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The PPAR γ agonist pioglitazone produces a female-predominant inhibition of hyperalgesia associated with surgical incision, peripheral nerve injury, and painful diabetic neuropathy

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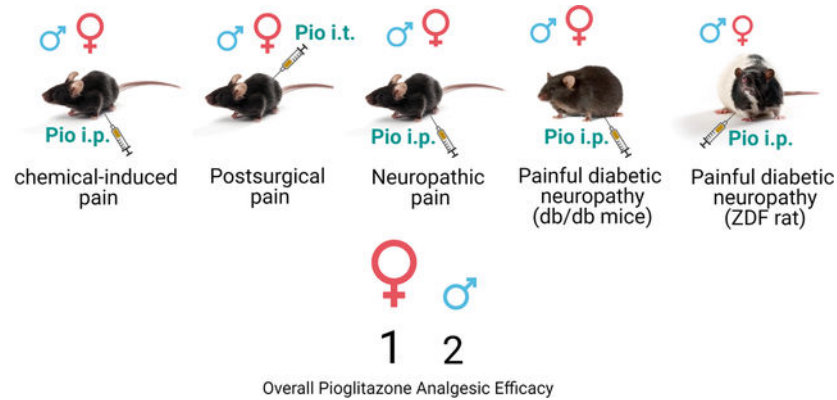
Abstract

Pioglitazone, an agonist at peroxisome proliferator-activated receptor gamma, is FDA-approved for the treatment of insulin resistance in type 2 diabetes. Numerous studies in male rodents suggest that pioglitazone inhibits inflammatory and neuropathic pain, but few included female subjects. To address this gap, we compared the effects of pioglitazone in both sexes in the intraplantar methylglyoxal model (MG) model of chemical pain and painful diabetic neuropathy (PDN), the plantar incision model (PIM) of postoperative pain, the spared nerve injury (SNI) model of traumatic nerve injury, and the ZDF rat and db/db mouse models of PDN. We administered pioglitazone by one-time intrathecal or intraperitoneal injection or by adding it to chow for 6 weeks, followed by measurement of hypersensitivity to non-noxious mechanical, noxious mechanical, heat, and/or cold stimuli. In all mouse models, injection of pioglitazone decreased pain-like behaviors with greater potency and/or efficacy in females as compared to males: heat and mechanical hypersensitivity in the MG model (0.1–10 mg/kg); mechanical hypersensitivity in the PIM model (10 μ g); mechanical and cold hypersensitivity in the SNI model (100 mg/kg); and heat hypersensitivity in the db/db model (100 mg/kg). Furthermore, co-administration of low doses of morphine (1 mg/kg) and pioglitazone (10 mg/kg) decreased SNI-induced mechanical and cold hypersensitivity in female but not male mice. In the ZDF rat, pioglitazone (100 mg/kg) decreased heat and mechanical hypersensitivity with no sex difference. In the db/db model, pioglitazone had no effect when given into chow for 6 weeks at 0.3, 3 or 30 mg/kg doses. We conclude that females exhibit greater anti-hyperalgesic responses to pioglitazone in mouse models of chemical-induced nociception, postsurgical pain, neuropathic pain, and PDN. These findings set the stage for clinical trials to determine whether pioglitazone has analgesic properties across a broad spectrum of chronic pain conditions, particularly in women.

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Graphical Abstract



Keywords

Pioglitazone; Morphine; PPAR gamma; Methylglyoxal; sex-difference; postsurgical pain; neuropathic pain; painful diabetic neuropathy

1. BACKGROUND

Pioglitazone is approved by the U.S. Food and Drug Administration for the treatment of insulin resistance in type 2 diabetes. Upon binding to peroxisome proliferator-activated receptors gamma (PPAR γ), a member of the nuclear receptor superfamily, pioglitazone stimulates glucose uptake into muscle cells, decreases adipocyte hypertrophy, and sensitizes insulin receptors to enhance insulin signaling (Takada and Genda, 2019). Furthermore, a steadily growing body of preclinical evidence indicates that PPAR γ agonists such as pioglitazone exert therapeutic actions in numerous neural disorders. For example, numerous studies indicate that pioglitazone decreases behavioral signs of hyperalgesia in animal models of inflammatory and peripheral neuropathic pain (de Guglielmo et al., 2014; Griggs et al., 2016; Griggs et al., 2015; Morgenweck et al., 2013; Okine et al., 2018; Zhong et al., 2019). For example, pioglitazone attenuates behavioral signs of neuropathic pain associated with peripheral nerve injury (Griggs et al., 2015) or chemotherapy (Khasabova et al., 2019). However, an overwhelming majority of these studies have been restricted to males.

Biological sex is an important risk factor for chronic pain. For example, the prevalence of chronic post-surgical pain (Pereira and Pogatzki-Zahn, 2015), neuropathic pain (Bouhassira et al., 2008), and painful diabetic neuropathy (Abraham et al., 2018; Raputova et al., 2017) is higher in women. Biological sex is also an important determinant of the analgesic efficacy of opioid analgesic drugs such as morphine (Abraham et al., 2018; Raputova et al., 2017). With regards to sex differences in the analgesic effects of pioglitazone, the available literature is limited, incomplete, and inconclusive. (Grace et al., 2021). Two studies in mouse models of peripheral nerve injury (Sorge et al., 2015) and spinal cord injury (Gensel et al., 2019) reported that pioglitazone reduced pain-like behavior in females but not males; however, these studies were limited to a single dose and a single route of administration. No studies have evaluated the interaction of sex and pioglitazone analgesia in

models of acute chemical pain or inflammatory pain. To address these gaps, we administered pioglitazone to males and females in the plantar incision model (PIM) of postsurgical pain, the spared nerve injury (SNI) model of traumatic nerve injury, the db/db mouse and ZDF rat models of painful diabetic neuropathy (PDN), and the intraplantar methylglyoxal (MG) model of chemical pain and PDN. Our results indicate that pioglitazone exerts a greater anti-nociceptive effect in female rodents in multiple pre-clinical models of pain, promoting pioglitazone as a potential pharmacological strategy for a broad spectrum of pain conditions, particularly in women.

2 METHODS

2.1 Animals

All experiments were carried out in accordance with guidelines from the International Association for the Study of Pain (Zimmermann, 1983) and with the approval of the Institutional Animal Care and Use Committees of the University of Pittsburgh and the University of Kentucky. Rodents were housed in a temperature- and humidity-controlled room with food and water available *ad libitum*. Mice were on a 14-hour light–10-hour dark cycle with lights on from 6:00 AM to 8:00 PM, while rats were on a 12:12 cycle from 7:00 am to 7:00 pm. Mice and rats were housed at a maximum five and two per cage, respectively. We restricted behavioral studies to the hours of 8:00 am – 6:00 pm to avoid steep changes in physiological rhythms surrounding lights-on or lights-off transitions. We acknowledge that hormonal changes during the estrous cycle can influence pain in women. However, we used females without consideration of their stage in the estrous cycle, in accordance with the consensus report of the Sex, Gender and Pain Special Interest Group of the International Association for the Study of Pain, which states that “The value of testing female rodents at different stages of the estrous cycle is debatable”, in part because “an influence of estrous cycle stage is not necessarily indicated by larger observed variance” and because “measurement of estrous cycle would require daily handling, and the ensuing stress could affect nociception or sensitivity to drugs” (Greenspan et al., 2007).

C57Bl/6 mice.—Mice ordered from Charles Rivers Laboratories (Wilmington, MA) at 8–12 weeks were used to evaluate any effects of pioglitazone in the MG, PIM and SNI models.

ZDF Rats.—ZDF rats (Strain #370; Obese) are homozygous for the loss-of-function “fatty” mutation in the leptin receptor (*fa/fa*) that results in the development of type 2 diabetes, as indicated by hyperglycemia, hyperlipidemia, impaired glucose tolerance, and insulin insensitivity. ZL rats (Strain #371; Lean *fa/+*) are the heterozygous (*fa/+*) genetic controls for ZDF (Clark et al., 1983). ZDF and ZL rats (Charles Rivers Laboratories) arrived at seven weeks of age and housed as same-sex littermates. Full development of the diabetes phenotype requires specific diets for male and female ZDF and ZL: males received the Formulab 5008 diet (TestDiets; Purina Mills, Richmond, IN, USA) containing 23% protein, 6.5% fat, 58.5% carbohydrates, 4% fiber and 8% vitamins and minerals; females received the D12468 diet (Research Diets, Inc; New Brunswick, NJ, USA) containing 12% protein, 25.5% fat, 51% carbohydrates, 5.93% fiber and 5.57% vitamins and minerals.

Db/db mice.—Diabetic db/db (BKS.Cg-Dock7m^{+/+}Lepr^{db}/J) mice are homozygous for the diabetes spontaneous mutation (Lepr^{db}) and manifest morbid obesity, chronic hyperglycemia, pancreatic beta cell atrophy, and hypoinsulinemia. BKS Dock7m^{+/+}Lepr^{db}, also referred as C57BLKS/J (BKS), are heterozygous (db/+), do not develop a diabetic phenotype, and are genetic controls for db/db mice (Coleman, 1978). Diabetic db/db mice were purchased from Jackson Laboratories (Bar Harbor, ME) for the studies conducted at University of Kentucky. Mice for the experiments conducted at University of Pittsburgh were bred in-house. To this end, we interbred the heterozygous mice (db/+) with C57BL/6 background purchased from Jackson Laboratories (Bar Harbor, ME) to generate +/+, db/+ and db/db mice as genotyped as previously described (Peng et al., 2018). Mice were housed with a maximum of 4 same-sex littermates per cage. No specific diet is required for db/db mice to develop type 2 diabetes.

2.2 Glucose Testing

We lightly restrained the animals in a towel and wiped the tail clean with an alcohol wipe. Using a #11 scalpel blade, we made a small nick in the side of the tip of the tail. For the non-fasting glucose test, we placed a single drop of blood on a standard glucose monitor strip, which we then inserted into the monitor (Truetrack, Walgreens). For the hemoglobin A1c measurement, drops of blood were loaded into a HbA1c cartridge and analyzed using a DCA Vantage Analyzer (Siemens, Munich, Germany).

2.3 Surgery

Surgical models of postsurgical and neuropathic pain we conducted under isoflurane anesthesia (5% induction followed by 1.5%–2.0% maintenance using a nose cone). After suturing of the skin, triple antibiotic ointment (Neosporin, Johnson and Johnson) was applied to the surgical area.

2.3.1 Plantar Incision—Post-operative hyperalgesia was induced by longitudinal incision of the plantaris muscle as previously described (Jang et al., 2011; Pogatzki and Raja, 2003). Following antisepsis of the left hind paw with Chlorascrub® then alcohol, a #11 scalpel blade was used to make a 5mm incision through the skin and fascia, beginning 2mm from the proximal edge of the heel, and extending towards the digits. The underlying muscle was raised with a curved forceps, extended 4 mm and then incised longitudinally with a #11 scalpel blade, leaving the origin and insertion of the muscle intact. The overlying skin was closed with synthetic 5–0 sutures (PDS*II, Ethicon). Surgery was typically completed within 5–10min.

2.3.2 Spared Nerve Injury (SNI) surgery—As previously described (Decosterd and Woolf, 2000; Fu et al., 2020), an incision of the inguinal skin was followed by retraction of muscles to expose the common peroneal, tibial, and sural branches of the left sciatic nerve. With care taken not to disturb the sural nerve, the common peroneal and tibial nerves were ligated with a 6–0 silk suture (Ethicon, Somerville, NJ) and then transected 1mm proximal and 1mm distal to the ligation. The muscle and skin were closed with loosely tied 5–0 absorbable sutures (Ethicon, Somerville, NJ) and 9-mm stainless steel wound clips, respectively. Clips were removed on postsurgical day (POD) 13, and animals were tested

on POD 14. All animals developed robust mechanical and cold hypersensitivity without adverse effects. Substantial doses of pioglitazone do not change mechanical thresholds or cold response duration in rats (Griggs et al., 2015) or mice (Maeda et al., 2008) with sham nerve injury surgery, and therefore we did not include sham groups here.

2.4 Drugs

Pioglitazone potassium salt (10028, Cayman Chemical, Ann Arbor, MI) was dissolved in either 0.9% saline for intraperitoneal administration, or in a mixture of 10% ETOH, 10% castor oil and 80% saline for intrathecal administration. Actos® tablets (25% pioglitazone hydrochloride; Pharmaceutical Takeda., Inc., Deerfield, IL, USA) were incorporated into mouse chow by TestDiets (Purina Mills, Richmond, IN, USA) and administered for 6 weeks at doses (0.3, 3 and 30 mg/kg/day) that we described previously (Griggs et al., 2016). Body weight and food consumption, measured by subtracting the amount of food left in the cage from that provided the previous day, were monitored weekly. The concentration of Actos® was calculated according to expected average daily body weight and food consumption over the course of the study. The control group received the same diet but without pioglitazone. “For single injection studies, pioglitazone or vehicle was administered in unanesthetized mice either by the intraperitoneal route (0.1–100 mg/kg in a volume of 10 ml/kg as we described (Griggs et al., 2015; Morgenweck et al., 2013), or by the intrathecal route using a 30-gauge needle at the maximum soluble dose (10 µg in 5µL of 10% DMSO, 10% castor oil, and 80% saline). To account for the rapid actions of pioglitazone (Griggs et al., 2015) and its 2.6 hour half-life (Maeshiba et al., 1997), we evaluated behavioral measures of mechanical and thermal sensitivity as early as 15 minutes and as late as 120 minutes. Morphine hydrochloride (Sigma, USA) was administered at a submaximal dose by the intraperitoneal route at 1 mg/kg as described by Zhao et al (Zhao et al. 2004).”

Methylglyoxal (MG; M0252, Sigma-Aldrich, St. Louis, MO) was diluted in 0.9% sterile saline. In unanesthetized mice using mild restraint, 30 µg MG was injected in a volume of 10µL under the skin of the plantar hindpaw using a 30-gauge needle as described previously (Griggs 2017).

2.5. Experiment Design and Timelines

The timelines of each experiment are illustrated with Panel A of each figure. Presurgical and/or pre-injection baselines are denoted as time =0. For the methylglyoxal studies, baseline mechanical and thermal baseline responses were immediately followed by an intraperitoneal injection of pioglitazone or vehicle. 30 min later, methylglyoxal was injected at t=0 followed by behavioral measurements at 15, 30, 45, 60 and 120 minutes in a repeated measures design. For the PIM and SNI studies, behavioral measurements at peak of hypersensitivity (Day 2 or Day 14 post-surgery, respectively), were following by drug injection and then repeated behavioral measurements. Other than the morphine plus pioglitazone study, we targeted N=6–10 *a priori* based on our previous publications using these pain models, behavioral assays, and experimental designs. The low variability observed in our SNI pioglitazone study justified the design of an experiment with smaller group sizes for the SNI morphine plus pioglitazone study. While keeping the experimenter blind to sex, pain condition and drug treatment, another experimenter randomly assigned animals to groups

with the caveat that this was done in a balanced fashion to avoid significant differences in baseline thresholds. Gross obesity in db/db mice and ZDF rats prevented blinding by strain in the painful diabetic neuropathy studies.

