,6) = 0.05; p = 0.83]. However, NB001 significantly attenuated heat hyperalgesia in db/db mice overall [F(1,6) = 7.35; p = 0.035] and at 45 min [p = 0.044].

5.4.5 Methylglyoxal scavenging reduces pain-like behavior in type 2 diabetes

We reasoned that if methylglyoxal is producing pain in type 2 diabetes, then strategies that reduce elevated methylglyoxal in diabetic subjects would alleviate pain-like behavior. Recently, the novel peptide GERP10 and its control GEAP10 were administered to methylglyoxal or streptozotocin treated mice. GERP10 peptide, which

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**Figure 5-4. AC1 mediates methylglyoxal-induced hyperalgesia in painful diabetic neuropathy.**

(A) Nociceptive, (B) hotplate, and (C) mechanical behavioral responses after intraplantar injection of saline or methylglyoxal in wild-type C57BL6J (+/+) or AC1 knockout (-/-) littermates. (D) Heat thresholds after intrathecal (i.t.) injection of the AC1 inhibitor NB001 (1.5 μg) in BKS and db/db mice at 13 wks of age. # p<0.05 as shown. ★ p<0.05 vs. all other groups.
contains ten repeats of the amino acid arginine that favors formation of MG-H1 residues (Allaman et al, 2015), but not GEAP10 (instead containing non-reactive alanine) was shown to reduce hyperalgesia and serum methylglyoxal levels (Bierhaus et al, 2012). Here we extended this experiment to the db/db model of type 2 diabetes, which progressively develop mechanical (Liao et al, 2011; Ren et al, 2012; Xu et al, 2014) and thermal (Xu et al, 2014) hypersensitivity as well as elevated blood MG (Bierhaus et al, 2012). As illustrated in Figure 5A, db/db mice developed heat hyperalgesia [F(4,112) = 11.49; p < 0.0001] at 7-10 wks of age [p < 0.0001] but were not hyperalgesic at 6 wks [p = 0.60]. As illustrated in Figure 5B-C, db/db mice had elevated blood glucose [F(4,112) = 9.092; p < 0.0001] at 6-10 wks [p < 0.0001] and increased MG-H1 AGE at 10 wks [p = 0.03]. As illustrated in Figure 5D, a single intraperitoneal injection of GERP10 but not GEAP10 increased heat thresholds in db/db mice [F(1,13) = 7.49; p = 0.02] without altering thresholds in BKS controls [F(1,13) = 0.134; p = 0.72]. GERP10 attenuation of heat hyperalgesia in db/db mice lasted for several days. As illustrated in Figure 5E-F, blood glucose [p < 0.0001] and HbA1c [p < 0.0001] were elevated in db/db compared to BKS mice 7 d after injection of the control peptide GEAP10. GERP10 did not alter blood glucose [F(1,12) = 0.1; p = 0.76] or HbA1c [F(1,8) = 1.49; p = 0.26] levels.
Figure 5-5. The methylglyoxal scavenger GERP10 reduces painful diabetic neuropathy.
Measurement of (A) heat thresholds, (B) blood glucose, and (C) serum methylglyoxal (11 wks of age) in BKS and db/db mice. (D) Heat thresholds, (E) blood glucose, and (F) blood HbA1c levels after a single intraperitoneal (i.p.) injection of the methylglyoxal scavenging peptide GERP10 (1 mg) or its control GEAP10 (1 mg) in BKS or db/db mice at 10-11 wks of age. ★ p<0.05 vs. all other groups.

5.5 Discussion

5.5.1 Methylglyoxal contributes to painful type 2 diabetes

Our current results are the first to show a number of related findings with regard to the spinal contribution of methylglyoxal to pain in diabetes. First, i.pl. administration of exogenous MG produces spontaneous nociceptive behavior in both mice and rats.
Second, MG produced transient, stimulus-evoked mechanical and heat hypersensitivity and negative affect as evidenced by conditioned place aversion. Third, we show that administration of MG to the spinal cord produces behavioral hypersensitivity, suggesting that spinal sites of action are involved in diabetic pain. Fourth, activation of spinal neurons and behavioral hypersensitivity depends upon both TRPA1 and AC1. Fifth, inhibition of spinal TRPA1 or AC1 attenuates pain-like behavior in db/db mice. Finally, scavenging of systemic methylglyoxal by GERP10 alleviates pain in a model of type 2 diabetes.

We provide evidence that the nociceptive response to MG is dose-dependent, occurs in rats, and persists for one hour at the highest dose tested (1000 µg). The level of nociceptive responses in mice were comparable to a previously used dose (18 µg; i.pl.) (Andersson et al, 2013). Nociception occurs rapidly, within several seconds, suggesting direct modulation (i.e. activation/depolarization) of primary afferent nociceptors. Indeed, MG produced a calcium response in cell culture (after transfection with human TRPA1) (Eberhardt et al, 2012; Ohkawara et al, 2012; Andersson et al, 2013) and mouse peripheral DRG neurons (Eberhardt et al, 2012; Andersson et al, 2013). This is in contrast to the notion that MG sensitizes nociceptors through a process mediated by keratinocytes, that could result in evoked hypersensitivity (Kwan et al, 2009).

Hindpaw administration of MG produced peripheral sensitization to further mechanical or heat stimulation that lasted for approximately one hour. Evoked hypersensitivity followed the initial chemical-induced spontaneous nociceptive responses and was maintained beyond the period of spontaneous pain-like responses. One explanation is suggested by previous findings, where MG mobilized calcium in isolated DRG neurons (Eberhardt et al, 2012; Andersson et al, 2013) to activate a substantial portion of polymodal nociceptors (Eberhardt et al, 2012), presumably contributing to the mechanical and heat hypersensitivity indicated by the current results. Evoked
hypersensitivity also occurs after hindpaw administration of the TRPV1 agonist capsaicin, both in humans (Simone et al, 1987; Simone et al, 1989) and rodents (Gilchrist et al, 1996). With capsaicin, initial spontaneous responses are mediated peripherally while the longer-lasting stimulus-dependent sensitization is due to a spinal mechanism (Zhang et al, 2006; Willis, 2009). These results taken together suggest that MG produces pain-like behavioral sensitization at least through a peripheral, if not also spinal, mechanism akin to activation of TRPV1 afferents. This hypothesis is further supported by our finding that intrathecal MG administration produced long-lasting mechanical hypersensitivity.

Strategies to block the effects of endogenous MG reveal its role in maintaining pain-like behavior in type 2 diabetes. It is clear that increases in circulating MG are associated with diabetes (Bierhaus et al, 2012; Kong et al, 2014). Further increases in circulating MG are associated with PDN (Bierhaus et al, 2012) and raising the intracellular MG concentration in freshly isolated DRG neurons from 1.5 µM to 2.2 µM (after 90 s application of 3 mM MG) induces neuronal activation (Eberhardt et al, 2012). Although these are examples of the effects of free (unbound) MG, MG-derived advanced glycation end-products (AGE), such as the formation of methylglyoxal-hydroimidazolone (MG-H1) moiety created by the modification of reactive protein residues by methylglyoxal (Ahmed, 2003), also contribute to painful neuropathy. AGEs such as MG-derived hydroimidazolone (MG-H1) are associated with diabetic pain in patients (Sveen et al, 2013) and result from the accumulation of free MG (Illien-Junger et al, 2015). These results suggest that even modest increases in global or local MG could contribute to painful sensitization.

Here we demonstrate that a single systemic administration of the free MG scavenging peptide GERP10 attenuates painful type 2 diabetes in db/db mice. We contribute to the growing body of work indicating that inhibition of MG, after exogenous
MG administration, in the STZ type 1 diabetes model (Bierhaus et al, 2012), and in type 2 diabetic db/db mice (current results) reduces pain-like behavior. This is in line with other methods of blocking MG, including administration of aminoguanidine or ALT-711 (Bierhaus et al, 2012), or by systemic overexpression of GLO1 (Bierhaus et al, 2012). Future studies are needed to determine whether GERP10 could alleviate painful diabetes in humans.

The major metabolic enzyme for MG, glyoxalase 1 (GLO1), plays a role in generating painful diabetes. For example, reduced GLO1 expression and activity is associated with pain in type 1 and type 2 patients (Skapare et al, 2013) as well as mice injected with MG or STZ (Jack et al, 2011; Bierhaus et al, 2012; Jack et al, 2012). Alternatively, administration of the GLO1 inhibitor Sr-p-Bromobenzylglutathionecyclopentyl diester (every 2nd day for 2 weeks, i.p. 50 mg/kg) resulted in hot, cold, pressure, and light-touch hypersensitivity (Andersson et al, 2013). Pain-like hypersensitivity presumably occurred via pharmacologically increasing endogenous MG, but elevated MG was not confirmed (Andersson et al, 2013). Finally, overexpression of GLO1 reduced painful diabetes in STZ (Bierhaus et al, 2012) and prevented MG-derived AGE formation (Berner et al, 2012). These results suggest that decreased GLO1 activity alone is sufficient to produce pain and that restoration of GLO1 activity could be beneficial for reducing the contribution of free MG and MG-AGEs to painful diabetes.

5.5.2 Methylglyoxal requires TRPA1 to produce painful diabetes

MG activates human (Eberhardt et al, 2012; Ohkawara et al, 2012), rat (Eberhardt et al, 2012), and mouse TRPA1 in both heterologous cell culture (Eberhardt et al, 2012; Ohkawara et al, 2012) and sensory neurons isolated from rodent DRGs (Eberhardt et al, 2012; Koivisto et al, 2012; Andersson et al, 2013). For the first time we report that either pharmacological inhibition or genetic knockout of TRPA1 abolished pain-related
responses (spontaneous nociceptive behavior and spinal pERK) and subsequent evoked behavioral hypersensitivity (hotplate). Along these lines, calcium influx or nociceptive behavior is abolished in cells (Eberhardt et al, 2012) or mice (Andersson et al, 2013) lacking TRPA1, respectively. Furthermore, the pain-like behavior that occurs after pharmacologically raising MG levels by repeated administration of the GLO1 inhibitor BrBz in wild-type mice is absent in TRPA1 knockouts (Andersson et al, 2013). These results taken together provide strong evidence that MG activates TRPA1 to produce pain.