2.6 Behavioral Testing

Before commencement of each von Frey and acetone testing session, we acclimated the animals within individual Plexiglas boxes placed on the top of a stainless-steel mesh platform for 45 min. Before commencement of the first hotplate or coldplate session, we acclimated the animals to the apparatus over 3 days. On the first day, they spent 5 minutes on the hotplate or coldplate in the OFF position. On the two consecutive days, we performed three trials with the devices turned to the ON position with an inter-trial interval of at least 10 min. Animals were removed immediately after a withdrawal response.

2.6.1 Mechanical sensitivity

Mouse: Sensitivity to a non-noxious stimulus was tested with an incremental series of 8 von Frey monofilaments of logarithmic stiffness (Stoelting, Wood Dale, IL), ranging in gram force from 0.008g to 6g. The location of stimulation at the plantar hindpaw varied with the model: the central region in the MG model; lateral to the suture line in the plantar incision model; and at the sural receptive field (lateral) in the spared nerve injury model. Filaments were applied to the skin with slightly bending for a maximum of 5 seconds. A clear withdrawal of the paw away from the stimulus was recorded as a positive response. The 50% withdrawal threshold was determined using the up-down method of Dixon (1965), modified by Chaplan (Chaplan et al., 1994).

Rat: Sensitivity to a noxious mechanical stimulus was tested in the ZDF model with an electronic force transducer (IITC Life Science Inc, Woodland Hills, CA) as previously described (Griggs et al., 2016). The force exerted on the surface of the hindpaw was gradually increased, and the gram force required to elicit a withdraw response was recorded. The force until paw withdraw (paw pressure threshold) was measured three times for each hindpaw and then averaged.

2.6.2 Cold sensitivity

2.6.2.1. Acetone test.: To test for cold sensitivity in the SNI model, 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 was used to apply a drop of acetone (surface tension maintained the volume of the drop to 10–12 μ L) to the sural receptive field (Griggs et al., 2015). The duration of time spent licking or flicking the ipsilateral hindpaw was recorded over a 1 min period. Three trials, with an inter-trial interval of at least 5 min, were averaged for each time point.

2.6.2.2. Cold plate test.: Cold sensitivity was assessed by placing the animal on a cooled surface ($4^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$) 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, as previously described (Griggs et al., 2015)

2.6.3 Heat sensitivity—Heat hypersensitivity was tested on a heated surface (52.5 °C) within an acrylic enclosure (Hotplate; Columbus Instruments, Columbus, OH). The latency to hindpaw withdraw response (jumping, hindpaw licking, or hindpaw flinching) was recorded as previously described (Griggs et al., 2019). Three trials, with an inter-trial interval of at least 10 min, were averaged for each time point. The rodents were immediately removed after paw withdraw or a cutoff of 25 s to avoid tissue injury.

2.7 Statistical Analysis

Data were graphed and analyzed using Prism software (version 8.4.0, GraphPad, La Jolla, CA). We conducted experimental designs that balanced multiple factors: Sex, Strain, Drug, and Time as a repeated measure. To analyze these results, we conducted analysis of variance (ANOVA). For each study, we tested post-drug injection data for normal distribution using the Shapiro-Wilkes test and for homogeneity of variance using Levene's test. In all cases, $p > 0.05$, thus supporting the null hypotheses of normal distribution and equal variance across groups. To analyze studies involving Sex, Drug and/or Time, we used 2-way ANOVA with Time integrated across timepoints using the trapezoidal method for calculation of area under the curve (AUC). Time was also treated as a repeated measure and if a main effect was found, these were followed by Sidak multiple comparisons test at each timepoint. To analyze studies involving Strain, Sex, Drug and/or Time, we began with 3-way ANOVA to evaluate interactions between Time, Sex and Strain or Time, Strain, and Drug, with Time integrated across timepoints using AUC. If a main effect was found, these were followed by 2-way ANOVA. Due to the large number of possible values using the up-down method, von Frey data were analyzed with parametric statistics (Scheff et al., 2002). Statistical significance was set at $p < 0.05$. Percentage of maximum possible effect (MPE) in the methylglyoxal study was calculated as: $(\text{post-drug threshold} - \text{post-vehicle threshold}) / (\text{baseline threshold} - \text{post-drug threshold}) * 100$. All data are presented as mean \pm SEM.

3 RESULTS

3.1 Pioglitazone inhibits chemical nociception with greater potency in female as compared to male mice

PDN is associated with increased levels of glucose-derived dicarbonyl metabolites such as MG (Becker et al., 2020; Bierhaus et al., 2012). In males, MG is both necessary and sufficient to the pain of PDN: methylglyoxal scavengers reduce behavioral signs of nociception in db/db mice, and either intraplantar or intrathecal administration of methylglyoxal induces nociception (Bierhaus et al., 2012; Griggs et al., 2019). Here we tested the hypothesis that pioglitazone would inhibit mechanical and heat hypersensitivity in the intraplantar methylglyoxal model of chemical pain. Figure 1 illustrates that MG induced a robust increase in both mechanical (Fig 1A–D) and heat (Fig 1E–H) sensitivity that lasted throughout the 120 min period of testing.

Mechanical hypersensitivity.—Pioglitazone reduced mechanical hypersensitivity following MG administration in both male and female mice, with a much stronger effect in females. In males, only the highest dose of pioglitazone (100 mg/kg, i.p.) prevented mechanical hypersensitivity from 15 to 120 minutes after intraplantar MG [Fig 1A; Dose \times

Time F (20, 175) = 8.0, $P < 0.0001$; Dose F (4, 35) = 107, $P < 0.0001$, Table 1 lines #1–3]. In females, pioglitazone dose-dependently reduced mechanical hypersensitivity from 15 to 120 minutes after intraplantar MG [Fig 1B; Dose \times Time F (20, 170) = 5.054 $P < 0.0001$; Dose F (4, 34) = 30.99 $P < 0.0001$, Table 1 lines #4–6]. As shown in Figs 1D–E, pioglitazone was 100-fold more potent in reducing MG-induced mechanical hypersensitivity in females (ED_{50} =0.17 mg/kg) than in males (ED_{50} =17.85 mg/kg).

Heat hypersensitivity.—Pioglitazone also reduced heat hypersensitivity following MG administration in both male and female mice, with a much stronger effect in females. In males, only the highest dose of 100 mg/kg prevented heat hypersensitivity from 20 to 40 minutes after intraplantar MG [Fig 1E; Dose \times Time F (12, 87) = 4.785 $P < 0.0001$; Dose F (4, 29) = 7.088 $P = 0.0004$, Table 1 lines #10–12]. In females, pioglitazone prevented heat hypersensitivity at all doses [Fig 1F; Dose \times Time F (12, 102) = 4.579 $P < 0.0001$; Dose F (4, 34) = 18.84 $P < 0.0001$, $n = 7$ –8, Table 1 lines #13–15]. As illustrated in Figs 1G–H, intraperitoneal administration of pioglitazone was 1000-fold more potent in reducing MG-induced mechanical hypersensitivity in females (ED_{50} =0.028 mg/kg) than in males (ED_{50} =28.8 mg/kg).

3.2 Pioglitazone decreases postsurgical hypersensitivity with greater efficacy in female mice

PPAR γ agonists reduce hypersensitivity in male models of inflammatory or incision pain. In males, the PPAR γ agonist 15d-PGJ2 decreased mechanical hypersensitivity in the rat intraplantar PGE2 model of inflammatory pain (Napimoga et al., 2008), and another PPAR γ agonist, rosiglitazone, attenuated heat and mechanical hypersensitivity in the mouse plantar incision model of postsurgical pain (Hasegawa-Moriyama et al., 2012). To determine the sex dependence of PPAR γ agonist analgesia in the PIM model, we administered pioglitazone in both male and female C57Bl/6 mice.

Figure 2 illustrates that PIM increased mechanical sensitivity at 2 days after incision, and intrathecal pioglitazone reduced this hypersensitivity. In males, intrathecal administration of pioglitazone (10 μ g) slightly decreased mechanical hypersensitivity with a significant effect of Treatment \times Time [Fig 2A; F (5, 90) = 3.137 $P = 0.0118$] and Treatment [F (1, 18) = 8.300 $P = 0.0099$, Table 1 lines #19–21]. Further analysis of Time collapsed across 15–120 min again suggest an overall effect of pioglitazone in males (Panel 2C), although this did not reach statistical significance at any individual timepoint. In females, pioglitazone (10 μ g, i.t.) also decreased mechanical hypersensitivity [Fig 2B; Treatment \times Time F (5, 90) = 3.491 $P = 0.0063$, Treatment F (1, 18) = 35.83 $P < 0.0001$, Table 1 lines #22–24]. With Time collapsed across the 15–120 measurements, Fig 2C reveals that pioglitazone reduced mechanical hypersensitivity to a greater extent in females as compared to males [Sex \times Treatment F (1, 18) = 6.431 $P = 0.0207$; Treatment F (1, 18) = 53.06 $P < 0.0001$, Table 1 lines #25–27].

3.3 Pioglitazone inhibits neuropathic pain with greater efficacy in female mice

Previous studies of the antihyperalgesic effects of pioglitazone after peripheral nerve injury were limited to either one sex or a single dose and yielded contradictory results (Sorge et al.,

2015)(Gensel et al., 2019). To resolve this discrepancy, we administered multiple doses of pioglitazone to both male and female C57Bl/6 mice. Figure 3 illustrates that SNI induced a robust increase in both mechanical and cold sensitivity at 14 days after surgery. Pioglitazone reduced mechanical and cold hypersensitivity in both male and female mice following SNI, with a relatively stronger effect in female mice.

Mechanical sensitivity.—In males, pioglitazone decreased mechanical hypersensitivity [Fig 3A; Dose F (1, 13) = 4.781 P=0.0477, Table 1 line #30] (albeit our alternative analysis using AUC did not reveal a difference between vehicle and drug), and post-hoc analysis revealed a significant effect of 100 mg/kg, i.p. at the 45- and 60-minute timepoints.” In females, 100 mg/kg pioglitazone significantly decreased mechanical hypersensitivity from 15 to 60 minutes after the injection [Fig 3B; Dose × Time F (6, 102) = 7.490 P<0.0001; Dose F (1, 17) = 28.00 P<0.0001, Table 1 lines #31–33]. In Fig 3C, we compared dose by sex and found that pioglitazone 100 mg/kg was more potent in reducing SNI-induced mechanical hypersensitivity in females than in males [Dose F (1, 30) = 33.18 P<0.0001; Sex F (1, 30) = 5.184 P=0.0301, Table 1 lines #34–36].

Cold sensitivity.—Pioglitazone also reduced cold hypersensitivity following SNI in female mice but had no effect in males. In males, pioglitazone did not decrease cold hypersensitivity [Fig 3D; P>0.05, Table 1 lines #37–39]. By contrast, in females, 100 mg/kg pioglitazone decreased cold hypersensitivity from 15 to 60 minutes after injection [Fig 3E; Dose × Time F (6, 96) = 2.553 P=0.0245; Dose F (1, 16) = 47.70 P<0.0001, Table 1 lines #40–42]. Further Dose × Sex analysis in Fig 3F confirmed that pioglitazone 100 mg/kg was more potent in reducing SNI-induced cold hypersensitivity in females than in males [Dose F (1, 28) = 35.91 P<0.0001; Sex F (1, 28) = 11.23 P=0.0023, Table 1 lines #43–45].

3.4 Pioglitazone plus morphine inhibits neuropathic pain with greater efficacy in female mice

Previous studies indicated that the analgesic effects of morphine and the anti-hyperalgesic actions of pioglitazone are both sex-dependent, suggesting that their combined effect would also be sexually dimorphic. Morphine exerts more potent analgesic effects in women volunteers (Craft, 2003; Niesters et al., 2010; Sarton et al., 2000), while pioglitazone exerts greater anti-hyperalgesic effects in female mice with spinal cord injury (Gensel et al., 2019). Pioglitazone delays the behavioral tolerance that develops after repeated morphine administration through a PPAR γ -dependent mechanism (de Guglielmo et al., 2014; Ghavimi et al., 2014), indicating that pioglitazone interacts with the morphine tolerance and withdrawal response in rodents. To test the hypothesis that pioglitazone and morphine interact to decrease mechanical and cold hypersensitivity in a sex-dependent manner, we administered them together to male and female mice in the SNI model. We combined a low dose of pioglitazone (10 mg/kg which is close to the therapeutic dose of 3 mg/kg used for clinical diabetes) with a low dose of morphine (1 mg/kg) to avoid adverse effects associated with high doses of pioglitazone (e.g. 500 mg/kg) such as ventricular hypertrophy and congestion of liver and kidneys (Chinnam et al., 2012), and adverse effects associated with high doses of morphine (10–50 mg/kg) such as sedation, inhibition of gastrointestinal transit, and respiratory suppression (Raehal et al., 2005).

Mechanical sensitivity.—Figure 4 again illustrates that SNI induced a robust increase in both mechanical and cold sensitivity at 14 days after surgery. In males, morphine (1 mg/kg) did not change mechanical hypersensitivity when given alone or in combination with 10 mg/kg pioglitazone [Fig 4A; $P>0.05$, Table 1 lines #46–48]. By contrast, in females, morphine (1 mg/kg) decreased mechanical hypersensitivity at 45 minutes after injection when given alone, and at 30 and 60 minutes after injection when given in combination with pioglitazone [Fig 4B; $F(2, 19) = 4.712$ $P=0.0218$, Table 1 lines #49–51]. Subsequent Treatment \times Sex analysis in Fig 4C confirmed that morphine + pioglitazone more efficaciously reduced SNI-induced mechanical hypersensitivity in females than in males [Sex: $F(1, 33) = 4.205$ $P=0.0483$, Table 1 lines #52–54].

Cold sensitivity.—Combined morphine + pioglitazone treatment also decreased cold hypersensitivity following SNI in female mice, but not in male mice. In males, morphine (1 mg/kg) did not change mechanical hypersensitivity when given alone or in combination with 10 mg/kg pioglitazone [Fig 4D; $P>0.05$, Table 1 lines #55–57]. In females, the combination of morphine with pioglitazone tended to decrease cold hypersensitivity 15 minutes after intraperitoneal injection [Fig 4E; Table 1 lines #58–60]. Subsequent analysis of Treatment \times Sex in Fig 4F confirmed that pioglitazone + morphine more efficaciously reduced cold hypersensitivity in females than in males [Treatment $F(2, 30) = 3.854$ $P=0.0324$, Table 1 lines #61–63].

3.5 Development of type 2 diabetes and cutaneous hypersensitivity in male and female ZDF rats

ZDF rats are homozygous for the fatty (*fa/fa*) leptin receptor mutation and develop a diabetic metabolic profile that includes an increase in obesity and hyperglycemia. We previously reported that male ZDF rats develop heat and mechanical hypersensitivity (Griggs et al., 2016). Here we determined whether hyperalgesia develops in a sex-dependent manner.

As illustrated in Figure 5, we first monitored the progression of type 2 diabetes in ZDF rats and their ZL controls. As expected, male and female ZDF rats showed time dependent increase at 7–16 weeks of age in body mass compared to male and female ZL rats, respectively [Fig 5A; Strain $F(1, 168) = 480.0$ $P<0.0001$, Table 2 lines #1–7]. Male and female ZDF rats showed time dependent increase at 7–16 weeks of age in blood glucose levels compared to male and female ZL rats, respectively [Fig 5B; Time \times Strain $F(5, 134) = 27.32$ $P<0.0001$, Table 2 lines #8–14]. Male and female ZDF rats showed time dependent increases in heat hypersensitivity at 15–16 weeks of age compared to male and female ZL rats, respectively [Fig 5C; Time \times Strain $F(5, 168) = 4.309$ $P=0.0010$, Table 2 lines #15–21]. Male and female ZDF rats also showed a time-dependent increase in mechanical hypersensitivity at 15–16 weeks of age compared to male and female ZL rats, respectively [Fig 5D; Time \times Strain $F(5, 168) = 4.309$ $P=0.0010$, Table 2 lines #22–28]. Interestingly, male control ZL rats also showed increased in body weight compared to ZL female control rats [Sex \times Strain $F(1, 168) = 63.98$ $P<0.0001$, Table 2 lines #1–7]. However, no sex difference was found in the development of blood glucose levels [$P>0.05$, Table 2 lines #8–14], heat hypersensitivity [$P>0.05$, Table 2 lines #15–21] or mechanical hypersensitivity [$P>0.05$, Table 2 lines #22–28].

3.6 Pioglitazone reduces heat and mechanical hyperalgesia in both male and female ZDF rats

Figure 6 illustrates that pioglitazone 10, 30 and 100 mg/kg decreased heat hypersensitivity assessed in hotplate (Fig 6A; Interaction F (3, 28) = 3.545 P=0.0271) and Hargreaves tests (Supplemental Figure S1), as well as cold hypersensitivity in a coldplate test (Fig 6B; Interaction F (3, 28) = 9.185 P=0.0002).

In male ZDF rats, repeated administration of pioglitazone provides the combined benefit of reduced hyperglycemia, hyperalgesia, and central sensitization (Griggs et al., 2016). Single administration of pioglitazone also reduces behavioral signs of neuropathic pain after peripheral nerve injury (Griggs et al., 2015; Iwai et al., 2008; Morgenweck et al., 2013), but these studies did not test the null hypothesis antihyperalgesic effects were secondary to changes in glucose levels. Fig 6C demonstrates that pioglitazone 10, 30 and 100 mg/kg did not change non-fasting glucose levels when tested one hour after injection (Interaction; F (3, 32) = 0.5121 P=0.6768). These results indicate that pioglitazone decreases behavioral signs of PDN, without changes in glucose levels, and establish a dose to compare not only heat hypersensitivity but also mechanical hypersensitivity in both sexes of ZDF rats. Pioglitazone (100 mg/kg) decreased heat hypersensitivity 60 minutes after intraperitoneal injection in both males [Fig 6D Treatment × Time F (2, 90) = 6.504 P=0.0023; Treatment F (1, 45) = 17.50 P=0.0001, Table 2 lines #29–35] and females [Fig 6E Treatment × Time F (2, 82) = 7.700 P=0.0009; Treatment F (1, 41) = 15.24 P=0.0003, Table 2 lines #36–42]; Fig 6F did not reveal a sex difference (Sex × Treatment P>0.05, Table 1 lines #64–66). Similarly, pioglitazone decreased mechanical hypersensitivity in both males [Fig 6G Treatment F (1, 43) = 12.69 P=0.0009, Table 2 lines #43–49] and females [Fig 6H Treatment F (1, 41) = 8.638 P=0.0054, Table 2 lines #50–56]; Figure 6I did not reveal a sex difference (P>0.05, Table 1 lines #67–69). These results indicate that pioglitazone reduced heat and mechanical hypersensitivity in ZDF rats as compared to ZL rats, but with no difference between male and female cohorts.

3.7 Development of diabetes and cutaneous hypersensitivity in male and female db/db mice

As is true for ZDF rats, db/db mice are homozygous for a leptin receptor mutation (*Lepr^{db}*) and develop obesity and hyperglycemia. In db/db mice, we previously reported that heat hypersensitivity typically develops within 8 weeks of age and lasts until at least 13 weeks of age, but these studies were restricted to male subjects (Griggs et al., 2019). Here we demonstrate in Figure 7 that not only male, but also female db/db mice exhibit a time-dependent increase across 5–11 weeks of age in body mass [Fig 7A Strain × Time F (2, 52) = 3.369 P=0.0421, Table 2 line #57–63], hemoglobin A1c levels [Fig 7B Strain × Time F (2, 54) = 168.3 P<0.0001, Table 2 lines #64–70], and heat hypersensitivity [Fig 7C Strain × Time F (2, 52) = 5.446 P=0.0071, Table 2 lines #71–77]. However, 3-way ANOVA did not reveal a sex difference in any of the outcome measures (P>0.05, Table 2 lines #57–77).

3.8 Pioglitazone decreased heat hypersensitivity with greater efficacy in females in the db/db model of PDN

Pioglitazone reduced heat hypersensitivity in both male and female db/db mice, with a stronger effect in females. Figure 8 illustrates that pioglitazone (100 mg/kg) decreased heat hypersensitivity 60 minutes after intraperitoneal injection in 12-week-old males [Fig 8A; Strain \times Treatment F (1, 32) = 23.10 P<0.0001; Treatment F (1, 32) = 17.42 0.0002 P=0.0002, Table 2 lines #78–84] and from 60 to 90 minutes in females, [Fig 8B; Strain \times Treatment F (1, 32) = 9.866 P=0.0036; Treatment F (1, 32) = 17.42 P=0.0002, Table 2 lines #85–91]. Subsequent analysis in Fig 8C indicates that pioglitazone more efficaciously reduced heat hypersensitivity in females than in males [Treatment F (1, 32) = 8.973 P=0.0053, Table 1 Lines #79–81].

3.9 Six weeks administration of pioglitazone does not change heat hypersensitivity in db/db mice

Griggs et al., 2016 reported that repeated administration of pioglitazone decreased the development of heat hypersensitivity in male ZDF rats (Griggs et al., 2016). Here we asked whether chronic administration of pioglitazone could exert the same effect in male and/or female db/db mice. To test this hypothesis, we provided pioglitazone in the food and evaluated heat response thresholds. Figure 9 illustrates that the 30 mg/kg/d dose of pioglitazone was bioactive as it decreased food intake in both males [Fig 9D; Time \times Dose F (12, 32) = 3.299 P=0.0034, Table 1 Lines #91–93] and females [Fig 9E; Time \times Dose F (12, 40) = 7.158 P<0.0001, Table 1 Lines #94–96], with greater effect in females [Fig 9F; Sex F (1, 16) = 5.945 P=0.0268, Table 1 Lines #97–99]. Pioglitazone 30 mg/kg/d also increased body weight in males Fig 9G; [Time \times Dose F (18, 114) = 28.95 P<0.0001, Table 1 Lines #100–102] and females [Fig 9H; Time \times Dose F (18, 108) = 2.660 P=0.001, Table 1 Lines #103–105] with greater effect in males [Fig 9I; Dose F (3, 40) = 26.60 P<0.0001, Table 1 Lines #106–108]. Pioglitazone 30 mg/kg/d also decreased blood glucose levels in in males [Fig 9J; Time \times Dose F (18, 114) = 5.692 P<0.0001, Table 1 Lines #109–111] and females [Fig 9L; Time \times Dose (18, 108) = 2.754 P=0.0006, Table 1 Lines #112–114] with greater effect in males [Fig 9M; Dose F (3, 40) = 8.885 P=0.0001]. However, neither 0.3, 3.0 nor 30 mg/kg/day pioglitazone decreased heat hypersensitivity in males [Fig 9A P>0.05, Table 1 lines #82–84] or females [Fig 9B Table 1 lines #85–87]. This contrasts with our previous study in ZDF rats that reported that oral pioglitazone (10 mg/kg/day for six weeks) reduced hypersensitivity and spinal pERK expression in male ZDF rats (Griggs et al., 2016); perhaps species differences in analgesic or pharmacokinetic profile might explain this discrepancy (Matheson and Le Foll, 2020; O'Brien et al., 2014). Thus, it remains possible that higher doses of pioglitazone will exert anti-hyperalgesic effects. Future studies are needed to determine the pharmacotherapeutic effectiveness of pioglitazone for the treatment of hyperalgesia and ongoing pain in PDN.

4 DISCUSSION

4.1 Pioglitazone inhibits acute chemical pain with greater potency in females

The current results indicate that systemic administration of pioglitazone inhibits MG-evoked mechanical and heat hypersensitivity. Remarkably, pioglitazone was 100-fold more potent in

females than in males in reducing MG-induced mechanical hypersensitivity, and 1000-fold more potent in reducing MG-induced heat hypersensitivity. Methylglyoxal is emerging as an important mediator of pain (Feldman et al., 2019). High serum levels of MG correlate with PDN in humans compared to diabetic patients without pain, as well as in multiple animal models of diabetes-associated cutaneous hypersensitivity including the db/db mouse (Bierhaus et al., 2012; Griggs et al., 2016; Griggs et al., 2019). Conversely, downregulation of spinal MG with intrathecal administration of an MG scavenger reduced heat hypersensitivity in db/db mice (Griggs et al., 2019). Intravenous, intrathecal, and intraplantar administration of MG evokes mechanical and thermal hypersensitivity as well as behavioral signs of the affective component of pain (Bierhaus et al., 2012; Griggs et al., 2019).

We previously demonstrated that pharmacological inhibitors of TRPA1, adenylyl cyclase type 1 (AC1), and Exchange protein directly activated by cAMP (Epac) prevent MG-induced hypersensitivity, indicating that MG triggers a TRPA1→AC1→Epac signaling pathway to produce nociception (Griggs et al., 2019). An important question raised by the current results is whether pioglitazone sex-dependently inhibits one or more of the TRPA1, AC1, or Epac signaling elements to inhibit MG-evoked pain.

4.2 Pioglitazone decreases behavioral signs of postsurgical pain in female mice

Antihyperalgesic actions of PPAR γ agonists have been reported in multiple rodent models of inflammatory pain, and the present results indicate for the first time that intrathecal administration of pioglitazone decreased mechanical hypersensitivity in an incision model of postsurgical pain, with greater efficacy in female mice as compared to male mice. This is analogous to humans studies that reported that opioid analgesics such as nalbuphine (Gear et al., 2003), butorphanol (Gear et al., 1996b), pentazocine alone (Gear et al., 1996a) or pentazocine combined with naloxone (Ryan et al., 2008) exert greater analgesia in women. Moreover, lower doses of nalbuphine are more effective in women than in men (Gear et al., 1999) and interestingly, in a Chinese population, women used significantly less morphine than men to decrease postsurgical pain (Chia et al., 2002). However, the sexual dimorphism of analgesic drugs for postsurgical pain remains controversial. For instance, women requested greater doses of morphine than men for postsurgical pain relief (Aubrun et al., 2005), while another study using Sprague Dawley rats that underwent to a model of postsurgical pain showed no sex differences to the analgesics used, including systemic or intrathecal administration of Morphine, gabapentin, clonidine or neostigmine (Kroin et al., 2003). The results are similarly unclear for the non-steroidal anti-inflammatory drugs (NSAIDs): while indomethacin was used more frequently in women to decrease postsurgical pain (Uchiyama et al., 2006), ibuprofen was not (Averbuch and Katzper, 2000). These discrepancies might be due to differences in experimental protocol, which includes diverse types of surgery, area of incision, dose of analgesic, use or not of patient-controlled analgesia, or side effect profile.

4.3 Pioglitazone inhibits chronic neuropathic pain with greater efficacy in female mice

We report that systemic administration of pioglitazone decreased SNI-induced mechanical and cold hypersensitivity in mice. These results are consistent with previous studies

describing the anti-allodynic actions of PPAR γ agonists in traumatic nerve injury models of neuropathic pain in mice (Iwai et al., 2008; Khasabova et al., 2019; Maeda et al., 2008; Takahashi et al., 2011) and rats (Churi et al., 2008; Griggs et al., 2015; Zhong et al., 2019). For example, we reported that a single administration of systemic pioglitazone rapidly and dose-dependently reduced mechanical and cold hypersensitivity in the rat SNI model of neuropathic pain (Griggs et al., 2015). Although PPAR γ is a transcription factor, the fast onset of action observed in our studies indicated that pioglitazone decreases hypersensitivity through a non-genomic mechanism of action (Griggs et al., 2015). We propose the following non-genomic mechanisms of action of the neuropathic pain-relieving actions of PPAR γ agonists: 1) inhibition of neuronal sensitization, consistent with prevention of p-p38 and touch stimulus-evoked pERK expression in dorsal horn (Griggs et al., 2015; Morgenweck et al., 2013); 2) inhibition of astrocyte activation, consistent with inhibition of injury-induced GFAP expression in dorsal horn (Griggs et al., 2015; Zhong et al., 2019); 3) inhibition of microglial activation, consistent with inhibition of injury-induced Iba1 expression in dorsal horn (Iwai et al., 2008; Park et al., 2007), though it must be kept in mind that spinal microglia may not be required for neuropathic mechanical hypersensitivity in females (Sorge et al., 2015); 4) inhibition of neuroinflammatory cytokines in the injured nerve, consistent with normalization of TNF α and IL6 levels (Jia et al., 2013; Murad and Ayuob, 2015; Zhong et al., 2019). Support for these mechanisms comes from studies conducted in male rodents, and so future studies are necessary to determine whether each of these mechanisms also operate in females.

An emerging body of literature is revealing sex differences in the mechanisms by which neuropathic pain develops in male and female mice (Sorge et al., 2015) and rats (Mapplebeck et al., 2018). Here we included both male and female subjects and demonstrate that pioglitazone more efficaciously reduced SNI-induced mechanical and cold hypersensitivity in female mice. Our findings are consistent with a previous report indicating that pioglitazone (albeit at a very high dose of 300 μ g, i.t.) reduced SNI-induced mechanical hypersensitivity in a PPAR γ -dependent manner by less than 20% in males but by more than 60% in females (Sorge et al., 2015). These findings are reminiscent of a recent study in a spinal cord injury model of central neuropathic pain by Gensel *et al* (Gensel et al., 2019), who found that systemic administration of pioglitazone (10 mg/kg) decreased heat hypersensitivity in female but not male mice. Sorge *et al* posited that T cells or microglia are required for mechanical hypersensitivity in female or male mice, respectively (Sorge et al., 2015). In support of this, the endogenous levels of sex hormones in females up-regulate the expression of PPAR γ in T cells to a greater extent in females as compared to males (Park and Choi, 2017; Park et al., 2016). However, the hypothesis that pioglitazone decreases neuropathic pain with greater efficacy in females due to the greater expression and activation of T cells requires definitive evidence of a causal link. This experimental direction has the potential to further our understanding of whether sex-specific differences in immune function contribute to the anti-allodynic mechanisms of pioglitazone in the setting of peripheral nerve injury.

4.4 Pioglitazone plus morphine inhibits neuropathic pain with greater efficacy in female mice

Pioglitazone may reduce dosage requirements of drugs that are commonly used for the treatment of pain. For example, the combination of pioglitazone with fluoxetine, a selective serotonin reuptake inhibitor, decreased behavioral and molecular signs of neuropathic pain after peripheral nerve injury (reversing heat hypersensitivity, spinal cord GFAP expression, serum TNF- α , IL-6, and MCP-1) to a greater degree as compared to either fluoxetine or pioglitazone alone (Murad and Ayuob, 2015). The current results indicate that pioglitazone might lower the dosage requirement for opioid analgesics as well. We found that the combination of pioglitazone with morphine decreased mechanical and cold hypersensitivity at doses that by themselves had no efficacy. This effect was restricted to females, consistent with sex differences in the analgesic actions of pioglitazone (Sorge et al., 2015) and opioids (Craft, 2003).