There are many lines of evidence that support the hypothesis that tonic activation of TRPA1 contributes to neuropathic pain in diabetes. (1) Intrathecal Chembridge-5861528 (TRPA1 antagonist; CHEM) attenuates pain-like hypersensitivity in STZ rats (Wei et al, 2010). (2) Inhibition of TRPA1 prevents the loss of intraepidermal nerve fibers that occurs after STZ-induced diabetes (Koivisto et al, 2012). (3) Activation of spinal TRPA1 by cinnemaldehyde (Wei et al, 2011) or methylglyoxal (current results) produces hindpaw hypersensitivity. (4) Spinal administration of CHEM reduces secondary hyperalgesia produced by intraplantar formalin or capsaicin (Wei et al, 2011). (5) Intrathecal TRPA1 inhibition reduces hypersensitivity after spinal nerve ligation, an alternative model of painful neuropathy (Wei et al, 2011). (6) TRPA1 modulates dorsal horn neuron responsivity in naïve wild-type rats (Kosugi et al, 2007). It is clear that both peripheral and spinal TRPA1 contribute to pain-like behavior in multiple pain models.

In the current study, we provide two additional experimental results that support the hypothesis that tonic MG produces painful diabetes through spinal TRPA1. First, blockade of spinal TRPA1 with HC reduces both nociceptive behavior and spinal pERK in response to peripheral MG administration. We suggest that activation of TRPA1 afferents by MG produces release of endogenous TPRA1 agonists in the spinal cord that induce subsequent evoked hypersensitivity in a feed forward process, probably through
sensitization of spinal nocireponsive neurons. We favor this over the alternative explanation that MG activates TRPA1 located in spinal dorsal horn neurons, as there is no convincing evidence from the literature that suggests TRPA1 is expressed in dorsal horn neurons. This idea is supported by studies showing spinal release of reactive oxygen species or arachidonic acid derivatives such as 4-hydroxynonenal that drive pain through TRPA1 (Koivisto et al., 2013). Second, spinal inhibition of TRPA1 attenuated heat hypersensitivity in db/db mice. This further suggests that endogenous activation and/or sensitization of TRPA1 mediates painful diabetes. Future studies could investigate whether spinal inhibition of MG, or overexpression of the MG detoxification enzyme glyoxalase 1 (GLO1), would also alleviate PDN.

The above findings indicate that sustained activation of spinal and/or peripheral TRPA1 contributes to painful diabetes. However, our enthusiasm for this mechanism is tempered by two studies that the STZ model in studies of diabetic pain is an inappropriate model for studying the role of TRPA1. First, there is a dissociation between the reduction of evoked hypersensitivity and the lack of conditioned place preference, a measure of the reward associated with pain relief, produced by spinal TRPA1 antagonism in STZ or SNI (Wei et al., 2013). Second, STZ directly activates TRPA1 to cause peripheral neuropathy that precedes STZ-induced type 1 diabetes (Andersson et al., 2015). Behavioral hypersensitivity produced by STZ was delayed in TRPA1 knockouts, suggesting that TRPA1 mediates rapid sensitization and degeneration of peripheral afferents following STZ administration. This confounds TRPA1 antagonist studies in STZ, because now these results could be interpreted as the antagonist is blocking STZ-evoked hypersensitivity and neuropathy rather than MG-evoked painful diabetes.

The mode of activation of TRPA1 by MG is covalent and produces long-lasting sensitization TRPA1-containing neurons. MG reacts with lysine and arginine residues to
form H1 residues, as well as facilitating the formation of disulfide bonds between reactive cysteines. Cys 621, Cys 641, and Cys 665 have been identified by site-directed mutagenesis to be critical for MG activation of TRPA1 (Eberhardt et al., 2012), as mutation of these residues abolished MG-induced calcium responses. Another study provided somewhat conflicting results, whereas mutation of these same cysteine residues to serines inhibited responses to AITC but not MG (Andersson et al., 2013). However, MG-evoked calcium responses are diminished by the disulfide bond breaker DTT (Eberhardt et al., 2012), indicating indeed reactive cysteines are involved. We hypothesize that the cell type of heterologous expression may explain the discrepancy. In any case, the publication of the crystal structure of TRPA1 indicated that TRPA1 channel function could be regulated by these same three critical cysteine residues (621, 641, 665) (Paulsen et al., 2015). In addition to cysteines, MG forms adducts with lysine and arginine residues. Indeed, mutation of lysine 710 to glutamine reduced MG-evoked responses (Andersson et al., 2013). Modification of proteins in this manner is also referred to as advanced glycation end products (AGEs), which, along with their cognate receptor RAGE, are implicated in the pathogenesis of painful diabetes (Toth et al., 2008; Yao and Brownlee, 2010). In addition to sensitization of TRPA1, MG may contribute to PDN through AGE-RAGE signaling, as RAGE specifically recognizes MG-derived AGEs (Xue et al., 2014), but this remains a future direction.

5.5.3 MG induces cross-modality sensitization to heat via a TRPA1-AC1 pathway

TRPA1 is activated by cold (Story et al., 2003), mechanical (Kwan et al., 2009), and chemical (Bautista et al., 2005; McNamara et al., 2007; Trevisani et al., 2007; Eberhardt et al., 2012) stimuli. Here, activation of TRPA1 by MG led to hypersensitivity, an effect that was abolished by TRPA1 knockout. In mice, TRPA1 is expressed in small, mostly unmyelinated, peptidergic peripheral DRG neurons (C/Aδ) and predominantly
colocalizes with TRPV1 (Story et al., 2003; Bautista et al., 2005; Kobayashi et al., 2005; Garcia-Anoveros and Duggan, 2007). Functional studies indicated that MG produced a calcium response in 72% of capsaicin (prototypical TRPV1 agonist) responsive DRG neurons (Andersson et al., 2013), suggesting that 72% of TRPV1 fibers also express TRPA1. Furthermore, sensitization of TRPA1 in peripheral neurons is suggested to fine tune mechanical sensitivity (Kwan et al., 2009; Brierley et al., 2011). Considering these lines of evidence, it is not surprising that TRPA1-dependent activation of a substantial portion of C-mechanoheat sensitive afferents by MG (Eberhardt et al., 2012) would result in sensitization to subsequent mechanical or heat stimulation.

At least three possible scenarios may explain this TRPA1-TRPV1 sensitization: (1) It is indirect, where sensitization occurs via TRPA1-dependent depolarization of nociceptors lowering the activation/response threshold; (2) It is mediated by direct interaction of TRPA1 and TRPV1 (Fischer et al., 2014) or through a scaffolding protein such as Tmem100 that was previously implicated in persistent pain (Weng et al., 2015); or (3) Sensitization is produced by stimulation of secondary messengers such as AC1, as cAMP activation of PKA can result in phosphorylation-dependent sensitization of TRPV1 (Spahn et al., 2014). Here we present evidence that AC1 plays a critical role in mechanoheat sensitization after MG administration, but not in the spontaneous responses produced by direct activation of TRPA1 nociceptors.

We are the first to implicate AC1 and its sensitization by an MG-TRPA1 mechanism in diabetic pain. We suggest that AC1 mediates spinal sensitization, because intrathecal administration of NB001 attenuated heat hypersensitivity in db/db mice. However, nociceptive responses evoked by peripheral MG administration in the current results, pERK induced by application of capsaicin or glutamate to spinal slices (Wei et al., 2006), and pERK after peripheral injection of formalin (Wei et al., 2006) were similar in wild-type and AC1 knockout mice. This suggests that AC1 is not necessary for acute activation of
nociceptive neurons, even after activation of TRPA1 with an alternative agonist such as formalin. We can conclude, however, that AC1 is required for subsequent hypersensitivity to both non-noxious mechanical and noxious heat stimuli as well as painful spinal sensitization in type 2 diabetes. If indeed AC1 activity is upregulated in painful diabetes, then a future study could investigate whether intrathecal forskolin-induced cAMP production is exacerbated in db/db mice compared to their non-diabetic controls.

5.5.4 Conclusions toward treatments for painful diabetes

Our results are the first study to implicate AC1 in the maintenance of painful type 2 diabetes. Furthermore, we confirm that TRPA1 mediates MG-induced nociception and extend these results to indicate MG evokes both hypersensitivity and affective pain. Finally, we provide further evidence that strategies aiming to inhibit the MG-related effect on painful neuropathy in diabetes, such as GERP10, are effective at reducing type 2 diabetic pain-like behavior.

Our results suggest that blocking TRPA1 in peripheral and spinal nociceptive neurons could be a strategy for alleviating painful diabetes. To this end several groups are developing new TRPA1 antagonists. Glenmark pharmaceuticals (Mumbai, India) holds several U.S. patents on various molecules that act as TRPA1 antagonists. Although the structure has not been revealed, one such molecule presumably covered by a Glenmark patent, GRC-17536, underwent a Phase 2a clinical trial with sites in India, Germany, Czech Republic, and United Kingdom in patients with painful diabetic neuropathy. GRC reduced average pain intensity and was determined safe, although the complete results have not yet been released (https://clinicaltrials.gov/ct2/show/study/NCT01726413). Preclinical studies presented in abstract form that preceded this clinical trial indicated GRC to be a potent and selective
TRPA1 antagonist (Anupindi et al, 2010), but we were unable to find peer-reviewed publications on this molecule. In addition, new compounds that block TRPA1 activity are being developed by rational drug design using structure-activity relationships based on the prototypical TRPA1 antagonist discovered in 2007, HC030031 (Preti et al, 2015), which we used to block TRPA1 in the current studies.

Thus, approaches that minimize availability of MG to produce sensitization (Andersson et al, 2013), MG-AGEs (Sveen et al, 2013), or directly activate TRPA1 (Eberhardt et al, 2012; Ohkawara et al, 2012) or Nav1.8 (Bierhaus et al, 2012) could benefit patients with painful diabetes. For example, the type 2 diabetes drug metformin reduces plasma MG and increases GLO1 activity in peripheral blood mononuclear cells from type 2 diabetic patients (Kender et al, 2014); however, whether metformin reduces diabetic pain in humans as it does in STZ rats (Byrne et al, 2015; Ma et al, 2015) remains unknown. Our manuscript under review at Journal of Pain indicates that the PPARγ agonist pioglitazone reduced PDN and spinal sensitization in type 2 diabetic rats that exhibited increased MG-AGEs. A future direction of our lab is to determine whether pioglitazone could reduce the MG-AGE formation, TRPA1 sensitization, and pain.
CHAPTER 6: Overall Discussion and Supplemental Data

6.1 Summary

The results discussed herein suggest multiple ways of targeting methylglyoxal or PPARγ to alleviate the neuropathic pain associated with type 2 diabetes. I began by discussing the background information that is necessary to understand preclinical pain research and the growing problem of painful type 2 diabetes. Next I showed published evidence in Chapter 2: that gabapentin produces conditioned place preference in rats with traumatic nerve injury, an important finding indicating that: 1) systemic injection of an analgesic drug is sufficient to produce conditioned place preference in rodents suggesting rapid alleviation of pain; and 2) I validated the use of conditioned place preference (or aversion as shown in section 5.4.1) to assess an aspect of motivational-affective pain in rodents. Below I propose several additional studies to utilize novel assays to assess motivational-affective pain in preclinical type 2 diabetes.