4.5 Pioglitazone decreased behavioral signs of PDN with greater efficacy in female db/db

Duloxetine and pregabalin are the only FDA-approved drugs available for the treatment of pain in PDN (Otto et al., 2011; Vincent et al., 2011), despite weak efficacy in just a small subset of patients, a new pharmacotherapeutic approach is needed. The current study indicates that a single injection of pioglitazone rapidly (within 30–60 minutes) decreased heat hypersensitivity in the ZDF rat and db/db mouse models of PDN in a sex-dependent manner, with greater efficacy in female db/db mice. Interestingly, pioglitazone accumulates in adipose tissue to a great extent in female rats compared to male rats after a single oral dose (10 mg/kg) or repeated oral administration (10 mg/kg for 21 consecutive days) (Fujita et al., 2003), perhaps leading to greater reductions in glycated hemoglobin (HbA1c) (Tajiri et al., 2007), and efficacy of pioglitazone in female as compared to male patients with nonalcoholic fatty liver disease or abnormal glucose metabolism in type 2 diabetes (Yan et al., 2021). Whether adipose tissue could be a target for the sex-dependent antihyperalgesic action of pioglitazone is an interesting question for future studies to address.

4.6 Neural sites of analgesic action of systemic pioglitazone

Pioglitazone has well-described binding affinity and efficacy at PPAR γ . Thus it is not surprising that PPAR γ -selective receptor antagonists such as GW9662 prevent the antihyperalgesic actions of pioglitazone in experimental models of neuropathic pain (Griggs et al., 2015), bone cancer pain (Gu et al., 2020), chemical-induced pain (Mansouri et al., 2017), inflammatory joint pain (Ruiz-Miyazawa et al., 2018), and inflammatory muscle pain (Santos et al., 2021). PPAR γ is expressed in numerous pain processing regions of the central nervous system that include peripheral nerves (Zhou et al., 2019 <https://pubmed.ncbi.nlm.nih.gov/31360147/>), spinal cord (Elkholy et al., 2020; Jiang et al., 2021) and supraspinal sites (Okine et al., 2018). Each of these sites could be a target of systemic pioglitazone. Pioglitazone is permeant through the blood-brain barrier, and therefore systemic administration can lead to significant concentrations not only in the periphery but also the spinal cord and brain.

Peripheral nerves.—PPAR γ agonists inhibit behavioral signs of persistent pain when delivered to the plantar site of inflammation. For example, in the plantar formalin test, systemic administration of pioglitazone reduced the persistent phase of nociceptive behaviors in mice, and both acute and persistent phase in rats (Oliveira et al., 2007). Likewise, local administration of rosiglitazone reduced mechanical and heat hypersensitivity in the plantar incision model in mice (Hasegawa-Moriyama et al., 2012), and mechanical hypersensitivity after intraplantar injection of CFA in rats (Hasegawa-Moriyama et al., 2013). Finally, local administration of the endogenous PPAR γ agonist 15d-PGJ2 reduced: 1) mechanical hyperalgesia following the injection of carrageenan under that plantar skin (Napimoga et al., 2008) or prior to the injection of carrageenan into the gastrocnemius muscle of rats (Santos et al., 2021); 2) mechanical hyperalgesia induced by intra-articular collagen in rat (Carregaro et al., 2016); and 3) the persistent phase of ongoing nociceptive behaviors after dilute formalin injection into the temporomandibular joint (but not into the paw) in rats (Napimoga et al., 2008; Pena-Dos-Santos et al., 2009).

In the setting of inflammation, immune cells can increase mechanical sensitivity at peripheral nerve terminals in the spinal cord through the release of pronociceptive neuropeptides and neurotransmitters (Pinho-Ribeiro et al., 2017). An alternative peripheral mechanism of analgesic action of PPAR γ agonists includes the downregulation of pro-inflammatory signaling in local macrophages, as observed in the intraplantar carrageenan model (Napimoga et al., 2008). Furthermore, rosiglitazone downregulated NF- κ B phosphorylation and decreased the M1-macrophage-associated marker IL-1 β , while increasing the M2-macrophage-associated marker interleukin IL-10 in the plantar incision model in male mice (Hasegawa-Moriyama et al., 2012). These results suggest that PPAR γ agonists may facilitate the polarization of macrophages from the M1 to the M2 phenotype, leading to inhibition of inflammation and pain. In summary, the antihyperalgesic actions of systemic pioglitazone, as we report here in the methylglyoxal and neuropathic pain models, might result from peripheral actions at the site of injury.

Dorsal horn.—Intrathecal administration of PPAR γ agonists decreases behavioral signs of persistent pain not only in the plantar incision model as described here, but also after nerve injury. For example, intrathecal pioglitazone decreased not only allodynia but also a natural stimulus-evoked activation of presumably nociceptive dorsal horn neurons (Griggs et al., 2015). After nerve injury, the development of allodynia is correlated with the downregulation of PPAR γ expression in the spinal cord dorsal horn (Jiang et al., 2021) in a time-dependent manner (Zhong et al., 2019). Further support for a spinal site of actions comes from reports that intrathecal administration of the PPAR γ antagonist BADGE (Fehrenbacher et al., 2009) and GW9662 inhibited the anti-hyperalgesic effect of rosiglitazone or 15d-PGJ2 (Churi et al., 2008). In summary, the antihyperalgesic actions of intrathecal or systemic pioglitazone as described here in the methylglyoxal, postsurgical and neuropathic pain models might result from actions at peripheral nerve.

Brain.—We previously reported that intracerebroventricular administration of the PPAR γ agonists rosiglitazone or 15d-PGJ2 reduced plantar carrageenan-induced paw edema, hypersensitivity and dorsal horn expression of the immediate-early gene c-fos (Morgenweck

et al., 2010). Since then, an emerging body of literature is beginning to identify specific brain regions (Okine et al., 2018). For example, microinjection of the PPAR γ inhibitor GW9662 in the anterior cingulate cortex reduced formalin-evoked nociceptive response, suggesting the endogenous PPAR γ agonists facilitate chemical pain (Okine et al., 2017).

4.7 Clinical Significance

More research is needed to identify peripheral, spinal and supraspinal mechanisms and sites of action of PPAR γ ligands in the setting of surgical incision, peripheral nerve injury, and painful diabetic neuropathy. Since pioglitazone is FDA approved and readily available, the current results promote its repurposing for chronic pain, particularly in women. Pioglitazone provides clinical benefit for patients with insulin resistance, and so repurposing it for patients who have PDN as well seems a reasonable goal.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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5 Declarations of interest:

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6 REFERENCES

- Abraham A, Barnett C, Katzberg HD, Lovblom LE, Perkins BA, Bril V, 2018. Sex differences in neuropathic pain intensity in diabetes. *J Neurol Sci* 388, 103–106. [PubMed: 29627001]
- Aubrun F, Salvi N, Coriat P, Riou B, 2005. Sex- and Age-related Differences in Morphine Requirements for Postoperative Pain Relief. *Anesthesiology* 103, 156–160. [PubMed: 15983468]
- Averbuch M, Katzper M, 2000. A Search for Sex Differences in Response to Analgesia. *Archives of Internal Medicine* 160, 3424–3428. [PubMed: 11112235]
- Becker AK, Auditore A, Pischetsrieder M, Messlinger K, Fleming T, Reeh PW, Sauer SK, 2020. Reactive dicarbonyl compounds cause Calcitonin Gene-Related Peptide release and synergize with inflammatory conditions in mouse skin and peritoneum. *J Biol Chem* 295, 6330–6343. [PubMed: 32198181]
- Bierhaus A, Fleming T, Stoyanov S, Leffler A, Babes A, Neacsu C, Sauer SK, Eberhardt M, Schnolzer M, Lasitschka F, Neuhuber WL, Kichko TI, Konrade I, Elvert R, Mier W, Pirags V, Lukic IK, Morcos M, Dehmer T, Rabbani N, Thornalley PJ, Edelstein D, Nau C, Forbes J, Humpert PM, Schwaninger M, Ziegler D, Stern DM, Cooper ME, Haberkorn U, Brownlee M, Reeh PW, Nawroth PP, 2012. Methylglyoxal modification of Nav1.8 facilitates nociceptive neuron firing and causes hyperalgesia in diabetic neuropathy. *Nat Med* 18, 926–933. [PubMed: 22581285]
- Bouhassira D, Lantéri-Minet M, Attal N, Laurent B, Touboul C, 2008. Prevalence of chronic pain with neuropathic characteristics in the general population. *Pain* 136, 380–387. [PubMed: 17888574]
- Carregaro V, Napimoga MH, Peres RS, Benevides L, Sacramento LA, Pinto LG, Grespan R, Cunha TM, da Silva JS, Cunha FQ, 2016. Therapeutic Treatment of Arthritic Mice with 15-Deoxy Delta(12,14)-Prostaglandin J2 (15d-PGJ2) Ameliorates Disease through the Suppression of Th17

- Cells and the Induction of CD4(+)CD25(-)FOXP3(+) Cells. *Mediators Inflamm* 2016, 9626427. [PubMed: 27872515]
- Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL, 1994. Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods* 53, 55–63. [PubMed: 7990513]
- Chia Y-Y, Chow L-H, Hung C-C, Liu K, Ger L-P, Wang P-N, 2002. Gender and pain upon movement are associated with the requirements for postoperative patient-controlled analgesia: a prospective survey of 2,298 Chinese patients. *Canadian Journal of Anesthesia* 49, 249. [PubMed: 11861342]
- Chinnam P, Mohsin M, Shafee LM, 2012. Evaluation of acute toxicity of pioglitazone in mice. *Toxicol Int* 19, 250–254. [PubMed: 23293462]
- Churi SB, Abdel-Aleem OS, Tumber KK, Scuderi-Porter H, Taylor BK, 2008. Intrathecal rosiglitazone acts at peroxisome proliferator-activated receptor-gamma to rapidly inhibit neuropathic pain in rats. *J Pain* 9, 639–649. [PubMed: 18387855]
- Clark JB, Palmer CJ, Shaw WN, 1983. The diabetic Zucker fatty rat. *Proc Soc Exp Biol Med* 173, 68–75. [PubMed: 6344096]
- Coleman DL, 1978. Obese and diabetes: two mutant genes causing diabetes-obesity syndromes in mice. *Diabetologia* 14, 141–148. [PubMed: 350680]
- Craft RM, 2003. Sex Differences in Opioid Analgesia: “From Mouse to Man”. *The Clinical Journal of Pain* 19, 175–186. [PubMed: 12792556]
- de Guglielmo G, Kallupi M, Scuppa G, Stopponi S, Demopoulos G, Gaitanaris G, Ciccocioppo R, 2014. Analgesic tolerance to morphine is regulated by PPARgamma. *Br J Pharmacol* 171, 5407–5416. [PubMed: 25048682]
- Decosterd I, Woolf CJ, 2000. Spared nerve injury: an animal model of persistent peripheral neuropathic pain. *Pain* 87, 149–158. [PubMed: 10924808]
- Dixon WJ, 1965. The Up-and-Down Method for Small Samples. *Journal of the American Statistical Association* 60, 967–978.
- Elkholy SE, Elaidy SM, El-Sherbeeny NA, Toraih EA, El-Gawly HW, 2020. Neuroprotective effects of ranolazine versus pioglitazone in experimental diabetic neuropathy: Targeting Nav1.7 channels and PPAR- γ . *Life Sci* 250, 117557. [PubMed: 32184124]
- Fehrenbacher JC, Loverme J, Clarke W, Hargreaves KM, Piomelli D, Taylor BK, 2009. Rapid pain modulation with nuclear receptor ligands. *Brain Res Rev* 60, 114–124. [PubMed: 19162071]
- Feldman EL, Callaghan BC, Pop-Busui R, Zochodne DW, Wright DE, Bennett DL, Bril V, Russell JW, Viswanathan V, 2019. Diabetic neuropathy. *Nat Rev Dis Primers* 5, 41. [PubMed: 31197153]
- Fu W, Wessel CR, Taylor BK, 2020. Neuropeptide Y tonically inhibits an NMDAR \rightarrow AC1 \rightarrow TRPA1/TRPV1 mechanism of the affective dimension of chronic neuropathic pain. *Neuropeptides* 80, 102024–102024. [PubMed: 32145934]
- Fujita Y, Yamada Y, Kusama M, Yamauchi T, Kamon J, Kadowaki T, Iga T, 2003. Sex differences in the pharmacokinetics of pioglitazone in rats. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 136, 85–94. [PubMed: 14522601]
- Gear RW, Gordon NC, Heller PH, Paul S, Miaskowski C, Levine JD, 1996a. Gender difference in analgesic response to the kappa-opioid pentazocine. *Neuroscience Letters* 205, 207–209. [PubMed: 8852594]
- Gear RW, Gordon NC, Miaskowski C, Paul SM, Heller PH, Levine JD, 2003. Sexual dimorphism in very low dose nalbuphine postoperative analgesia. *Neuroscience Letters* 339, 1–4. [PubMed: 12618286]
- Gear RW, Miaskowski C, Gordon NC, Paul SM, Heller PH, Levine JD, 1996b. Kappa-opioids produce significantly greater analgesia in women than in men. *Nature Medicine* 2, 1248–1250.
- Gear RW, Miaskowski C, Gordon NC, Paul SM, Heller PH, Levine JD, 1999. The kappa opioid nalbuphine produces gender- and dose-dependent analgesia and antianalgesia in patients with postoperative pain. *Pain* 83.
- Gensel JC, Donahue RR, Bailey WM, Taylor BK, 2019. Sexual Dimorphism of Pain Control: Analgesic Effects of Pioglitazone and Azithromycin in Chronic Spinal Cord Injury. *Journal of neurotrauma* 36, 2372–2376. [PubMed: 30618345]

- Ghavimi H, Hassanzadeh K, Maleki-Dizaji N, Azarfardian A, Ghasami S, Zolali E, Charkhpour M, 2014. Pioglitazone prevents morphine antinociception tolerance and withdrawal symptoms in rats. *Naunyn Schmiedebergs Arch Pharmacol* 387, 811–821. [PubMed: 24899385]
- Grace PM, Tawfik VL, Svensson CI, Burton MD, Loggia ML, Hutchinson MR, 2021. The Neuroimmunology of Chronic Pain: From Rodents to Humans. *J Neurosci* 41, 855–865. [PubMed: 33239404]
- Greenspan JD, Craft RM, LeResche L, Arendt-Nielsen L, Berkley KJ, Fillingim RB, Gold MS, Holdcroft A, Lautenbacher S, Mayer EA, Mogil JS, Murphy AZ, Traub RJ, Consensus Working Group of the Sex, G., Pain, S. I. G. o. t. I., 2007. Studying sex and gender differences in pain and analgesia: a consensus report. *Pain* 132 Suppl 1, S26–S45. [PubMed: 17964077]
- Griggs RB, Donahue RR, Adkins BG, Anderson KL, Thibault O, Taylor BK, 2016. Pioglitazone Inhibits the Development of Hyperalgesia and Sensitization of Spinal Nociceptive Neurons in Type 2 Diabetes. *J Pain* 17, 359–373. [PubMed: 26687453]
- Griggs RB, Donahue RR, Morgenweck J, Grace PM, Sutton A, Watkins LR, Taylor BK, 2015. Pioglitazone rapidly reduces neuropathic pain through astrocyte and nongenomic PPAR γ mechanisms. *Pain* 156, 469–482. [PubMed: 25599238]
- Griggs RB, Santos DF, Laird DE, Doolen S, Donahue RR, Wessel CR, Fu W, Sinha GP, Wang P, Zhou J, Brings S, Fleming T, Nawroth PP, Susuki K, Taylor BK, 2019. Methylglyoxal and a spinal TRPA1-AC1-Epac cascade facilitate pain in the db/db mouse model of type 2 diabetes. *Neurobiol Dis.*
- Gu W, Sun Y, Gu W, Huang Y, Bo J, Zhou L, Ma Z, Gu X, Zhang W, 2020. The analgesic effects of pioglitazone in the bone cancer pain rats via regulating the PPAR γ /PTEN/mTOR signaling pathway in the spinal dorsal horn. *Biomed Pharmacother* 131, 110692. [PubMed: 32942156]
- Hasegawa-Moriyama M, Kurimoto T, Nakama M, Godai K, Kojima M, Kuwaki T, Kanmura Y, 2013. Peroxisome proliferator-activated receptor-gamma agonist rosiglitazone attenuates inflammatory pain through the induction of heme oxygenase-1 in macrophages. *Pain* 154, 1402–1412. [PubMed: 23707273]
- Hasegawa-Moriyama M, Ohnou T, Godai K, Kurimoto T, Nakama M, Kanmura Y, 2012. Peroxisome proliferator-activated receptor-gamma agonist rosiglitazone attenuates postincisional pain by regulating macrophage polarization. *Biochemical and biophysical research communications* 426, 76–82. [PubMed: 22910418]
- Iwai S, Maeda T, Kiguchi N, Kobayashi Y, Fukazawa Y, Ozaki M, Kishioka S, 2008. Pioglitazone attenuates tactile allodynia and microglial activation in mice with peripheral nerve injury. *Drug Discov Ther.* 2, 353–356. [PubMed: 22504745]
- Jang JH, Liang D, Kido K, Sun Y, Clark DJ, Brennan TJ, 2011. Increased local concentration of complement C5a contributes to incisional pain in mice. *J Neuroinflammation* 8, 80. [PubMed: 21736743]
- Jia HB, Wang XM, Qiu LL, Liu XY, Shen JC, Ji Q, Yang JJ, 2013. Spinal neuroimmune activation inhibited by repeated administration of pioglitazone in rats after L5 spinal nerve transection. *Neurosci Lett* 543, 130–135. [PubMed: 23583338]
- Jiang P, Jiang Q, Yan Y, Hou Z, Luo D, 2021. Propofol ameliorates neuropathic pain and neuroinflammation through PPAR γ up-regulation to block Wnt/ β -catenin pathway. *Neurol Res* 43, 71–77. [PubMed: 32985377]
- Khasabova IA, Khasabov SG, Olson JK, Uhelski ML, Kim AH, Albino-Ramírez AM, Wagner CL, Seybold VS, Simone DA, 2019. Pioglitazone, a PPAR γ agonist, reduces cisplatin-evoked neuropathic pain by protecting against oxidative stress. *Pain* 160, 688–701. [PubMed: 30507781]
- Kroin JS, Buvanendran A, Nagalla SKS, Tuman KJ, 2003. Postoperative pain and analgesic responses are similar in male and female Sprague-Dawley rats. *Canadian Journal of Anesthesia* 50, 904–908. [PubMed: 14617587]
- Maeda T, Kiguchi N, Kobayashi Y, Ozaki M, Kishioka S, 2008. Pioglitazone attenuates tactile allodynia and thermal hyperalgesia in mice subjected to peripheral nerve injury. *J Pharmacol Sci* 108, 341–347. [PubMed: 19008646]

- Maeshiba Y, Kiyota Y, Yamashita K, Yoshimura Y, Motohashi M, Tanayama S, 1997. Disposition of the new antidiabetic agent pioglitazone in rats, dogs, and monkeys. *Arzneimittelforschung* 47, 29–35. [PubMed: 9037440]
- Mansouri MT, Naghizadeh B, Ghorbanzadeh B, Rajabi H, Pashmforoush M, 2017. Pharmacological evidence for systemic and peripheral antinociceptive activities of pioglitazone in the rat formalin test: Role of PPAR γ and nitric oxide. *European Journal of Pharmacology* 805, 84–92. [PubMed: 28286124]
- Mapplebeck JCS, Dalgarno R, Tu Y, Moriarty O, Beggs S, Kwok CHT, Halievski K, Assi S, Mogil JS, Trang T, Salter MW, 2018. Microglial P2X4R-evoked pain hypersensitivity is sexually dimorphic in rats. *Pain* 159, 1752–1763. [PubMed: 29927790]
- Matheson J, Le Foll B, 2020. Therapeutic Potential of Peroxisome Proliferator-Activated Receptor (PPAR) Agonists in Substance Use Disorders: A Synthesis of Preclinical and Human Evidence. *Cells* 9.
- Morgenweck J, Griggs RB, Donahue RR, Zadina JE, Taylor BK, 2013. PPAR γ activation blocks development and reduces established neuropathic pain in rats. *Neuropharmacology* 70, 236–246. [PubMed: 23415633]
- Murad H, Ayuob N, 2015. Co-Administration of Pioglitazone Improves Fluoxetine's Antinociceptive, Neuroprotective, and Antidepressant Effects in Chronic Constriction Injury in Rats. *Pain Physician* 18, 609–620. [PubMed: 26606013]
- Napimoga MH, Souza GR, Cunha TM, Ferrari LF, Clemente-Napimoga JT, Parada CA, Verri WA Jr., Cunha FQ, Ferreira SH, 2008. 15d-prostaglandin J2 inhibits inflammatory hypernociception: involvement of peripheral opioid receptor. *J Pharmacol Exp Ther* 324, 313–321. [PubMed: 17928570]
- Niesters M, Dahan A, Kest B, Zacny J, Stijnen T, Aarts L, Sarton E, 2010. Do sex differences exist in opioid analgesia? A systematic review and meta-analysis of human experimental and clinical studies. *Pain* 151, 61–68. [PubMed: 20692097]
- O'Brien PD, Sakowski SA, Feldman EL, 2014. Mouse models of diabetic neuropathy. *ILAR journal* 54, 259–272. [PubMed: 24615439]
- Okine BN, Gaspar JC, Finn DP, 2018. PPARs and pain. *Br J Pharmacol*.
- Okine BN, Gaspar JC, Madasu MK, Olango WM, Harhen B, Roche M, Finn DP, 2017. Characterisation of peroxisome proliferator-activated receptor signalling in the midbrain periaqueductal grey of rats genetically prone to heightened stress, negative affect and hyperalgesia. *Brain Res* 1657, 185–192. [PubMed: 27916440]
- Oliveira ACP, Bertollo CM, Rocha LTS, Nascimento EB, Costa KA, Coelho MM, 2007. Antinociceptive and antiedematogenic activities of fenofibrate, an agonist of PPAR alpha, and pioglitazone, an agonist of PPAR gamma. *European Journal of Pharmacology* 561, 194–201. [PubMed: 17343847]
- Otto KJ, Wyse BD, Cabot PJ, Smith MT, 2011. Longitudinal study of painful diabetic neuropathy in the Zucker diabetic fatty rat model of type 2 diabetes: impaired basal G-protein activity appears to underpin marked morphine hyposensitivity at 6 months. *Pain Med* 12, 437–450. [PubMed: 21332939]
- Park HJ, Choi JM, 2017. Sex-specific regulation of immune responses by PPARs. *Exp Mol Med* 49, e364. [PubMed: 28775365]
- Park HJ, Park HS, Lee JU, Bothwell AL, Choi JM, 2016. Gender-specific differences in PPAR γ regulation of follicular helper T cell responses with estrogen. *Sci Rep* 6, 28495. [PubMed: 27335315]
- Park SW, Yi JH, Miranpuri G, Satriotomo I, Bowen K, Resnick DK, Vemuganti R, 2007. Thiazolidinedione class of peroxisome proliferator-activated receptor gamma agonists prevents neuronal damage, motor dysfunction, myelin loss, neuropathic pain, and inflammation after spinal cord injury in adult rats. *J Pharmacol Exp Ther* 320, 1002–1012. [PubMed: 17167171]
- Pena-Dos-Santos DR, Severino FP, Pereira SAL, Rodrigues DBR, Cunha FQ, Vieira SM, Napimoga MH, Clemente-Napimoga JT, 2009. Activation of peripheral [kappa]/[delta] opioid receptors mediates 15-deoxy-[Delta]12,14-prostaglandin J2 induced-antinociception in rat temporomandibular joint. *Neuroscience* 163, 1211–1219. [PubMed: 19647045]

- Peng BY, Wang Q, Luo YH, He JF, Tan T, Zhu H, 2018. A novel and quick PCR-based method to genotype mice with a leptin receptor mutation (db/db mice). *Acta Pharmacol Sin* 39, 117–123. [PubMed: 28748911]
- Pereira MP, Pogatzki-Zahn E, 2015. Gender aspects in postoperative pain. *Curr Opin Anaesthesiol* 28, 546–558. [PubMed: 26308516]
- Pinho-Ribeiro FA, Verri WA Jr., Chiu IM, 2017. Nociceptor Sensory Neuron-Immune Interactions in Pain and Inflammation. *Trends in immunology* 38, 5–19. [PubMed: 27793571]
- Pogatzki EM, Raja SN, 2003. A mouse model of incisional pain. *Anesthesiology* 99, 1023–1027. [PubMed: 14508341]
- Raehal KM, Walker JK, Bohn LM, 2005. Morphine side effects in beta-arrestin 2 knockout mice. *J Pharmacol Exp Ther* 314, 1195–1201. [PubMed: 15917400]
- Raputova J, Srotova I, Vlckova E, Sommer C, Üçeyler N, Birklein F, Rittner HL, Reborn C, Adamova B, Kovalova I, Kralickova Nekvapilova E, Forer L, Belobradkova J, Olsovsky J, Weber P, Dusek L, Jarkovsky J, Bednarik J, 2017. Sensory phenotype and risk factors for painful diabetic neuropathy: a cross-sectional observational study. *Pain* 158, 2340–2353. [PubMed: 28858986]
- Ruiz-Miyazawa KW, Staurengo-Ferrari L, Pinho-Ribeiro FA, Fattori V, Zaninelli TH, Badaro-Garcia S, Borghi SM, Andrade KC, Clemente-Napimoga JT, Alves-Filho JC, Cunha TM, Fraceto LF, Cunha FQ, Napimoga MH, Casagrande R, Verri WA, 2018. 15d-PGJ2-loaded nanocapsules ameliorate experimental gout arthritis by reducing pain and inflammation in a PPARgamma-sensitive manner in mice. *Scientific Reports* 8, 13979. [PubMed: 30228306]
- Ryan JL, Jureidini B, Hodges JS, Baisden M, Swift JQ, Bowles WR, 2008. Gender Differences in Analgesia for Endodontic Pain. *Journal of Endodontics* 34, 552–556. [PubMed: 18436033]
- Santos DFS, Melo-Aquino B, Jorge CO, Clemente-Napimoga JT, Taylor BK, Oliveira-Fusaro MCG, 2021. Prostaglandin 15d-PGJ2 targets PPAR γ and opioid receptors to prevent muscle hyperalgesia in rats. *Neuroreport* 32, 238–243. [PubMed: 33470759]
- Sarton E, Olofsen E, Romberg R, den Hartigh J, Kest B, Nieuwenhuijs D, Burm A, Teppema L, Dahan A, 2000. Sex differences in morphine analgesia: an experimental study in healthy volunteers. *Anesthesiology* 93, 1245–1254; discussion 1246A. [PubMed: 11046213]
- Scheff SW, Saucier DA, Cain ME, 2002. A statistical method for analyzing rating scale data: the BBB locomotor score. *Journal of neurotrauma* 19, 1251–1260. [PubMed: 12427332]
- Sorge RE, Mapplebeck JC, Rosen S, Beggs S, Taves S, Alexander JK, Martin LJ, Austin JS, Sotocinal SG, Chen D, Yang M, Shi XQ, Huang H, Pillion NJ, Bilan PJ, Tu Y, Klip A, Ji RR, Zhang J, Salter MW, Mogil JS, 2015. Different immune cells mediate mechanical pain hypersensitivity in male and female mice. *Nat Neurosci* 18, 1081–1083. [PubMed: 26120961]
- Tajiri Y, Takei R, Mimura K, Umeda F, 2007. Indicators for the efficacy of pioglitazone before and during treatment in Japanese patients with type 2 diabetes. *Diabetes Technol Ther* 9, 429–437. [PubMed: 17931051]
- Takada N, Genda K, 2019. Pioglitazone (Actos, Glustin). In: Nagaoka S, (Ed), *Drug Discovery in Japan: Investigating the Sources of Innovation*. Springer Singapore, Singapore, pp. 169–181.
- Takahashi Y, Hasegawa-Moriyama M, Sakurai T, Inada E, 2011. The macrophage-mediated effects of the peroxisome proliferator-activated receptor-gamma agonist rosiglitazone attenuate tactile allodynia in the early phase of neuropathic pain development. *Anesth Analg* 113, 398–404. [PubMed: 21490083]
- Uchiyama K, Kawai M, Tani M, Ueno M, Hama T, Yamaue H, 2006. Gender differences in postoperative pain after laparoscopic cholecystectomy. *Surgical Endoscopy And Other Interventional Techniques* 20, 448–451. [PubMed: 16432649]
- Vincent AM, Callaghan BC, Smith AL, Feldman EL, 2011. Diabetic neuropathy: cellular mechanisms as therapeutic targets. *Nature reviews. Neurology* 7, 573–583. [PubMed: 21912405]
- Yan H, Wu W, Chang X, Xia M, Ma S, Wang L, Gao J, 2021. Gender differences in the efficacy of pioglitazone treatment in nonalcoholic fatty liver disease patients with abnormal glucose metabolism. *Biology of Sex Differences* 12, 1. [PubMed: 33397443]
- Zhong Y, Chen J, Chen J, Chen Y, Li L, Xie Y, 2019. Crosstalk between Cdk5/p35 and ERK1/2 signalling mediates spinal astrocyte activity via the PPARgamma pathway in a rat model of chronic constriction injury. *J Neurochem* 151, 166–184. [PubMed: 31314915]

Zimmermann M, 1983. Ethical guidelines for investigations of experimental pain in conscious animals. *Pain* 16, 109–110. [PubMed: 6877845]

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Highlights

- Pioglitazone inhibits chemical nociception with greater potency in female mice
- Pioglitazone inhibits post-surgical pain with greater potency in female mice
- Pioglitazone inhibits neuropathic pain with greater efficacy in female mice
- Pioglitazone inhibits PDN with greater efficacy in female db/db mice
- These results promote the repurposing of pioglitazone for chronic pain in women