I presented another publication indicating that PPARγ agonists rapidly reduce nociceptive and neuropathic pain-like behaviors through a non-genomic, astrocyte-related mechanism in Chapter 3: . In addition to rapid analgesia, my manuscript under review at the Journal of Pain shows that genomic PPARγ mechanisms also mediate inhibition of type 2 diabetic neuropathic pain and associated sensitization of spinal nocireponsive neurons. These results, along with unpublished studies shown below, indicate a differential effect of acute versus chronic administration of PPARγ agonists such as pioglitazone on Fos and/or pERK markers of painful central sensitization in the setting of nerve injury or type 2 diabetes. Furthermore, data below suggest that pioglitazone is sufficient to both prevent and reverse pain associated with type 2 diabetes, independent of its glucose lowering effect. Below I speculate on how
pioglitazone, through a PPARγ-dependent or independent mechanism, may alleviate neuropathic pain.

Finally, in Chapter 5: I implicated methylglyoxal in the generation and maintenance of pain associated with type 2 diabetes. I showed that methylglyoxal is sufficient to produce nociceptive, evoked, and affective pain-like behavior. The production of pain evoked by methylglyoxal requires TRPA1 and AC1, as knockout of these proteins in normal, non-diabetic mice or administration of their pharmacological inhibitors to db/db mice alleviated pain-like behaviors. Several lines of investigation pointed toward a spinal mode of action, whereby activation of dorsal horn nocireceptive neurons produces pain-like behavior. I showed that inhibition of methylglyoxal using the scavenging peptide GERP10 alleviated hypersensitivity in diabetic db/db mice. Future directions outlined below propose to target glyoxalase 1, the catabolic enzyme for methylglyoxal, in order to reduce the contribution of methylglyoxal to neuropathic pain in type 2 diabetes.

6.2 How does type 2 diabetes lead to painful neuropathy?

I propose that pre-diabetic conditions in type 2 diabetes lead to peripheral insulin resistance feeding forward to an increase in blood glucose that results in chronic glucose neurotoxicity. This causes permanent changes in somatosensory processing that cannot be reversed by glucose management alone. I also believe the mechanisms of PDN differ in type 1 versus type 2 models of diabetes and this is supported by simultaneous studies of type 1 and type 2 diabetic models (Schmidt et al, 2003; Schmidt et al, 2004; Kamiya et al, 2005; Schuelert et al, 2015). Although there has been extensive research into the streptozotocin-induced type 1 diabetic model in both mice and rats this method fails to investigate mechanisms of pain in type 2 diabetes, and type 2 diabetes has greater prevalence in the human diabetic patient population. The greater number of publications studying pain in STZ compared to ZDF or db/db is disproportionate to the prevalence of
type 1 diabetes (just 10%) and is inappropriate with the recent publication indicating that
STZ activates nociceptors through agonist action at TRPA1 (Andersson et al, 2015).
Therefore more work is needed to replicate studies in STZ models using models specific
to painful type 2 diabetes.

6.2.1 Motivational-affective pain in models of painful type 2 diabetes

We are the first to show negative affect in type 2 diabetes using MCS. We also
provide evidence using CPP that gabapentin, a common treatment for PDN (Wodarski et
al, 2009; Rauck et al, 2013), alleviates affective pain in SNI, an alternate model of
neuropathic pain. Therefore we propose to investigate in a reverse translation study
whether administration of gabapentin reverses affective pain in type 2 diabetes. This
could be accomplished in several models including MG-induced CPA in wild-type mice,
MCS in ZDF, or CPP in db/db or ZDF. As we are the first to measure negative affect in a
model of type 2 diabetes using MCS, we attempted to extend this finding to an
alternative model of type 2 diabetes using a complementary assay. We began to test the
hypothesis that systemic gabapentin would produce conditioned place preference in
diabetic db/db mice.

Figure 6-1 illustrates preliminary results (n=8) indicating baseline preconditioning
preferences in db/db mice. As shown, db/db spent the majority of time in the center (C)
grey neutral chamber, as opposed to exploration of the pairing chambers on the left (L)
and right (R) of the center chamber. This is in contrast to the normal preconditioning
preferences in non-diabetic sham or SNI rats (see Figure 2-3), where the majority of the
time is spent in the white or black pairing chambers. Because diabetic animals at this
stage of diabetes exhibit decreased exploratory locomotor activity (see Figure 4-10) and
the mice were initially placed in the center chamber to assess initial preferences, it is not
surprising that the majority of time was spent in the neutral start chamber. CPP could be
performed at an earlier age, when locomotor activity is not diminished, however this is
unadvisable because db/db mice do not exhibit behavioral hyperalgesia until several
weeks after hyperglycemia begins. Therefore an alternative approach to assess affective
pain in type 2 diabetes would be to use a two-chamber CPP apparatus, the MCS assay,
or test the hypothesis that gabapentin administration reverses MG-induced CPA in wild-
type mice.

![Graph showing baseline time in chambers for db/db mice](image)

**Figure 6-1. Preliminary preconditioning results in db/db mice using CPP.**
Time spent in the left (L), center (C), and right (R) chambers in a conditioned place
preference assay using a three-chamber box. Mice were placed into the center,
normal chamber to begin assessment of preconditioning baseline. The db/db mice
failed to explore the left and right drug-pairing chambers during preconditioning.

6.2.2 *Inhibition of TRPA1 to reduce painful type 2 diabetes*

Our current results implicate spinal TRPA1 in the mediation of pain-like behavior
associated with type 2 diabetes. Therefore we propose several strategies of inhibiting
TRPA1 in order to reduce painful diabetes. First, reduction of endogenous activation of
TRPA1 by methylglyoxal in type 2 diabetes could be achieved by masking agonist
binding sites using antibodies. This was demonstrated in a previous study where a TRPA1 antibody blocked calcium mobilization induced by AITC (Lee et al., 2014). This could prevent the tonic activation of TRPA1 by compounds such as methylglyoxal and 4-HNE that are endogenously generated in diabetes (Koivisto et al., 2012). Second, we could prevent the TRPA1-dependent sensitization of TRPV1 in nociceptors by blocking their interaction through modulation of the peptide Tmem100. A recent study showed that the Tmem100 mediates an optimal interaction of TRPA1 with TRPV1 that contributes to pain after injury. The authors developed a mutant peptide, Tmem100-Q3 that facilitates a strong interaction between TRPA1 and TRPV1 thereby inhibiting pain. Strikingly, a cell permeable peptide mimicking the 3Q region of Tmem100-3Q had a similar effect of reducing pain sensitization in vivo (Weng et al., 2015). Therefore we hypothesize that administration of this Tmem100-Q3 peptide derivative would alleviate painful type 2 diabetes by blocking the cross-sensitization between TRPA1 and TRPV1 in peripheral nociceptors.

Previous studies show a dissociation between evoked and non-evoked pain measures. The TRPA1 antagonist HC reduced evoked hypersensitivity, but failed to produce conditioned place preference in STZ or SNI after spinal administration (Wei et al., 2013) or in CFA after systemic administration (Okun et al., 2011). As affective pain is mediated by CNS and PNS mechanisms, it is possible that reducing TRPA1 activity peripherally is necessary to produce CPP, similar to studies showing peripheral lidocaine induces CPP in inflammatory (Okun et al., 2011) or incisional (Navratilova et al., 2012) models. Therefore we propose a future study to test the hypothesis that peripheral nerve block inhibits CPA to intraplantar methylglyoxal administration. Alternatively, the ability of TRPA1 antagonists to produce CPP or reduce MCS-related pain behavior could be tested in ZDF or db/db. This experiment could inform whether activation of nociceptors by MG is sufficient to produce negative affect.
Increased methylglyoxal in diabetes is known to sensitize sensory neurons through TPRA1 (Wei et al, 2009; Koivisto et al, 2012; Wei et al, 2013). Similarly, in the streptozotocin model of type 1 diabetes, drugs such as HC-030031 and Chembridge-586152 inhibit stimulus-dependent pain-like behavior (Wei et al, 2010; Koivisto et al, 2012) but not ongoing aversion in the CPP paradigm (Wei et al, 2013). Therefore we wanted to test the hypothesis that TRPA1-dependent activation of peripheral sensory neurons in the dorsal root ganglia is potentiated by type 2 diabetes. Figure 6-2 illustrates that AITC (TRPA1 agonist; 100μM) stimulation produced DRG neuron responses in both ZL and ZDF as evidenced by ratiometric Fura-2 calcium imaging. Neuronal cells were identified by their response to K+ and capsaicin (100μM) (data not shown). The percentage of the DRG neurons that responded to AITC, and therefore were peripheral neurons putatively containing TRPA1, was 35-38%. This is in line with previous functional expression studies (Kobayashi et al, 2005; Hjerling-Leffler et al, 2007). The response in ZL and ZDF was similar, indicating that painful type 2 diabetes did not alter TRPA1-dependent activation of peripheral nociceptors. This suggests that TRPA1 sensitization in peripheral neurons may not mediate the pain associated with type 2 diabetes.
Figure 6-2. TRPA1-dependent calcium response in DRG neurons from ZL and ZDF.

Data courtesy of Suzanne Doolen. I formulated the hypotheses, designed the experiments, and analyzed the data.

Neurons were dissociated from L4/5 dorsal root ganglia harvested from control (ZL) and diabetic (ZDF) rats. Quantification of (A) peak height, (B) peak height as a % of the response to potassium, and (C) percentage of the cells analyzed that responded to AITC. There was no difference between ZL and ZDF responses in any of the parameters measured.