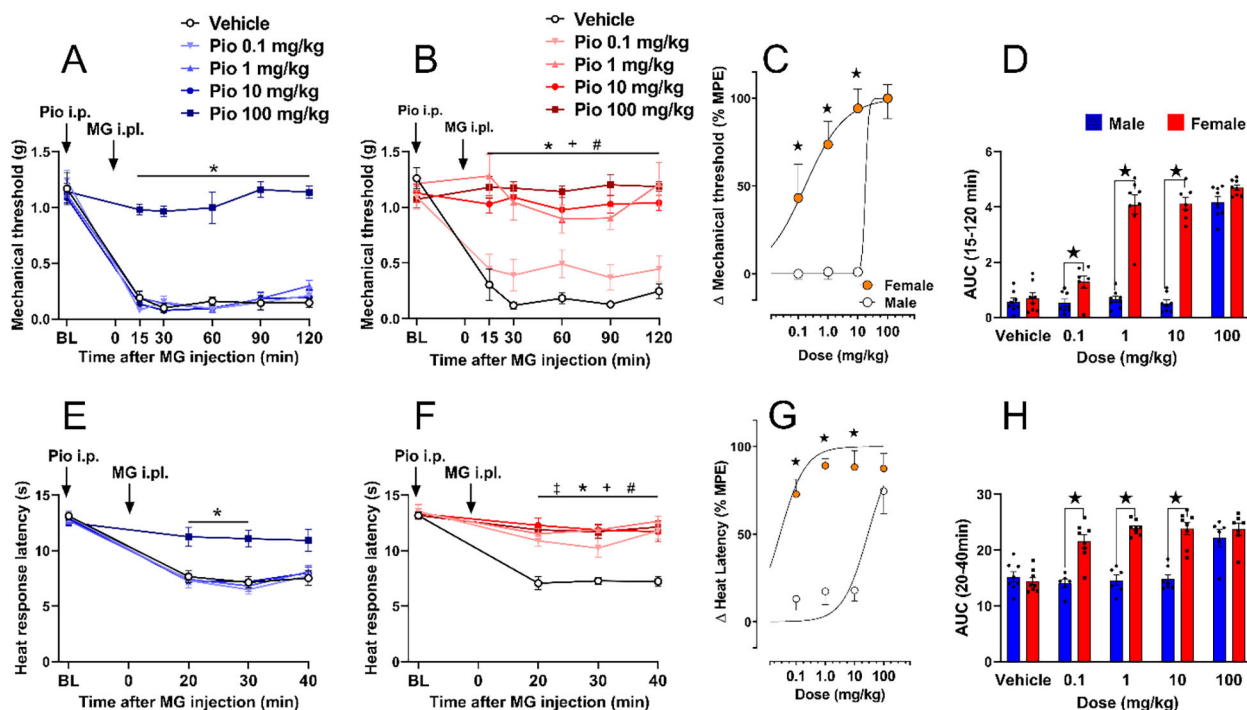


Figure 1. Pioglitazone inhibits chemical pain with greater potency in female as compared to male mice.

(A) Only the 100 mg/kg dose of pioglitazone prevented mechanical hypersensitivity in male mice. (B) 1–100 mg/kg pioglitazone decreased mechanical hypersensitivity in female mice. (C) Dose-response curve indicating that pioglitazone decreased MG-induced mechanical hyperalgesia in a dose-dependent manner with a greater effect in females (ED₅₀: 0.17 mg/kg) as compared to males (ED₅₀: 17.85 mg/kg). (D) Analysis of the data with Time collapsed across post-injection timepoints illustrates that pioglitazone exerted greater decreases in mechanical hypersensitivity in females as compared to males at 0.1, 1, and 10 mg/kg. (E) Only the 100 mg/kg dose of pioglitazone prevented heat hypersensitivity in male mice. (F) Pioglitazone doses of 1, 10, and 100 mg/kg decreased heat hypersensitivity in female mice. (G) Dose-response curve showing that pioglitazone decreased MG-induced heat hyperalgesia in a dose-dependent manner with a greater effect in females (ED₅₀: 0.028 mg/kg) as compared to males (ED₅₀: 28.8 mg/kg). (H) Analysis of the data with Time collapsed across post-injection timepoints illustrates that pioglitazone exerted greater decreases in mechanical hypersensitivity in females as compared to males at 0.1, 1, and 10 mg/kg. Two-way ANOVA was performed followed by Sidak's multiple comparisons test for figures 1B–C, 1E–G and 1H. Non-linear regression analysis log(agonist) vs. normalized response was performed for figures 1D and 1H. %MPE = maximum possible effect. Symbol * P<0.05 Pio 100mg vs vehicle; + P<0.05 Pio 10mg vs vehicle; # P<0.05 Pio 1mg vs vehicle; ‡ P<0.05 Pio 0.1mg vs vehicle. ★ P<0.05 male vs female. Values represent mean ± SEM. N=6–8.

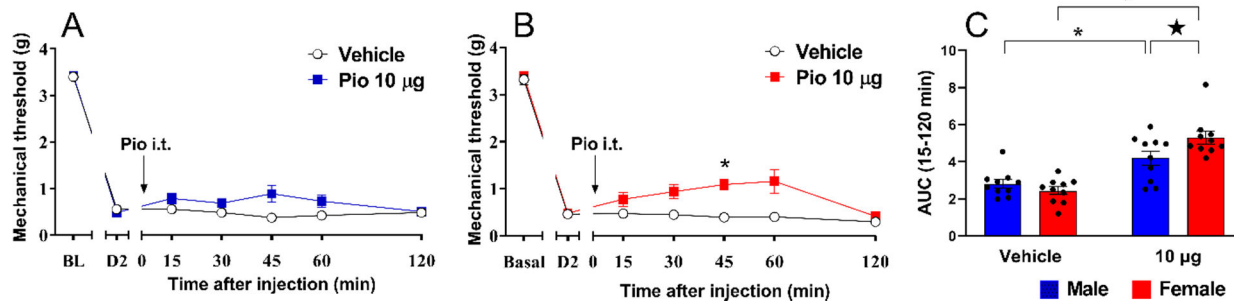


Figure 2. Pioglitazone inhibits postsurgical hypersensitivity with greater efficacy in female mice. Pioglitazone decreased mechanical hypersensitivity in (A) male and (B) female mice. (C) Analysis of the data with Time collapsed across post-injection timepoints indicates greater efficacy in females at the 10 µg dose. Two-way ANOVA followed by Sidak's multiple comparisons test). Symbol * $P < 0.05$ Pio 10 µg vs vehicle; ★ $P < 0.05$ male vs female. Values represent mean \pm SEM. $N = 10$.

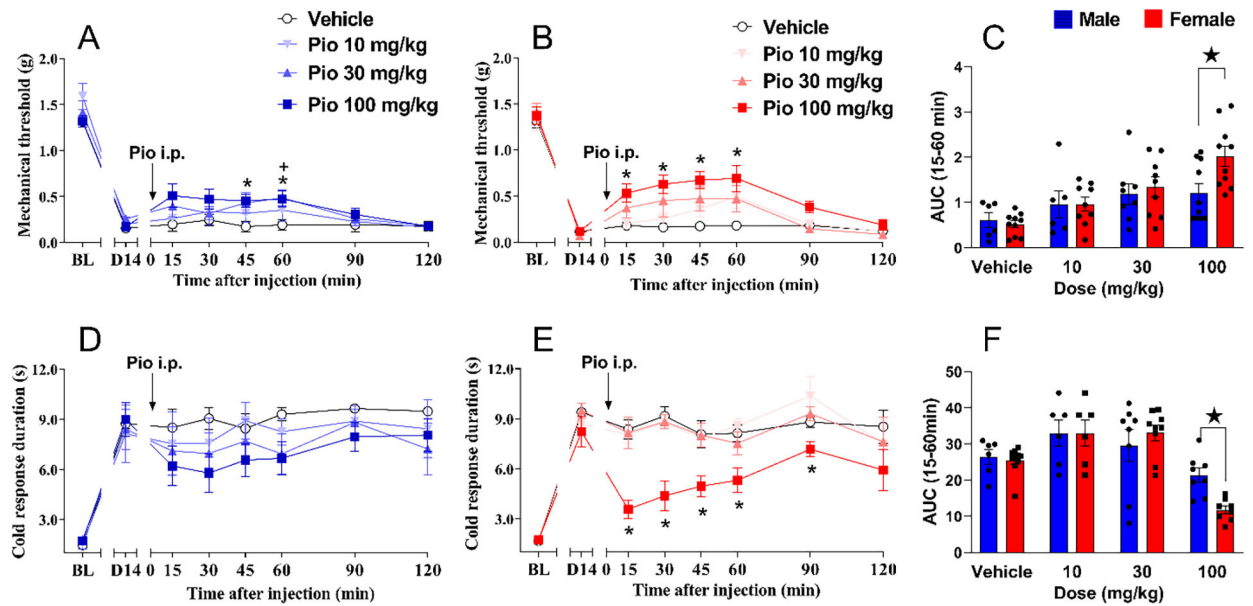


Figure 3. Pioglitazone inhibits neuropathic pain with greater efficacy in female mice. (A) 100 mg/kg pioglitazone decreased mechanical hypersensitivity in male mice. (B) 100 mg/kg pioglitazone decreased mechanical hypersensitivity in female mice. (C) Analysis of the data with Time collapsed across post-injection timepoints illustrates that 100 mg/kg pioglitazone exerted a greater decrease in mechanical hypersensitivity in females as compared to males. (D) Pioglitazone did not decrease cold hypersensitivity in male mice. (E) 100 mg/kg pioglitazone decreased cold hypersensitivity in female mice. (F) Analysis of the data with Time collapsed across post-injection timepoints illustrates that 100 mg/kg pioglitazone exerted a greater decrease in cold hypersensitivity in females as compared to males. Two-way ANOVA was performed followed by Sidak's multiple comparisons test. Symbol * $P < 0.05$ Pio 100mg/kg vs vehicle; ★ $P < 0.05$ male vs female. Values represent mean \pm SEM. $N = 6-10$.

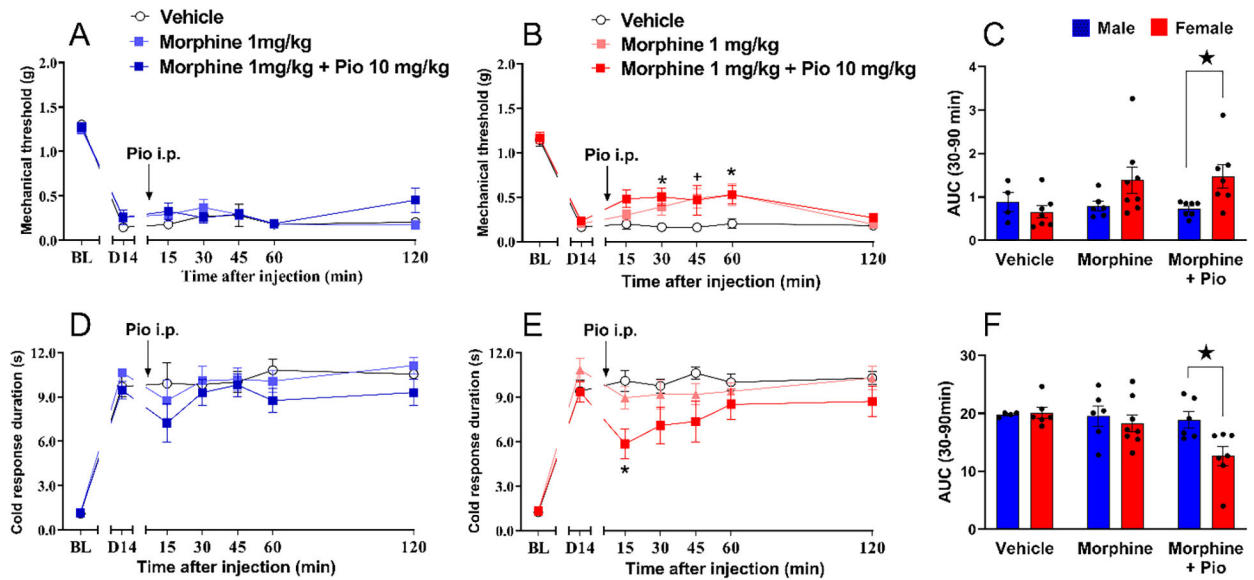


Figure 4. Pioglitazone / morphine inhibits neuropathic pain with greater efficacy in female mice.

(A) Neither morphine 1 mg/kg alone nor morphine 1 mg/kg combined with pioglitazone 10 mg/kg decreased mechanical hypersensitivity in male mice. (B) Morphine alone or combined with pioglitazone decreased mechanical hypersensitivity in female mice. (C) Analysis of the data with Time collapsed across post-injection timepoints illustrates that the combination of morphine and pioglitazone exerted greater decrease in mechanical hypersensitivity in females as compared to males. (D) Neither morphine 1 mg/kg alone nor morphine 1 mg/kg combined with pioglitazone 10 mg/kg decreased cold hypersensitivity in male mice. (E) Morphine alone or combined with pioglitazone decreased cold hypersensitivity in female mice. (F) Analysis of the data with Time collapsed across post-injection timepoints illustrates that the combination of morphine and pioglitazone exerted greater decrease in cold hypersensitivity in females as compared to males. Two-way ANOVA was performed followed by Sidak's multiple comparisons test for figures 4A–B and, Tukey's multiple comparison test for figures 4D–E and Sidak's multiple comparisons test for figure 3C and 3F. Symbol: + $P < 0.05$ morphine 1 mg/kg alone vs vehicle; * $P < 0.05$ morphine 1 mg/kg combined with pio 10mg/kg vs vehicle; ★ $P < 0.05$ male vs female. Values represent mean \pm SEM. $N = 4-8$.

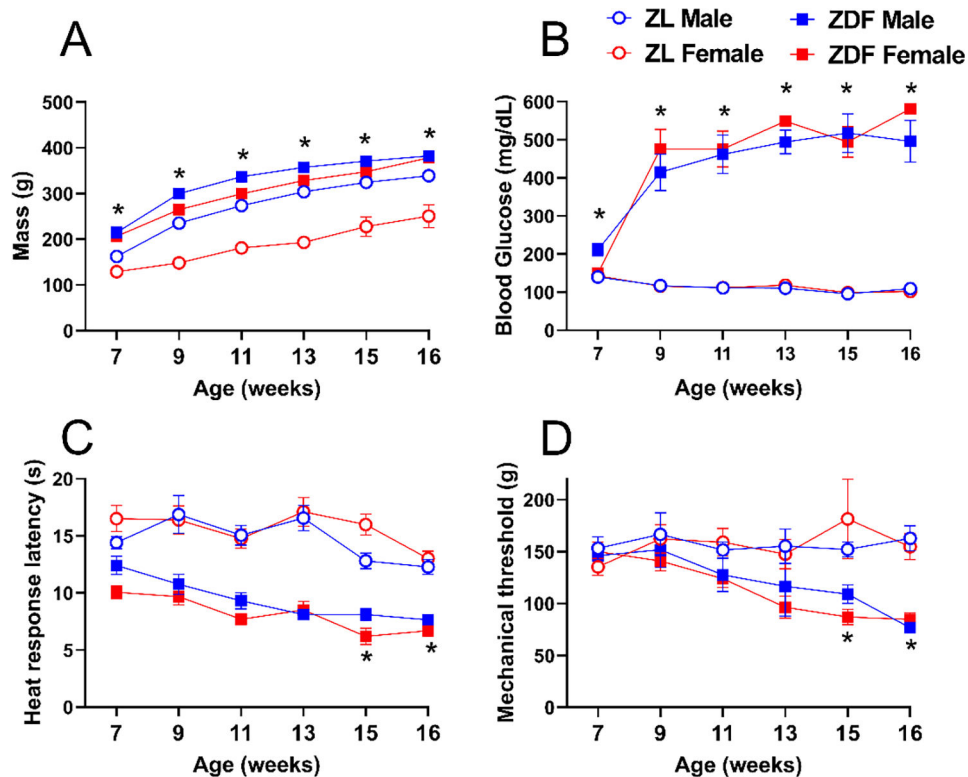


Figure 5. Development of type 2 diabetes and cutaneous hypersensitivity in male and female ZDF rats.

Compared to Zucker Lean (ZL) controls, male and female Zucker Diabetic Fatty (ZDF) rats exhibited a time-dependent increase in (A) body mass, (B) blood glucose, (C) heat hypersensitivity, and (D) mechanical hypersensitivity. Symbol * $P < 0.05$ same-sex ZL vs ZDF, three-way ANOVA followed by Tukey's multiple comparison test. Values represent mean \pm SEM. $N=8$.

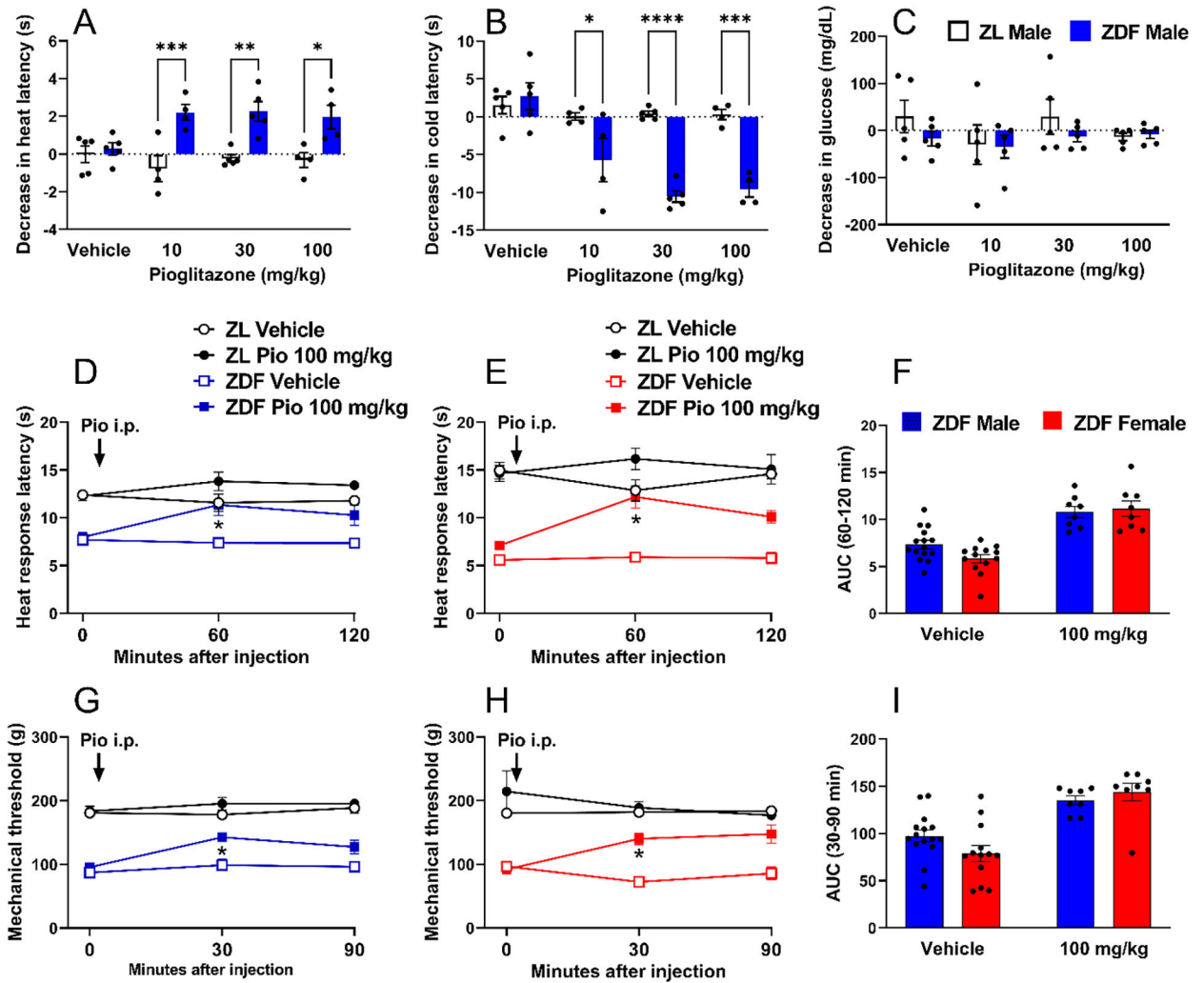


Figure 6. Pioglitazone reduced heat and mechanical hyperalgesia in male and female ZDF rats. Values are plotted as the difference between pre-drug baseline values and 1–2 hour post-drug values collected in ZDF and ZL genetic controls after i.p. injection of pioglitazone at doses of 0, 100, 300 mg/kg, with respect to changes from baseline in (A) Hotplate latency, (B) cold plate responses and (C) glucose. Further studies comparing males and females illustrate that 100 mg/kg pioglitazone decreased heat hypersensitivity in (D) male and (E) female ZDF rats. (F) Analysis of the data with Time collapsed across post-injection timepoints illustrates that pioglitazone exerted a similar decrease in heat hypersensitivity in ZDF females and males at 100 mg/kg. 100 mg/kg pioglitazone decreased mechanical hypersensitivity in (G) male and female (H) ZDF rats. (I) Analysis of the data with Time collapsed across post-injection timepoints illustrates that pioglitazone exerted a similar decrease in mechanical hypersensitivity in males and females. Three-way ANOVA and Tukey's multiple comparisons test for figures 6D–E and 6G–H. Two-way ANOVA and Sidak's multiple comparisons test for figures 6A–C, 6F and 6I. Symbol: Panels A–C * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ pre- vs post-injection value; Panels D, E, G, H * $p < 0.05$ ZDF vehicle vs ZDF Pio 100 mg/kg. Panels F and I * $P < 0.05$ vehicle vs Pio 100 mg/kg. Values represent mean \pm SEM. N=8–15.

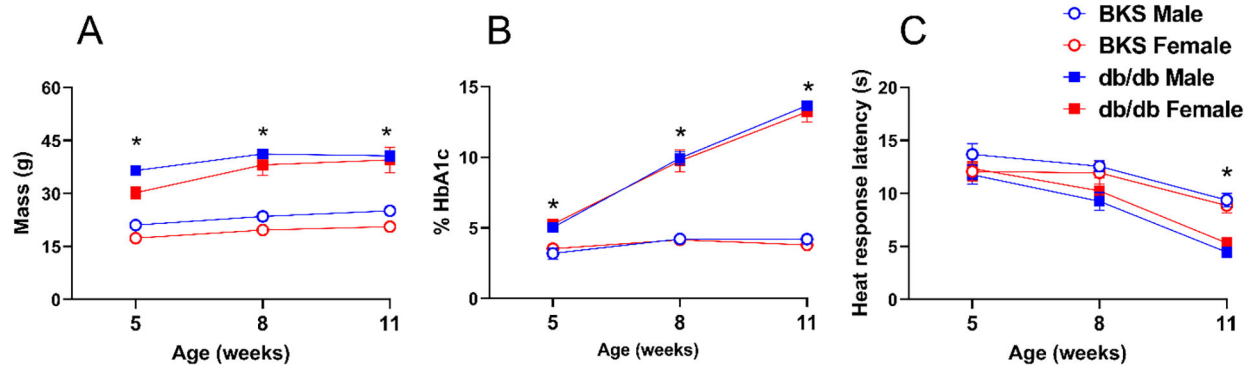


Figure 7. Development of diabetes and cutaneous hypersensitivity in male and female db/db mice.

Male and female db/db exhibited a time-dependent increase in (A) body mass, (B) blood glucose, and (C) heat hypersensitivity. Symbol * $P < 0.05$ same-sex C57BLKS/J (BKS) vs db/db, three-way ANOVA followed by Tukey's multiple comparison test. Values represent mean \pm SEM. $N = 7-9$.

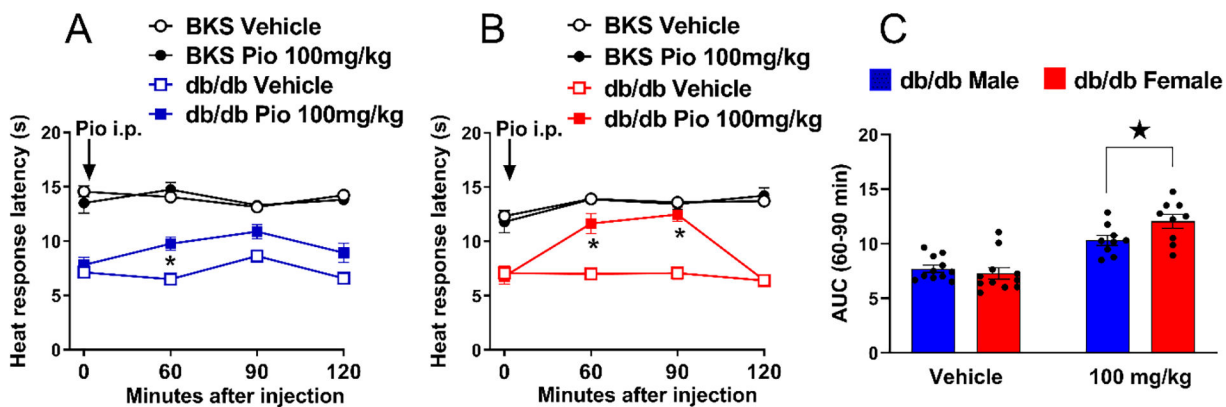


Figure 8. Pioglitazone decreased heat hypersensitivity in db/db mice with greater efficacy in female mice.

100 mg/kg pioglitazone decreased heat hypersensitivity in male (A) and female (B) db/db mice. Three-way ANOVA was performed followed by Tukey's multiple comparisons test. (C) pioglitazone exerted a greater decrease in heat hypersensitivity in db/db females as compared to males. Sidak's multiple comparisons test. Symbol * $P < 0.05$ ZDF Pio 100 mg/kg vs vehicle; ★ $P < 0.05$ ZDF male vs ZDF female. Values = mean \pm SEM. N=8–15.

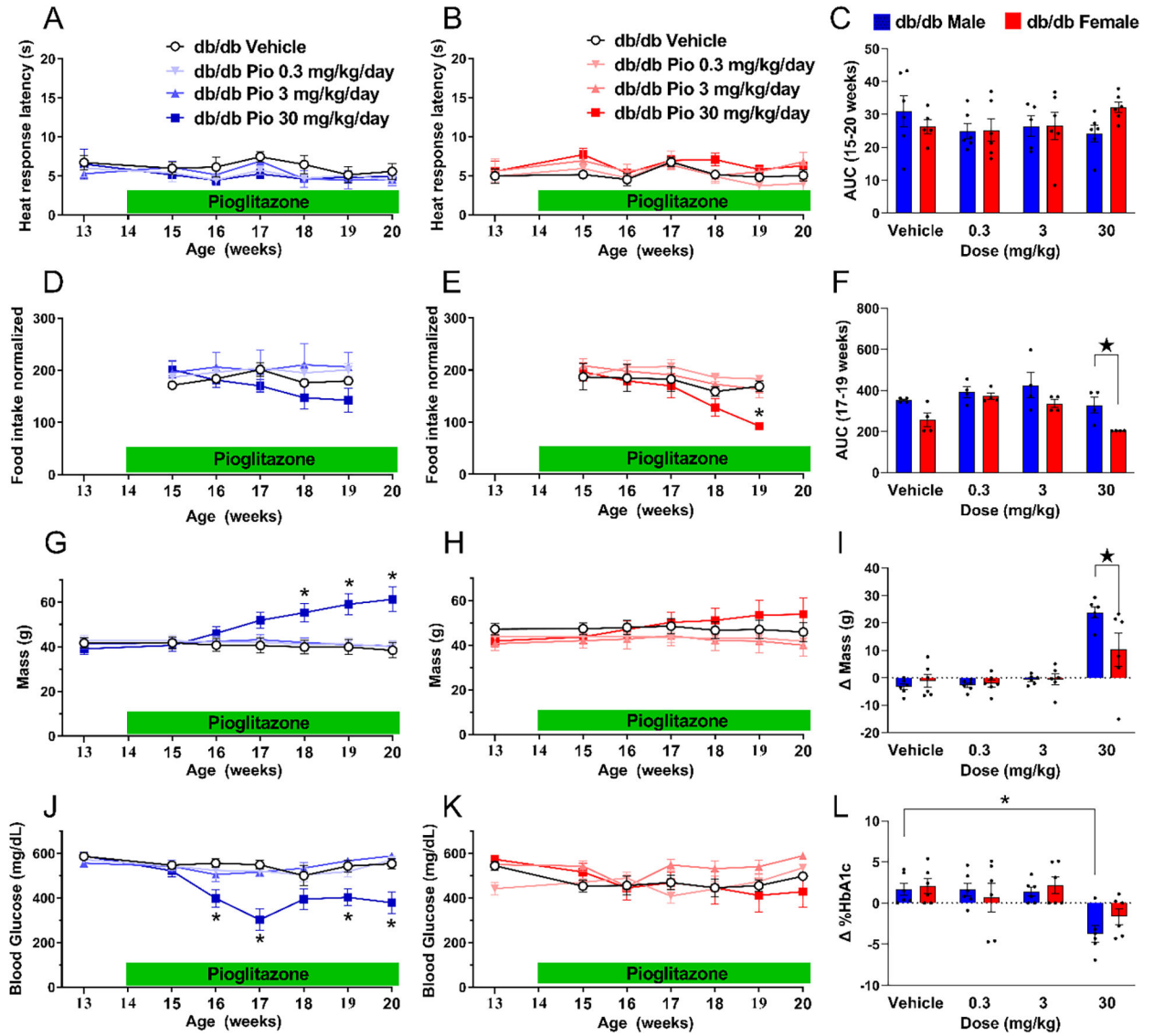


Figure 9. Six weeks of pioglitazone does not change heat hypersensitivity in db/db mice. Pioglitazone did not change heat hypersensitivity in (A) male or (B) female db/db mice. (C) Analysis of the data with Time collapsed across post-injection timepoints illustrates that pioglitazone did not change heat hypersensitivity. Pioglitazone decreased food consumption in (D) males and (E) female mice. (F) Analysis of the data with Time collapsed across post-injection timepoints illustrates that the 30 mg/kg/day dose of pioglitazone decreased food consumption in females. Pioglitazone significantly increased body weight in (G) males but not (H) females. (I) Pioglitazone 30 mg/kg/d increased body weight (change from 13 to 20 weeks of age) to a greater extent in males as compared to females. Pioglitazone decreased non-fasting glucose levels in (J) males but not females (K). (L) Pioglitazone 30 mg/kg/d increased HbA1c (change from 13 to 20 weeks of age) in males but not females. 30 mg/kg/d produced greater increases in males. Two-way ANOVA was performed followed by Sidak’s multiple comparisons test for figures 9A–B, 9D–E, G–H and J–k. Tukey’s multiple

comparisons test was performed for figure 9C, F, I and L. * $p < 0.05$, ** $p < 0.01$. Values = mean \pm SEM. N=5–6.