Next we tested the hypothesis that MG would produce an exacerbated pain response when administered to diabetic db/db mice. Figure 6-3 illustrates that intraplantar methylglyoxal produces equal nociceptive response in control BKS and diabetic db/db. An alternative approach to this result revealing an absence of peripheral TRPA1 sensitization in painful type 2 diabetes is to further investigate the role of spinal TRPA1 in naïve animals. As such, we propose to test the hypothesis that the activation of spinal neurons by MG is attenuated by TRPA1 inhibition. This is based upon several results: (1) MG-induced nociceptive behavior and pERK in the dorsal horn was inhibited by intrathecal HC and TRPA1 genetic knockout; (2) spinal administration of HC reduced painful type 2 diabetes in db/db mice; and (3) MG activation of DRGs is absent in TRPA1-/- mice (Eberhardt et al, 2012).
Figure 6-3. Nociceptive behavior after MG administration in type 2 diabetic mice.

Nociceptive response after intraplantar methylglyoxal (MG; 3 μg) administration in control BKS and type 2 diabetic db/db mice at age 11 wks. There was no difference in BKS and db/db mice in nociceptive response evoked by i.pl. MG.

Several possible results exist for the proposed experiment that TRPA1 inhibition reduces MG-evoked spinal neuron activation. The TRPA1 antagonist HC030031 could reduce the MG-induced response indicating that TRPA1 plays a role in spinal neuron activation. Alternatively, the results could indicate that inhibition of presumably presynaptic TRPA1 in the dorsal horn is not sufficient to reduce MG-evoked calcium responses. This provides for the possibility that MG activates post-synaptic neurons directly, independent of its effects on TRPA1. A clue comes from previous work, where MG was shown to activate Nav1.8 in primary afferents (Bierhaus et al, 2012). Care must be taken in the experimental design so that spinal neurons are not exposed to MG more than once, as MG covalently binds to lysine, arginine, or cysteine residues within TRPA1 and therefore it is reasonable to suspect that this produces long-lasting sensitization that
could alter the efficacy of the HC antagonist during future MG stimulation (see 5.5.2 ).

This is supported by a study showing the calcium response to a low dose of MG is biphasic, suggesting sustained sensitization of TRPA1 (Koivisto et al, 2012). These studies could be performed using dorsal root stimulation (DRS) to begin to separate pre versus post synaptic activation of neurons by MG. A possible expected result is that MG activates post synaptic neurons independent of TRPA1, while pre synaptic effects are indeed regulated, at least in part, by TRPA1. DRS studies could test this idea.

6.2.3 AC1 and mechanisms of spinal sensitization

In addition to the pERK evidence presented herein, studies are needed to further investigate functional spinal sensitization in models of type 2 diabetic pain. One approach is to test the hypothesis that glutamate evoked neuron activation, by using a combination of calcium imaging and electrophysiological studies, is exacerbated in the spinal cord dorsal horn from ZDF or db/db. A similar approach is to use methylglyoxal as the activation stimulus. We predict that spinal neuron responses would be greater in ZDF or db/db compared to their respective controls, which would be consistent with previous results from our colleagues in inflammatory and nerve injury models (Doolen et al, 2012; Corder et al, 2013). Follow-up studies could test whether GERP10, HC, pioglitazone, or NB001 inhibits glutamate- or methylglyoxal-evoked spinal sensitization.

Another approach is to bypass the downstream targets of methylglyoxal in diabetes by testing whether spinal inhibition of MG directly could reduce painful type 2 diabetes. As spinal inhibition of TRPA1 attenuated heat hypersensitivity in db/db mice, but not calcium mobilization induced by TRPA1 activation ex vivo, one method to clarify the role of spinal MG in PDN would be to test whether inhibition of MG in the spinal cord would reduce painful diabetes. Our results suggest that endogenous activation and/or sensitization of TRPA1 in the spinal cord, presumably on presynaptic terminals,
facilitates evoked hypersensitivity. Therefore if elevated MG is contributing to this process, then intrathecal administration of GERP10 (or other MG scavenging compound) would reduce behavioral hypersensitivity in db/db mice.

Contrary to the results above that suggests that sensitization of peripheral TRPA1 does not mediate PDN, we show pain-like behavior is reduced by spinal inhibition of both TRPA1 and AC1 in db/db mice and in AC1/-/- after MG administration. This leads us to speculate that there is an upregulation of AC1 activity, or the AC1 system is primed and that endogenous activation of TRPA1 by MG produces pain through revealing this calcium-dependent AC1 sensitization. Along this line of thinking, there would be no difference between control and diabetic animals in the calcium response mediated by artificial activation of DRG neurons with AITC (as above). However, we would speculate that the production of cAMP would be greater in diabetic animals after TRPA1 activation, indicating a primed AC1 system. Since we suspect that activation of TRPA1 produces sensitization of AC1, and that this sensitization leads to a potentiated cAMP, one possible mechanism of the generation of heat hypersensitivity in type 2 diabetes is PKA-dependent phosphorylation/sensitization of TRPV1 (Spahn et al., 2013). To test this we could stimulate DRG neurons with MG or saline followed by capsaicin in the presence or absence of NB001 pretreatment. Several results are possible in this experiment. First, we predict that initially stimulating the DRGs with MG (as opposed to saline) would potentiate the response to capsaicin, revealing a functional link between TRPA1 and TRPV1. Second, we predict that inhibition of AC1 with NB001 would reduce the capsaicin-evoked calcium response subsequent to MG stimulation, indicating that MG sensitizes TRPV1 through AC1.
6.3 Methylglyoxal and glyoxalase 1 in painful type 2 diabetes

6.3.1 Developing a chronic model of MG-induced PDN

To test the hypothesis that methylglyoxal is sufficient to produce diabetic-like pain a chronic administration model is needed. Several reports indicate that a diabetic-like phenotype develops after repeated or chronic administration of MG to rodents, but pain-like behavior in these models has not been tested. A recent review of modeling type 2 diabetes in rodents suggested that a major culprit of diabetic dysfunction is MG, and suggested chronic MG administration as a suitable model for type 2 diabetes (Dornadula et al., 2015). A chronic MG model may better model the type 2 diabetic condition when compared to intraplantar MG administration, because MG is elevated in the plasma rather than at local sites such as at subcutaneous nociceptors in the dermis or epidermis. Therefore we summarize a few chronic MG studies as a basis for future experimentation.

Intraperitoneal administration of methylglyoxal (17.25 mg/kg) produced a modest increase in tissue MG concentrations 3 h after administration and increased plasma MG within 5-15 min (Dhar et al., 2010). Coadministration with the MG scavenging compound Alagebrium (100 mg/kg) attenuated increases in MG. Both i.p. and i.v. (50 mg/kg) administration of MG acutely resulted in decreased glucose tolerance. MG administration decreased the levels of GSH and increased D-Lactate, indicating that the glyoxalase system was activated by exogenous MG administration (see Figure 1-2). Plasma MG levels remained elevated two fold at 2 h after administration, although further timepoints were not assessed. This result suggests that repeated (e.g. twice daily) i.p. injection of MG could be sufficient to increase circulating and tissue concentrations of MG and possibly induce a pain-like phenotype.

Chronic MG treatment by addition to the drinking water (50-75 mg/kg/d) in Wistar (control, W) and Goto-Kakizaki (nonobese type 2 diabetes, GK) aged 6 months (young,
control, C) or 14 months (aged, A) resulted in impaired glucose tolerance and increased MG (Rodrigues et al, 2014). Results are shown in the figure below adapted from the manuscript. Kidney immunohistochemical expression of AGEs and RAGE appeared greater in the GKC and in the W and GK rats that were aged or treated with MG, indicating that MG induces the AGE-RAGE system.

Chronic minipump infusion (60 mg/kg/d) for 28 d in Sprague-Dawley rats resulted in changes in fasting plasma glucose (33% increase), insulin (60% decrease), and GSH (50% decrease). Glucose and GSH were also lowered in pancreatic, skeletal muscle, and adipose tissues. Impaired oral glucose tolerance and fasting measures were alleviated by the MG scavenger and AGE breaker Alagebrium (Dhar et al, 2011).

Figure 6-4. Diabetic measures after chronic administration of methylglyoxal. Adapted from (Rodrigues et al, 2014). Methylglyoxal (MG) is elevated in a type 2 diabetes model (GK), in aged (A) rats, or after administration of exogenous MG in drinking water. [KEY: Wistar (W), Goto-Kakizaki (GK), young Control (C), Aged (A), methylglyoxal administered (MG)]. Blood glucose in (A) fasted or (B) at 2 h in an oral glucose tolerance test. (C) Serum methylglyoxal levels are shown.
Based on the above findings, we attempted to test the hypothesis that chronic administration of MG using Alzet minipumps would produce pain-like mechanical and/or heat hypersensitivity. Figure 6-5 illustrates the effect of MG minipump administration on blood glucose, weight, and behavioral hypersensitivity. After 7 d of MG administration we found no difference in mechanical or heat thresholds. It should be noted that all mice in the high dose (100 mg/kg/d) had removed their minipumps by the second week of the experiment. By the third week almost all the mice subjected to MG administration had removed their minipumps. All of the minipumps in the saline mice remained implanted at week 3. We speculate that chronic release of MG in the subcutaneous space along the lumbar dorsal region cause an irritation or hypersensitivity that resulted in scratching and eventual removal of the minipumps. Minipump MG administration by Dhar et al as described was also subcutaneous, but at a lower dose of 60 mg/kg/d using a larger minipump (2ML4) and the experiment was performed in rats over a 28 d administration period. The authors anecdotally reported no inflammatory response associated with the minipump implant (Dhar et al, 2011), but it is unclear whether any minipumps had been removed during the experiment. We suspect that species or dose differences could explain the aversive effect of MG minipumps in our study. It remains a future direction for us to develop a chronic model of MG-induced pain through minipump, daily injections, or drinking water administration of MG in mice and rats.
Figure 6-5. Pain-like sensitivity is unchanged by MG minipump administration. Alzet minipumps (#2004) were implanted subcutaneously in the dorsal-lumbar region. Concentrations in the minipump were calculated to deliver 0 (saline vehicle), 1, 30, or 100 mg/kg/d of methylglyoxal (MG). (A) Blood glucose was measured immediately following implant. (B) Weight, (C) mechanical, and (D) heat hypersensitivity were monitored before (BL; baseline) and at several timepoints following minipump implant. Chronic administration of MG for one week did not affect weight gain or evoked pain-like behaviors. (n=8)

6.3.2 Manipulating GLO1 expression/function to reduce painful diabetes

Several lines of evidence suggest that the activity and/or expression of GLO1 regulates the contribution of MG to painful diabetes. (1) Both type 1 and type 2 diabetic patients whose neuropathy symptom score was rated as severe, compared to moderate or mild ratings, exhibited decreased blood Glo1 activity (Skapare et al, 2013). (2) Aging
impairs GLO1 expression and function and impairs wound healing, which can be restored by administration of aminoguanidine (Fleming et al., 2013). (3) Strain differences in the copy number variants of Glo1 results in protection from STZ-induced diabetic neuropathy in BALB/cByJ mice compared to BALB/cJ mice, in which GLO1 expression is decreased tenfold (Jack et al., 2012). (4) GLO1 polymorphisms regulate GLO1 activity (Peculis et al., 2013), but a link between SNPs and diabetic pain risk has not been established. (5) Repeated administration of BrBz resulted in behavioral hypersensitivity to mechanical, heat, and cold stimulation ( Andersson et al., 2013). (6) GLO1 overexpression reduced STZ-mediated hypersensitivity (Bierhaus et al., 2012). The above findings indicate that strategies aiming to increase GLO1 activity in type 2 diabetes could potentially alleviate pain.

We propose several methods to overexpress GLO1 by both systemic and targeted vectors. (1) Somatic gene transfer using the pCMV-Neo overexpression vector for GLO1 (i.p. injection in db/db mice) provided to us by our colleagues Thomas Fleming and Peter Nawroth. (2) AAV overexpression of GLO1 by insertion of the pCMV-Neo overexpression cassette into an AAC serotype. Local injections to the spinal cord, brain, or peripheral nerve could be used to determine the site of MG-related hyperalgesia and/or affective pain. For example, we hypothesize that overexpression of GLO1 peripherally would reduce hyperalgesia and affective pain, as negative pain affect requires both the central and peripheral nervous systems (Qu et al., 2011). (3) Cross BAC-GLO1-transgenic mice (Distler and Palmer, 2012; Distler et al., 2012; Distler et al., 2013) with a transgenic mouse that has neuronal promoter-driven CRE to specifically overexpress GLO1 in neurons.

A method alternative to overexpression of GLO1 could be promoting degradation of methylglyoxal using substances in addition to already known MG scavengers and AGE breakers. For example, we hypothesize that administration of glutathione would facilitate
MG degradation, even in type 2 diabetes where GLO1 activity is low, by providing a surplus of the required cofactor for GLO1. Glutathione has antioxidant properties and could also act to scavenge ROS, prevent MG-AGE formation, and ameliorate mitochondrial dysfunction, all mechanisms that contribute to PDN.

6.4 Mechanisms of PPARγ-related analgesia

6.4.1 The rapid effects on pain by PPARγ agonists

Three of our findings led us to conclude that PPARγ agonists exhibit non-genomic, possibly receptor-independent effects on pain-like behavior. First, we found that both rosiglitazone and pioglitazone attenuate mechanical hyperalgesia in rats with nerve injury within five to ten minutes of intrathecal administration. Second, the rapid antihyperalgesic effect was unchanged by the translation inhibitor anisomycin, whereas the antihyperalgesic effects at sixty to ninety minutes were reduced by anisomycin. Third, pioglitazone attenuated heat hyperalgesia in ZDF and db/db mice within one hour of administration. We suspect that PPARγ mediates this rapid effect as the PPARγ antagonist GW9662 inhibited pioglitazone antihyperalgesia in SNI within thirty minutes of administration. However, several of these timepoints are in the ambiguous time frame between anisomycin insensitive (10 min) and sensitive (60 min) periods, so future studies could seek to determine the antihyperalgesic effect of pioglitazone at rapid timepoints in alternative pain models or in transgenic animals where PPARγ has been temporally and/or spatially eliminated.

The exact mechanism by which pioglitazone rapidly reduces pain in nociceptive (intraplantar capsaicin and methylglyoxal administration), diabetic (ZDF and db/db models), or nerve injury conditions still remains unknown. We provide correlative evidence that pioglitazone reduces markers of central sensitization. Acute pioglitazone administration reduces astrocyte activation within one hour (section 3.4.9 ). Chronic
administration of pioglitazone reduces both activation of glia in SNI (Morgenweck et al., 2013) as well as inhibits spinal neuron sensitization to noxious pressure in painful type 2 diabetes (section 4.4.3) and non-noxious light-touch in SNI (see Figure 6-6 below). But, these latter findings were in chronic administration scenarios, and new data below reveals that acute administration of pioglitazone does not alter stimulus-evoked markers of neuronal sensitization in the spinal cord (as evidenced by light-touch evoked Fos and pERK).

A growing body of evidence suggests that the mechanisms of pioglitazone analgesia are different between acute (rapid) and chronic (sustained) administration. For example, acute administration of pioglitazone did not reduce light-touch evoked pERK or Fos in SNI (Figure 6-7 below), even though chronic administration in both diabetic and nerve injury models reduced stimulus-evoked pERK. Similarly, pioglitazone did not reduce spinal Fos expression associated with capsaicin injection (see Figure 3-6). Furthermore, although acute intrathecal pioglitazone rapidly reduced evoked mechanical hypersensitivity, it failed to produce CPP in SNI rats (Figure 6-8 below). In fact, we found that sham rats exhibited CPP to intrathecal pioglitazone while preferences in SNI rats were unchanged by pioglitazone conditioning. This finding that uninjured animals perceived pioglitazone as rewarding is puzzling because we see no effect of pioglitazone on sensory thresholds in sham or naïve animals. However, it could be that the activation of reward pathways in sham animals by pioglitazone is diminished after peripheral nerve injury, although this is unfounded speculation.
Figure 6-6. Chronic pioglitazone administration reduces light touch-evoked pERK.
Adapted from (Morgenweck et al, 2013).
Representative images of phosphorylated ERK (p-ERK) in spared nerve injury rats that were administered (A) saline or (B) pioglitazone for seven weeks. (C) Pioglitazone reduced p-ERK in the dorsal horn evoked by light touch stimulation of the sural receptive field of the injured hindpaw using a cotton swab. ★ significantly different from "0" (unpaired). n = 5-8.
Figure 6-7. Acute pioglitazone does not reduce evoked spinal pERK or Fos in SNI rats.

Two weeks after SNI surgery rats were injected i.p. with either saline vehicle or pioglitazone (100 mg/kg) one hour prior to stimulation of the injured hindpaw as described above. Rats were perfusion-fixated and processed for immunohistochemical staining of phosphorylated ERK (pERK) and expression of Fos in the lumbar dorsal horn. (A) Quantification of ipsilateral and contralateral pERK in vehicle animals. (B) Quantification of ipsilateral pERK in vehicle or pioglitazone animals (n=3-4). (C-F) Representative images of pERK in the indicated
groups. (G) Quantification of ipsilateral and contralateral Fos in vehicle animals. (H) Quantification of ipsilateral Fos expression in vehicle or pioglitazone animals (n=8). (I-L) Representative images of Fos expression in the indicated groups. Light-touch stimulation of the injured hindpaw in SNI rats produced an increase in pERK and expression of Fos in the spinal dorsal horn ipsilateral to stimulation. Pretreatment with pioglitazone prior to stimulation did not change evoked pERK or expression of Fos. ★ p<0.05 vs. ipsilateral.

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**Figure 6-8. Pioglitazone produces conditioned place preference in sham but not SNI rats.**

(A) Mechanical withdraw thresholds in sham and SNI rats before injury (BL), before the CPP preconditioning (PRE), and after CPP postconditioning (POST). (B) Preconditioning and postconditioning time spent in the saline or pioglitazone paired chamber. (C) Difference scores (preconditioning time spent in chamber minus postconditioning time spent in chamber) for sham and SNI rats are shown. ★ p<0.05 vs. preconditioning in B or vs. sham saline in C. n=6-8.

6.4.1.1 pERK and Fos

Evidence from our pioglitazone studies indicate that pERK and Fos markers of spinal sensitization are differentially affected by acute versus chronic administration. With regard to these pERK and Fos studies, we did not perform colabeling with neuronal or glial markers. The cell type where pERK and Fos are labeled via immunohistochemistry is unclear as is the excitatory or inhibitory nature these cells. Therefore it is possible that pioglitazone produced activation of inhibitory neurons or increased the phosphorylation of ERK (pERK) or expression of Fos in glial cells, even though those glial cells may have been contributing to the anti-hypersensitive effect of pioglitazone. pERK has previously
been shown to be expressed in glial cells (Gao and Ji, 2010), along with activity-dependent phosphorylation of other MAPK in nerve injury (Tsuda et al., 2004; Ji et al., 2009) or diabetes (Daulhac et al., 2006). ERK activation is suggested to facilitate pain (Ji, Gereau et al., 2009) and several studies suggest that PPARγ agonists may inhibit ERK phosphorylation. For example, activated PPARγ prevents MEK, the MAPK kinase upstream of ERK, from phosphorylating ERK (Burgermeister, Chuderland et al. 2007, Burgermeister and Seger 2007), although contrary to the finding that increased pERK is associated with decreased PPARγ expression in a microbial sepsis model (Kaplan et al., 2010). PPARγ reduces ERK phosphorylation after TNFα or IFNγ stimulation (Lombardi, Cantini et al. 2008, Lombardi, Cantini et al. 2009) and administration of these cytokines in vivo can directly mediate hyperalgesia (Tsuda, Masuda et al. 2009, Gruber-Schoffnegger, Drdla-Schutting et al. 2013). Taken together, our results and the literature indicate a direct interaction between PPARγ and the MAPK pathway (Gardner et al., 2005).