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Table 1.

Two-Way ANOVA

Figure	Sex	Assay	Two-way ANOVA			
			Line #	Factors	F	P value
1A	Male	vF	1	Time × Dose	(20, 175) = 8.035	<0.0001
			2	Time	(3, 111) = 153.0	<0.0001
			3	Dose	(4, 35) = 107.4	<0.0001
1B	Female	vF	4	Time × Dose	(20, 170) = 5.054	<0.0001
			5	Time	(4, 137) = 14.56	<0.0001
			6	Dose	(4, 34) = 30.99	<0.0001
1D	Male vs Female	vF	7	Dose × Sex	(4, 68) = 39.70	<0.0001
			8	Dose	(4, 68) = 129.6	<0.0001
			9	Sex	(1, 68) = 198.3	<0.0001
1E	Male	HP	10	Time × Dose	(12, 87) = 4.785	<0.0001
			11	Time	(2, 71) = 154.9	<0.0001
			12	Dose	(4, 29) = 7.088	0.0004
1F	Female	HP	13	Time × Dose	(12, 102) = 4.579	<0.0001
			14	Time	(2, 79) = 33.11	<0.0001
			15	Dose	(4, 34) = 18.84	<0.0001
1H	Male vs Female	HP	16	Dose × Sex	(4, 62) = 11.76	<0.0001
			17	Dose	(4, 62) = 18.31	<0.0001
			18	Sex	(1, 62) = 74.83	<0.0001
2A	Male	vF	19	Time × Drug	(5, 90) = 3.137	P=0.0118
			20	Time	(3, 62) = 1.276	0.29
			21	Drug	(1, 18) = 8.300	0.0099
2B	Female	vF	22	Time × Drug	(5, 90) = 3.491	0.0063
			23	Time	(2, 44) = 4.177	0.0158
			24	Drug	(1, 18) = 35.83	<0.0001
2C	Male vs Female	vF	25	Drug × Sex	(1, 18) = 6.431	0.0207
			26	Drug	(1, 18) = 53.06	<0.0001
			27	Sex	(1, 18) = 1.410	0.2505
3A	Male	vF	28	Time × Dose	(6, 78) = 1.584	0.163
			29	Time	(2, 38) = 2.513	0.073
			30	Dose	(1, 13) = 4.781	0.0477
3B	Female	vF	31	Time × Dose	(6, 102) = 7.490	<0.0001
			32	Time	(2, 43) = 10.87	<0.0001
			33	Dose	(1, 17) = 28.00	<0.0001

Figure	Sex	Assay	Two-way ANOVA			
			Line #	Factors	F	P value
3C	Male vs Female	vF	34	Dose × Sex	(1, 30) = 7.854	0.0088
			35	Dose	(1, 30) = 33.18	<0.0001
			36	Sex	(1, 30) = 5.184	0.0301
3D	Male	Act	37	Time × Dose	(6, 72) = 0.4550	0.8392
			38	Time	(3, 36) = 0.9631	0.4214
			39	Dose	(1, 12) = 2.480	0.1413
3E	Female	Act	40	Time × Dose	(6, 96) = 2.553	0.0245
			41	Time	(3, 54) = 4.441	0.0054
			42	Dose	(1, 16) = 47.70	<0.0001
3F	Male vs Female	Act	43	Dose × Sex	(1, 28) = 7.723	0.0096
			44	Dose	(1, 28) = 35.91	<0.0001
			45	Sex	(1, 28) = 11.23	0.0023
4A	Male	vF	46	Time × Drug	(10, 70) = 0.4995	0.8847
			47	Time	(2, 31) = 1.181	0.3246
			48	Drug	(2, 14) = 1.331	0.2956
4B	Female	vF	49	Time × Drug	(10, 95) = 1.212	0.2936
			50	Time	(3, 63) = 4.286	0.0063
			51	Drug	(2, 19) = 4.712	0.0218
4C	Male vs Female	vF	52	Sex × Drug	(2, 33) = 2.495	0.0979
			53	Drug	(2, 33) = 1.286	0.2898
			54	Sex	(1, 33) = 4.205	0.0483
4D	Male	Act	55	Time × Drug	(10, 60) = 0.6296	0.7827
			56	Time	(2, 31) = 2.162	0.1186
			57	Drug	(2, 12) = 1.014	0.3919
4E	Female	Act	58	Time × Drug	(10, 90) = 1.247	0.273
			59	Time	(3, 61) = 2.143	0.0966
			60	Drug	(2, 18) = 2.662	0.0971
4F	Male vs Female	Act	61	Sex × Drug	(2, 30) = 2.418	0.1062
			62	Drug	(2, 30) = 3.854	0.0324
			63	Sex	(1, 30) = 3.383	0.0758
6A	Male	HP	64	Strain × Drug	(3, 28) = 3.545	P=0.0271
			65	Drug	(3, 28) = 1.502	P=0.2358
			66	Strain	(1, 28) = 38.03	P<0.0001
6B	Male	CP	67	Strain × Drug	(3, 28) = 9.185	P=0.0002
			68	Drug	(3, 28) = 13.28	P<0.0001
			69	Strain	(1, 28) = 45.09	P<0.0001

Figure	Sex	Assay	Two-way ANOVA			
			Line #	Factors	F	P value
6C	Male	Glucose	70	Strain × Drug	(3, 32) = 0.5121	P=0.6768
			71	Drug	(3, 32) = 1.046	P=0.3856
			72	Strain	(1, 32) = 1.417	P=0.2426
6F	Male vs Female	HP	73	Sex × Drug	(1, 40) = 2.695	0.1085
			74	Drug	(1, 40) = 59.53	<0.0001
			75	Sex	(1, 40) = 1.096	0.3015
6I	Male vs Female	PP	76	Sex × Drug	(1, 40) = 2.946	0.0938
			77	Drug	(1, 40) = 40.99	<0.0001
			78	Sex	(1, 40) = 0.3639	0.5497
8C	Male vs Female	HP	79	Sex × Drug	(1, 36) = 4.876	0.0337
			80	Drug	(1, 36) = 56.38	<0.0001
			81	Sex	(1, 36) = 1.812	0.1867
9A	Male	HP	82	Time × Dose	(15, 95) = 0.9696	0.4928
			83	Time	(3, 71) = 5.808	0.0006
			84	Dose	(3, 19) = 0.7648	0.5278
9B	Female	HP	85	Time × Dose	(15, 90) = 1.232	0.2633
			86	Time	(3, 68) = 5.721	0.0006
			87	Dose	(3, 18) = 1.805	0.1824
9C	Male vs Female	HP	88	Dose × Sex	(3, 38) = 1.329	0.2793
			89	Dose	(3, 38) = 0.5167	0.6733
			90	Sex	(1, 38) = 0.1684	0.6839
9D	Male	Food intake	91	Time × Dose	(12, 32) = 3.299	0.0034
			92	Time	(1, 12) = 2.826	0.1042
			93	Dose	(3, 8) = 0.5317	0.6732
9E	Female	Food intake	94	Time × Dose	(12, 40) = 7.158	<0.0001
			95	Time	(1, 12) = 5.565	0.0313
			96	Dose	(3, 10) = 1.177	0.3667
9F	Male vs Female	Food intake	97	Dose × Sex	(3, 24) = 0.9784	0.4194
			98	Dose	(3, 24) = 6.669	0.002
			99	Sex	(1, 24) = 13.65	0.0011
9G	Male	Weight	100	Time × Dose	(18, 114) = 28.95	<0.0001
			101	Time	(1, 32) = 13.10	0.0001
			102	Dose	(3, 19) = 2.476	0.0926
9H	Female	Weight	103	Time × Dose	(18, 108) = 2.660	0.001
			104	Time	(1, 23) = 2.000	0.1685

Figure	Sex	Assay	Two-way ANOVA			
			Line #	Factors	F	P value
			105	Dose	(3, 18) = 0.7926	0.5138
9I	Male vs Female	Weight	106	Dose × Sex	(3, 40) = 4.008	0.0138
			107	Dose	(3, 40) = 26.60	<0.0001
			108	Sex	(1, 40) = 2.142	0.1512
9J	Male	Glucose	109	Time × Dose	(18, 114) = 5.692	<0.0001
			110	Time	(4, 80) = 13.36	<0.0001
			111	Dose	(3, 19) = 6.770	0.0027
9K	Female	Glucose	112	Time × Dose	(18, 108) = 2.754	0.0006
			113	Time	(3, 68) = 4.003	0.0065
			114	Dose	(3, 18) = 1.110	0.3708
9L	Male vs Female	Glucose	115	Dose × Sex	(3, 40) = 0.7509	0.5283
			116	Dose	(3, 40) = 8.885	0.0001
			117	Sex	(1, 40) = 0.6510	0.4245

vF: von Frey; HP: Hotplate; Act: Acetone testing; PP: Paw pressure

Table 2.

Three-Way ANOVA

Figure	Sex	Assay	Line #	Factors	F	P value
5A	Male vs Female	Body mass	1	Time	(5, 168) = 150.6	<0.0001
			2	Sex	(1, 168) = 192.5	<0.0001
			3	Strain	(1, 168) = 480.0	<0.0001
			4	Time × Sex	(5, 168) = 3.609	0.004
			5	Time × Strain	(5, 168) = 1.257	0.2848
			6	Sex × Strain	(1, 168) = 63.98	<0.0001
			7	Time × Sex × Strain	(5, 168) = 1.413	0.2221
5B	Male vs Female	Blood Glucose	8	Time	(5, 134) = 18.54	<0.0001
			9	Sex	(1, 134) = 0.8059	0.371
			10	Strain	(1, 134) = 692.4	<0.0001
			11	Time × Sex	(5, 134) = 0.8510	0.516
			12	Time × Strain	(5, 134) = 27.32	<0.0001
			13	Sex × Strain	(1, 134) = 0.6879	0.4083
			14	Time × Sex × Strain	(5, 134) = 0.9537	0.4487
5C	Male vs Female	HP	15	Time	(5, 140) = 12.81	<0.0001
			16	Sex	(1, 28) = 0.1101	0.7425
			17	Strain	(1, 28) = 214.0	<0.0001
			18	Time × Sex	(5, 140) = 0.6481	0.6634
			19	Time × Strain	(5, 140) = 3.431	0.0059
			20	Sex × Strain	(1, 28) = 6.489	0.0166
			21	Time × Sex × Strain	(5, 140) = 1.671	0.1454
5D	Male vs Female	PP	22	Time	(5, 168) = 2.939	0.0143
			23	Sex	(1, 168) = 0.3942	0.5309
			24	Strain	(1, 168) = 41.49	<0.0001
			25	Time × Sex	(5, 168) = 0.2074	0.959
			26	Time × Strain	(5, 168) = 4.309	0.001
			27	Sex × Strain	(1, 168) = 0.3558	0.5517
			28	Time × Sex × Strain	(5, 168) = 0.7538	0.5844
6D	Male	HP	29	Time	(2, 90) = 2.437	0.0932
			30	Strain	(1, 45) = 79.43	<0.0001
			31	Drug	(1, 45) = 17.50	0.0001
			32	Time × Strain	(2, 90) = 0.8989	0.4106
			33	Time × Drug	(2, 90) = 6.504	0.0023
			34	Strain × Drug	(1, 45) = 1.735	0.1944
			35	Time × Strain × Drug	(2, 90) = 0.3000	0.7415
			36	Time	(2, 82) = 2.594	0.0809
			37	Strain	(1, 41) = 108.2	<0.0001
			38	Drug	(1, 41) = 15.24	0.0003

Figure	Sex	Assay	Line #	Factors	F	P value
6E	Female	HP	39	Time × Strain	(2, 82) = 3.787	0.0267
			40	Time × Drug	(2, 82) = 7.700	0.0009
			41	Strain × Drug	(1, 41) = 4.647	0.037
			42	Time × Strain × Drug	(2, 82) = 0.4271	0.6538
6G	Male	PP	43	Time	(2, 86) = 5.997	0.0036
			44	Strain	(1, 43) = 268.9	<0.0001
			45	Drug	(1, 43) = 12.69	0.0009
			46	Time × Strain	(2, 86) = 2.513	0.087
			47	Time × Drug	(2, 86) = 2.950	0.0576
			48	Strain × Drug	(1, 43) = 2.641	0.1114
			49	Time × Strain × Drug	(2, 86) = 0.6298	0.5351
6H	Female	PP	50	Time	(2, 82) = 0.08181	0.9215
			51	Strain	(1, 41) = 82.62	<0.0001
			52	Drug	(1, 41) = 8.638	0.0054
			53	Time × Strain	(2, 82) = 3.253	0.0437
			54	Time × Drug	(2, 82) = 1.064	0.3496
			55	Strain × Drug	(1, 41) = 2.831	0.1001
			56	Time × Strain × Drug	(2, 82) = 7.442	0.0011
7A	Male vs Female	Weight	57	Time	(2, 52) = 24.79	<0.0001
			58	Strain	(1, 26) = 206.3	<0.0001
			59	Sex	(1, 26) = 10.81	0.0029
			60	Time × Strain	(2, 52) = 3.369	0.0421
			61	Time × Sex	(2, 52) = 1.008	0.3719
			62	Strain × Sex	(1, 26) = 0.05295	0.8198
			63	Time × Strain × Sex	(2, 52) = 1.790	0.1771
7B	Male vs Female	HbAlc	64	Time	(1, 50) = 233.8	<0.0001
			65	Strain	(1, 27) = 393.6	<0.0001
			66	Sex	(1, 27) = 0.1071	0.746
			67	Time × Strain	(2, 54) = 168.3	<0.0001
			68	Time × Sex	(2, 54) = 1.468	0.2394
			69	Strain × Sex	(1, 27) = 0.02360	0.879
			70	Time × Strain × Sex	(2, 54) = 0.006281	0.9937
7C	Male vs Female	HP	71	Time	(1, 50) = 60.23	<0.0001
			72	Strain	(1, 26) = 20.03	0.0001
			73	Sex	(1, 26) = 0.007916	0.9298
			74	Time × Strain	(2, 52) = 5.446	0.0071
			75	Time × Sex	(2, 52) = 0.2999	0.7422
			76	Strain × Sex	(1, 26) = 2.385	0.1346
			77	Time × Strain × Sex	(2, 52) = 0.08583	0.9179
			78	Time	(2, 82) = 1.276	0.2877

Figure	Sex	Assay	Line #	Factors	F	P value
8A	Male	HP	79	Strain	(1, 32) = 565.8	<0.0001
			80	Drug	(1, 32) = 17.42	0.0002
			81	Time × Strain	(3, 96) = 5.855	0.001
			82	Time × Drug	(3, 96) = 2.172	0.0963
			83	Strain × Drug	(1, 32) = 23.10	<0.0001
			84	Time × Strain × Drug	(3, 96) = 0.1530	0.9275
8B	Female	HP	85	Time	(2, 77) = 16.24	<0.0001
			86	Strain	(1, 32) = 174.4	<0.0001
			87	Drug	(1, 32) = 8.973	0.0053
			88	Time × Strain	(3, 96) = 9.445	<0.0001
			89	Time × Drug	(3, 96) = 7.931	<0.0001
			90	Strain × Drug	(1, 32) = 9.866	0.0036
			91	Time × Strain × Dru	(3, 96) = 8.165	<0.0001

HP: Hotplate; PP: Paw pressure