6.4.1.2 Cytokines and chemokines

Rapid analgesia after pioglitazone administration may result from decreased chemokine/cytokine release. For example, reduction of neuropathic pain is associated with a decrease in MCP1/CCL2 (Gao, Zhang et al. 2009) or TNFα (Ohtori, Takahashi et al. 2004), which can contribute to astrocyte pain sensitization (Zhang, Berta et al. 2011) and spinal LTP (Gruber-Schoffnegger, Drdla-Schutting et al. 2013). Repeated PPARγ activation reduces TNFα in the spinal cord and DRG after nerve injury (Maeda, Kiguchi et al. 2008) and decreases astrocyte TNFα release after inflammatory activation in vitro (Storer, Xu et al. 2005). Furthermore, pioglitazone rapidly decreases astrocyte TNFα release in vitro (Gurley, Nichols et al. 2008). As cytokines can directly and quickly modulate the responsivity of nociceptive neurons, reduction of their release by TZDs is
one possible mechanism of rapid analgesia. Furthermore, increased TNFα is associated with pain in diabetic patients (Purwata, 2011).

6.4.1.3 mitoNEET

Receptor-independent functions of PPARγ agonists at mitochondria was first proposed ten years ago by Feinstein and colleagues (Feinstein et al, 2005). Mitochondrial dysfunction resulting in alterations in calcium homeostasis contributes to hyperactivity of sensory processing (Flatters, 2015), painful neuropathy (Fernyhough and Calcutt, 2010; Barrière et al, 2012; Verkhratsky and Fernyhough, 2014), and neurodegenerative disease (Feinstein et al, 2005; Geldenhuys et al, 2010; Yonutas and Sullivan, 2013). TZDs (Geldenhuys et al, 2010; Yonutas and Sullivan, 2013) or exogenous ligands such as TT01001 (Takahashi et al, 2015) reduce diabetic mitochondrial dysfunction by targeting the mitochondrial protein mitoNEET. Functional and expressional evidence indicate that pioglitazone binds to and stabilizes mitoNEET within the outer mitochondrial membrane (Bolten et al, 2007; Paddock et al, 2007), making mitoNEET a potential target for the rapid antihyperalgesic effect of TZDs. Therefore we hypothesize that pioglitazone or other TZDs act at mitoNEET to rapidly restore calcium homeostasis in PDN (Latham et al, 2009; Cao et al, 2011; Todorovic and Jevtovic-Todorovic, 2014; Verkhratsky and Fernyhough, 2014) or after experimental traumatic nerve injury and that this could be a mechanism of acute PPARγ agonist analgesia.

6.4.1.4 GPR40

The rapid effects of pioglitazone could be mediated by “off-target” effects at GPR40, a G-protein coupled receptor recently implicated in the regulation of hyperalgesia associated with formalin, CFA, SNL, and stroke (Nakamoto et al, 2012; Nakamoto et al,
Activation of GPR40 by endogenous (e.g. DHA, polyunsaturated fatty acids) and exogenous (GW9508) ligands reduces inflammatory (Nakamoto et al, 2012; Nakamoto et al, 2013), neuropathic (Karki et al, 2015), and post-stroke (Harada et al, 2014) pain. Analgesic effects were inhibited by the GPR40 antagonist GW1100. GPR40 is expressed in POMC and β-endorphin neurons in the hypothalamus (Nakamoto et al, 2013), serotonergic and noradrenergic neurons in the RVM and LC (Nakamoto et al, 2015), and neurons but not glia in the spinal cord (Karki et al, 2015). Therefore both functional and expressional evidence implicate GPR40 in various pain models. Furthermore, calcium mobilization produced by rosiglitazone is abolished by siRNA knockdown of GPR40, suggesting that TZDs may also activate GPR40 (Gras et al, 2009). This is intriguing, given the notion that GPR40 functions to facilitate descending inhibition (Harada et al, 2014; Karki et al, 2015; Nakamoto et al, 2015), that TZDs may activate GPR40, and TZDs rapidly inhibit hypersensitivity. Both inflammatory (carrageenan and CFA) and nerve injury (SNL) models increase the expression of GRP40 in the spinal cord (Karki et al, 2015). There's even evidence that activation of GPR40 by GW9508 increases the expression of Fos after injury even with simultaneous reduction of formalin nociception (Nakamoto et al, 2013; Nakamoto et al, 2015), which would explain the lack of effect of pioglitazone on Fos expression in capsaicin and SNI models – pioglitazone decreased Fos in nociceptive neurons while simultaneously increasing Fos in inhibitory neurons resulting in no net change in Fos expression. Finally, hyperalgesia after injury and decreased endogenous activity of GPR40 in the descending inhibitory system, either through decreased expression of GPR40 or lesser availability of endogenous PUFA agonists, is associated with changes in GFAP (Harada et al, 2014) further supporting the interplay of pioglitazone, GPR40, and astrocytes. Thus GPR40 is an intriguing candidate for the regulation of rapid analgesia produced by TZDs.
6.4.1.5 TRP channels

PPARγ agonists may reduce hypersensitivity via interactions with TRP channels. 15d-PGJ2 activates TRPA1 by covalent modification of reactive cysteine residues (Cruz-Orengo et al, 2008; Takahashi et al, 2008; Weng et al, 2012). Peripheral administration of 15d-PGJ2 results in immediate nociceptive behavior and calcium mobilization in TRPA1 containing DRG neurons. Paradoxically, although 15d-PGJ2 activates TRPA1 to produce immediate nociception, we and others have shown that 15d-PGJ2 reduces evoked hypersensitivity in an inflammatory pain model (Morgenweck et al, 2010; Alves et al, 2011). These antihyperalgesic effects are thought to be mediated by PPARγ, as they are blocked by a PPARγ antagonist. However, a possible mechanism of the rapid analgesic effects of PPARγ agonists such as 15d-PGJ2 is through the acute desensitization of TRPA1 (Weng et al, 2012). Indeed, rosiglitazone is suggested to directly inhibit TRP channels (Majeed et al, 2011), suggesting a possible mechanism for the rapid inhibition of pain produced by TZDs. A future direction is to test the hypothesis that the antihyperalgesic effect of pioglitazone is abolished in TRPA1 or TRPV1 knockouts, which would suggest that TRP channels play a role in the rapid analgesic effect of both endogenous and exogenous PPARγ agonists.

6.4.2 How does pioglitazone reduce painful type 2 diabetes?

Therapeutic management of hyperglycemia does not always reverse PDN (Calcutt, 2002), indicating the need for dual therapies that treat both the hyperglycemia and insulin desensitization as well as the neuronal mechanisms that contribute to pain. Here we show that chronic oral administration of pioglitazone reduced both hyperglycemia and hypersensitivity in ZDF. One could conclude that the mechanism of analgesia is mediated by normalizing blood glucose and therefore reducing the neurotoxic effects of hyperglycemia on nociceptive processing. However, Figure 6-9 illustrations that acute
administration of pioglitazone rapidly increased heat response thresholds (within one hour) in both db/db mice and ZDF rats but not in their respective normoglycemic controls. Furthermore, pioglitazone reduced hypersensitivity without changing blood glucose. This result provides strong evidence that the reduction of PDN by pioglitazone is not mediated through its role as an insulin-sensitizer, but likely facilitated by one of the rapid action mechanisms described above.

Figure 6-9. Effect of acute systemic pioglitazone on heat hyperalgesia and blood glucose in type 2 diabetes models. 
*Data courtesy of Renee Donahue and Tommaso Iannitti.*

Pain-like heat withdraw latencies and blood glucose were measured before and after intraperitoneal (i.p.) injection of saline or pioglitazone (Pio) in rat and mouse models of type 2 diabetes. Heat sensitivity is shown in (A) control ZL and (B) diabetic ZDF rats (n=4-5) as well (C) control BKS and (D) diabetic db/db mice (n=8-16). Glucose levels are shown in (E) control ZL and (F) diabetic ZDF rats (n=4-5) as well (G) control BKS and (H) diabetic db/db mice (n=8-16). Acute administration of pioglitazone attenuated heat hypersensitivity without changing blood glucose levels.
We provide evidence that pioglitazone may alleviate painful type 2 diabetes through a methylglyoxal mechanism. Not only did acute administration of pioglitazone rapidly attenuate heat hyperalgesia in type 2 diabetic mice and rats, but it also acutely reduced MG-evoked nociception and hyperalgesia. Figure 6-10 illustrates that systemic or spinal administration of pioglitazone reduced MG-evoked nociceptive behavior. These data indicate that spinal administration of pioglitazone is more effective than systemic administration at reducing peripherally evoked nociception. We conclude that pioglitazone reduced the contribution of MG to pain-like behavior in type 2 diabetes through a spinal mechanism. I speculate that chronic administration of pioglitazone could act as an AGE breaker (Rahbar et al, 2000) to reduce PDN in ZDF.

Figure 6-10. Pioglitazone reduces MG-evoked pain-like behavior.
Data in Panels A, C, D courtesy of Renee Donahue. I formulated the hypotheses, designed the experiments, and analyzed the data.

(A) Intraperitoneal (100 mg/kg; mice) or (B) intrathecal (300 μg; rats) administration of pioglitazone but not saline vehicle 30 min prior to intraplantar methylglyoxal (100 μg) reduced nociceptive responses in wild-type animals (n=3-4). Nociceptive licking, lifting, and flinching were quantified for the first five minutes after intraplantar administration of methylglyoxal (MG). Intraperitoneal administration of pioglitazone (100 mg/kg; mice) reduced (C) mechanical and (D) heat hyperalgesia. ★ p<0.05, † p<0.05 vs. Veh-Saline, ¥ p<0.05 vs. i.p. pioglitazone + i.pl. methylglyoxal.
Enough evidence exists for us to speculate that pioglitazone inhibits the development of tolerance to the endogenous opioid system that normally acts to inhibit pain after injury. Corder et al established that behavioral hypersensitivity after inflammatory, nerve, or incisional injury recovers due to the body’s endogenous opioid system. They showed that endogenous mu opioid receptors mediate the restoration of normal sensory thresholds (Corder et al, 2013) by administration of opioid receptor antagonists. In the setting of type 2 diabetes, ZDF rats became hyposensitive to morphine after a prolonged period of behavioral hypersensitivity, suggesting that the ability of the body to recover from injury in diabetes is impaired, possibly due to impaired G-protein coupling of the mu opioid receptor (Otto et al, 2011). Therefore strategies that mitigate changes in the endogenous opioid system could be beneficial pain therapies. To this end, a recent study showed that pioglitazone delayed the behavioral tolerance that develops after repeated morphine administration (de Guglielmo et al, 2014; Ghavimi et al, 2014). This effect was reduced by GW9662 inhibition of PPARγ or by Cre recombinase knockout of PPARγ in nestin-positive neurons. reduced the effect of pioglitazone on delaying tolerance to morphine (de Guglielmo et al, 2014). Therefore we suggest that pain in type 2 diabetes occurs by desensitization of and/or tolerance to the body’s endogenous pain inhibitory system, and speculate that chronic oral administration of pioglitazone reduced the development of hyperalgesia by mitigating (in the case of mechanical pressure hyperalgesia) or delaying (in the case of heat hyperalgesia) endogenous opioid tolerance. This hypothesis could help explain why pressure hyperalgesia was completely prevented by pioglitazone, while the onset of heat hyperalgesia was delayed several weeks but not abolished.
6.5 Conclusions

Figure 6-11 summarizes this work. My working model is that hyperglycemia and decreased GLO1 activity in type 2 diabetes leads to accumulation of methylglyoxal. Methylglyoxal tonically activates TRPA1 \textit{in vivo}, priming the AC1 system resulting in peripheral and spinal sensitization through a yet to be determined mechanism, but I speculate that cAMP/PKA and TRPV1 as well as spinal plasticity mechanisms are involved. This proposed pathway of pain in type 2 diabetes can be altered at many different junctions to reduce pain sensitization. MG is inhibited by GERP10 and by pioglitazone, probably through activation of PPARγ activation, although PPARγ-independent mechanisms could be involved in TZD analgesia. Inhibition of TRPA1 and AC1 activity through pharmacologic and genetic methods reduced spinal sensitization and pain. I propose a multitude of future studies to further investigate this overall mechanism. (1) Overexpression and inhibition of GLO1 activity as well as chronic MG administration as tools to manipulate MG levels either systemically or within specific tissues such as the spinal cord dorsal horn. (2) Inhibition of MG by alternative scavenging compounds such as ALT-711, AG, and GSH. (3) CHEM inhibition of TRPA1 or using dorsal root stimulation, Tmem100 cell permeable peptide, blocking antibodies, and TRPV1 knockouts (-/-) to investigate the interplay between TRPA1 and TRPV1. (4) Investigate the mechanism of rapid pioglitazone antihyperalgesia through TRP channels, mitoNEET, or GPR40. (5) Use electrophysiology and CPP to uncover the mechanisms of MG-dependent spinal sensitization and affective pain, respectively.
Type 2 diabetes is a growing problem in the United States and throughout the world. Pain associated with type 2 diabetes is a complex process involving both peripheral and central sensitization. My results in combination with the literature provide evidence that this pain cannot be reversed by management of blood glucose and insulin dysfunction alone. Therefore I reasoned that the known antihyperalgesic and antihyperglycemic effects of TZDs could be combined to reduce painful type 2 diabetes. I provide the first preclinical evidence that pioglitazone reduces hyperglycemia and pain-like behavior associated with type 2 diabetes. The antihyperalgesic influence of pioglitazone in type 2 diabetes is independent of its glucose lowering effect because acute administration of pioglitazone reversed hypersensitivity without changing blood glucose. I show that acute
pioglitazone rapidly reduces astrocyte gliosis but not markers of neuronal activation, and therefore provide insight into future studies determining the exact mechanisms by which pioglitazone rapidly reduces neuropathic pain, a phenomena we showed in several models including nociception induced by capsaicin and methylglyoxal, traumatic nerve injury, and type 2 diabetes. Drugs such as pioglitazone and other TZDs that inhibit glucose neurotoxicity, are anti-inflammatory, reduce gliosis, improve insulin signaling and glucose metabolism, and reduce pain are likely to provide a greater benefit to patients than treating the symptoms of pain or hyperglycemia independently.

In preclinical pain studies it is important to include measures of negative affect. This work highlights the importance of measuring “pain” in rodents using mechanical conflict avoidance and conditioned place preference/aversion assays, in addition to stimulus-evoked measures of hypersensitivity. For the first time we show negative affect in a model of type 2 diabetes and implicate methylglyoxal in the generation of this motivational-affective pain in diabetes. Measurement of evoked and non-evoked, affective pain in preclinical studies will be essential to enhance the translation of new analgesic drugs.

We extend previous studies investigating the effects of methylglyoxal on peripheral nociceptors and in the STZ model of type 1 diabetes by showing methylglyoxal induces spinal sensitization and pain-like behavior that is dependent upon TRPA1 in painful type 2 diabetes. We identified a new target in painful type 2 diabetes, AC1, and suggest future studies aiming to reduce the methylglyoxal-TRPA1-AC1 sensitization pathway. We show for the first time that blocking methylglyoxal by administering GERP10 has long-lasting antihyperalgesia after a single dose. Finally, we indicate that pioglitazone reduces methylglyoxal-evoked pain and suggest future studies to investigate this mechanism. We conclude that both PPARγ and methylglyoxal represent promising targets for reducing the pain associated with type 2 diabetes.
APPENDIX

Description of abbreviations used.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>15d</td>
<td>15d-PGJ2, endogenous prostaglandin PPARγ agonist</td>
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<tr>
<td>AC1</td>
<td>adenylyl cyclase 1, calcium activated enzyme that produces cAMP</td>
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<tr>
<td>AG</td>
<td>aminoguanidine, methylglyoxal scavenger and AGE breaker</td>
</tr>
<tr>
<td>AGE</td>
<td>advanced glycation end-product</td>
</tr>
<tr>
<td>ALT-711</td>
<td>alagebrium, methylglyoxal scavenger and AGE breaker</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance, statistical test</td>
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<td>BKS</td>
<td>C57BLK6S, mouse strain that is a control for db/db mice</td>
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<tr>
<td>BrBz</td>
<td>S-p-bromobenzylglutathioine cyclopentyl diester, GLO1 inhibitor</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<tr>
<td>CHEM</td>
<td>Chembridge-5861528, TRPA1 antagonist derived from HC</td>
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<tr>
<td>CIG</td>
<td>ciglitazone, PPARγ agonist</td>
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<tr>
<td>CPA</td>
<td>conditioned place aversion</td>
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<tr>
<td>CPP</td>
<td>conditioned place preference</td>
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<td>db/db</td>
<td>Lepr/- mouse model of type 2 diabetes</td>
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<td>dorsal horn</td>
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<td>DRG</td>
<td>dorsal root ganglia sensory neuron</td>
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<td>DRS</td>
<td>dorsal root stimulation</td>
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<td>DTT</td>
<td>dithiothreitol, disulfide bond reducing agent</td>
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<td>GEAP</td>
<td>control peptide containing 10 repeats of G,E,A,P amino acids</td>
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<tr>
<td>GERP</td>
<td>MG scavenging peptide containing 10 repeats of G,E,A,P amino acids</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein, an astrocyte marker</td>
</tr>
<tr>
<td>GLO1</td>
<td>glyoxalase 1, primary enzyme involved in MG detoxification</td>
</tr>
<tr>
<td>GLO2</td>
<td>glyoxalase 2, secondary enzyme involved in MG detoxification</td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione, GLO1 cofactor</td>
</tr>
<tr>
<td>H1</td>
<td>hydroimidazolone residue</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>i.pl.</td>
<td>intraplantar</td>
</tr>
<tr>
<td>i.t.</td>
<td>intrathecal</td>
</tr>
<tr>
<td>IASP</td>
<td>International Association for the Study of Pain</td>
</tr>
<tr>
<td>Iba1</td>
<td>ionized calcium-binding adapter molecule 1, a microglia marker</td>
</tr>
<tr>
<td>MCS</td>
<td>mechanical conflict avoidance system</td>
</tr>
<tr>
<td>MG</td>
<td>methylglyoxal</td>
</tr>
<tr>
<td>MG-AGE</td>
<td>methylglyoxal-derived advanced glycation end-product</td>
</tr>
<tr>
<td>PDN</td>
<td>painful diabetic neuropathy</td>
</tr>
<tr>
<td>pERK</td>
<td>phosphorylated extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>Pio</td>
<td>pioglitazone, TZD PPARγ agonist</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A, activated by cAMP</td>
</tr>
<tr>
<td>PPARγ</td>
<td>peroxisome proliferator-activated receptor gamma</td>
</tr>
<tr>
<td>PUFA</td>
<td>polyunsaturated fatty acids</td>
</tr>
<tr>
<td>Rosi</td>
<td>rosiglitazone, TZD PPARγ agonist</td>
</tr>
<tr>
<td>s.c.</td>
<td>subcutaneous</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>STZ</td>
<td>streptozotocin model of type 1 diabetes</td>
</tr>
<tr>
<td>TRPA1</td>
<td>Transient Receptor Potential cation channel A1</td>
</tr>
<tr>
<td>TRPV1</td>
<td>Transient Receptor Potential cation channel V1</td>
</tr>
<tr>
<td>TZD</td>
<td>thiazolidinedione, structural class of PPARγ agonists</td>
</tr>
<tr>
<td>vF</td>
<td>von Frey, method of determining mechanical sensitivity</td>
</tr>
<tr>
<td>ZDF</td>
<td>Zucker Diabetic Fatty rat (fa/fa) model of type 2 diabetes</td>
</tr>
</tbody>
</table>
IASP definitions of terms used to describe pain phenomena.

<table>
<thead>
<tr>
<th>TERM</th>
<th>DEFINITION</th>
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<tbody>
<tr>
<td>Allodynia</td>
<td>Pain due to a stimulus that does not normally provoke pain.</td>
</tr>
<tr>
<td>Hyperalgesia</td>
<td>Increased pain from a stimulus that normally provokes pain.</td>
</tr>
<tr>
<td>Hypoalgesia</td>
<td>Diminished pain from a stimulus that normally provokes pain.</td>
</tr>
<tr>
<td>Paresthesia</td>
<td>An abnormal sensation, whether spontaneous or evoked.</td>
</tr>
<tr>
<td>Dysesthesia</td>
<td>An unpleasant abnormal sensation, whether spontaneous or evoked.</td>
</tr>
<tr>
<td>Hyperesthesia</td>
<td>Increased sensitivity to stimulation, excluding the special senses.</td>
</tr>
<tr>
<td>Hypoesthesia</td>
<td>Decreased sensitivity to stimulation, excluding the special senses.</td>
</tr>
<tr>
<td>Neuropathic Pain</td>
<td>Pain caused by a lesion or disease of the somatosensory nervous system.</td>
</tr>
<tr>
<td>Neuropathy</td>
<td>A disturbance of function or pathological change in a nerve: in one nerve, mononeuropathy; in several nerves, mononeuropathy multiplex; if diffuse and bilateral, polyneuropathy.</td>
</tr>
<tr>
<td>Nociception</td>
<td>The neural process of encoding noxious stimuli.</td>
</tr>
<tr>
<td>Nociceptive Neuron</td>
<td>A central or peripheral neuron of the somatosensory nervous system that is capable of encoding noxious stimuli.</td>
</tr>
<tr>
<td>Nociceptive Pain</td>
<td>Pain that arises from actual or threatened damage to non-neural tissue and is due to the activation of nociceptors.</td>
</tr>
<tr>
<td>Nociceptive Stimulus</td>
<td>An actually or potentially tissue-damaging event transduced and encoded by nociceptors.</td>
</tr>
<tr>
<td>Nociceptor</td>
<td>A high-threshold sensory receptor of the peripheral somatosensory nervous system that is capable of transducing and encoding noxious stimuli.</td>
</tr>
<tr>
<td>Noxious Stimulus</td>
<td>A stimulus that is damaging or threatens damage to normal tissues.</td>
</tr>
<tr>
<td>Central Sensitization</td>
<td>Increased responsiveness of nociceptive neurons in the central nervous system to their normal or subthreshold afferent input.</td>
</tr>
<tr>
<td>Peripheral Sensitization</td>
<td>Increased responsiveness and reduced threshold of nociceptive neurons in the periphery to the stimulation of their receptive fields.</td>
</tr>
</tbody>
</table>


Anupindi, R., I. Mukhopadhyay, A. Thomas, S. Kumar, S. Chaudhari, A. Kulkarni, G. Gudi and N. Joshi (2010). GRC 17536, a novel, selective TRPA1 antagonist for
potential treatment of respiratory disorders. European Respiratory Society Annual Congress, Barcelona.


Gao, Y. J. and R. R. Ji (2009). "c-Fos and pERK, which is a better marker for neuronal activation and central sensitization after noxious stimulation and tissue injury?" Open Pain J 2: 11-17 PMID: 2773551 DOI: 10.2174/187638630902010011.

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235


Wei, H., M. Karimaa, T. Korjamo, A. Koivisto and A. Pertovaara (2012). "Transient receptor potential ankyrin 1 ion channel contributes to guarding pain and
mechanical hypersensitivity in a rat model of postoperative pain." Anesthesiology 117(1): 137-148 DOI: 10.1097/ALN.0b013e31825adb0e.


the effects on glycaemic control and β-cell function in newly diagnosed type 2 diabetes patients of treatment with exenatide, insulin or pioglitazone: a multicentre randomized parallel-group trial (the CONFIDENCE study)." Journal of Internal Medicine 277(1): 137-150 DOI: 10.1111/joim.12293.


mechanical allodynia in this neuropathic pain model." Pain 114(1-2): 149-159
DOI: S0304-3959(04)00588-3 [pii]


VITA

Ryan B. Griggs  
Birthplace: Columbus, Ohio

EDUCATION

<table>
<thead>
<tr>
<th>Year</th>
<th>Degree</th>
<th>Field</th>
<th>Institution</th>
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<tr>
<td>2015</td>
<td>PhD</td>
<td>Physiology</td>
<td>University of Kentucky</td>
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<tr>
<td>2009</td>
<td>MS</td>
<td>Biotechnology</td>
<td>University of Pennsylvania</td>
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<tr>
<td>2007</td>
<td>BS</td>
<td>Unified Science</td>
<td>Drexel University</td>
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PROFESSIONAL EXPERIENCE

<table>
<thead>
<tr>
<th>Year</th>
<th>Company</th>
<th>Position</th>
<th>Responsibilities</th>
</tr>
</thead>
<tbody>
<tr>
<td>2008</td>
<td>Merck &amp; Co., Inc</td>
<td>Research Biochemist</td>
<td>RNAi Drug Screening</td>
</tr>
<tr>
<td>2004-2005</td>
<td>Sunoco, Inc</td>
<td>Industrial Hygienist</td>
<td></td>
</tr>
</tbody>
</table>

PUBLICATIONS

2016  
*Methylglyoxal produces pain in type 2 diabetes via sensitization of TRPA1 and AC1.*  

2015  
*Pioglitazone inhibits the development of hyperalgesia and sensitization of spinal nocireponsive neurons in type 2 diabetes.*  

*Short-lived diabetes in the young-adult ZDF rat does not exacerbate neuronal Ca(2+) biomarkers of aging.*  

*Gabapentin alleviates affective pain after traumatic nerve injury.*  

*Pioglitzone rapidly reduces neuropathic pain through astrocyte and non-genomic PPARγ mechanisms.*  

2013  
*PPARγ activation blocks development and reduces established neuropathic pain in rats.*  
GRANTS

2013 – 2015 **F31 Individual Fellowship** – NIH NINDS NS083292
*Methyglyoxal and TRPA1 contribution to neuropathic pain in type 2 diabetes*

2012 – 2013 **T32 Institutional Fellowship** – NIH NINDS NS077889
*Neurobiology of CNS Injury & Repair*

ORAL PRESENTATIONS AS INVITED SPEAKER

2012 **Society for Neuroscience 42nd Annual Meeting** – Nanosymposium.

MENTORSHIP

*Graduate Students*
Weisi Fu (PhD Candidate, 2011-2015); Lilian Goncalves Custodio, MD (PhD Student, 2014-2015); Maks Gold (MD Student, 2014-2015); Stuart Cowley (MD Candidate, 2014); Clayton Bright (MS, 2011-2012)

*Undergraduate Students*

TEACHING EXPERIENCE

2015 **Graduate Certificate in Teaching Physiology**
*University of Kentucky – Department of Physiology*

2014 – 2015 **Concepts of Biology Laboratory I: Cell & Molecular (BIO 151L)**
**General Biology Laboratory I (BIO 101L)**
Adjunct Faculty, *University of Dayton – Department of Biology*

2012 – 2013 **Human Anatomy & Physiology Lab (BIO 137)** – Instructor
*Bluegrass Community and Technical College – Natural Sciences*

**Critical Scientific Readings (IBS 610)** – Senior Student Leader
*University of Kentucky – Integrated Biomedical Sciences*

**Human Physiology (PGY 206 / PGY 412G / PGY 502)** – Tutor
*University of Kentucky – Department of Physiology*

2011 – 2012 **Elementary Human Physiology Recitation (PGY 207)** – Instructor
*University of Kentucky – Biological Sciences*
FINANCIAL AWARDS EARNED

2014  Conference Travel Award (UK), $800
      Competitive Financial Aid (IASP), $900
      15th World Congress on Pain, Buenos Aires, Argentina

2013  Graduate Student Incentive Program (UK Graduate School), $1100
      Conference Travel Award (UK), $400
      Society for Neuroscience Annual Meeting, San Diego, USA

2012  Competitive Financial Aid (IASP), 800€
      14th World Congress on Pain, Milan, Italy
      Conference Travel Award (UK), $400
      American Pain Society Annual Meeting, New Orleans, USA

2011  Conference Travel Award (UK), $400
      Society for Neuroscience Annual Meeting, Washington D.C., USA

2010  Conference Travel Award (UK), $800
      13th World Congress on Pain, Montreal, Canada

JOURNAL PEER-REVIEWING

2014  Current Topics in Behavioral Neuroscience
2014  Frontiers in Cellular Neuroscience
2013  PLOS One
2012  Journal of Neuroscience

LEADERSHIP, SERVICE, OUTREACH

2014  Neuroscience Researcher visit to Career Fair – Outreach Coordinator
      Lori Vogel, Northern Elementary (Grades 2-5) in Lexington, KY

2012 – 2013  Graduate Student Department Representative (Peer Elected)
      University of Kentucky – Department of Physiology

2011 – 2013  TEAM Student Leader and Organizer (Peer Elected)
      Department of Physiology – Teaching Education And Mentoring Group

2011 – 2012  Science Explorers – Neuroscience Coordinator and Volunteer
      Katherine Bullock, Living Arts and Sciences Center in Lexington, KY

2010 – 2014  BGSFN Neuroscience Outreach – Coordinator and Volunteer
      Fayette County Public School Annual Science Fair in Lexington, KY
MEMBERSHIP IN PROFESSIONAL ORGANIZATIONS

International Association for the Study of Pain (IASP)
Society for Neuroscience (SFN)
American Pain Society (APS)
Bluegrass Society for Neuroscience (BGSFN)
University of Kentucky Department of Physiology Teaching, Educating, and Mentoring (TEAM)

CONFERENCE PRESENTATIONS

2015
American Pain Society Annual Meeting (Palm Springs, CA)
Early Career Forum – Invited Panel Member (Hosted By: Jennifer Rabbitts, M.B.,Ch.B)

2014
IASP 15th World Congress on Pain (Buenos Aires, Argentina)
Methylglyoxal produces pain and activates spinal neurons via TRPA1
9th Annual CCTS & 30th Annual BGSFN Spring Neuroscience Day
Elevated methylglyoxal in diabetes produces nociception via spinal TRPA1 activation.

2013
Society for Neuroscience 43rd Annual Meeting (San Diego, CA)

Midwest Regional Pain Interest Group Meeting (Cincinnati, OH)

3rd Annual Barnstable Brown Obesity & Diabetes Research Day
8th Annual CCTS & 29th Annual BGSFN Spring Neuroscience Day (Lexington, KY)
19th Annual KSCHIRT Symposium (Louisville, KY)

2012
Society for Neuroscience 42nd Annual Meeting (New Orleans, LA)
Behavioral signs of painful diabetic neuropathy and stimulus-induced spinal pERK expression in the ZDF rat model of Type 2 diabetes.

IASP 14th World Congress on Pain (Milan, Italy)
<table>
<thead>
<tr>
<th>Year</th>
<th>Conference</th>
<th>Details</th>
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<tbody>
<tr>
<td>2010</td>
<td>IASP 13th World Congress on Pain&lt;br&gt;(Montreal, Canada)</td>
<td>PPARγ contributes to the rapid effects of low- and high-dose 15d-PGJ₂ on Ca²⁺ influx in DRG cultures. S. Doolen, <strong>R.B. Griggs</strong>, I. Parikh, B.K. Taylor.</td>
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