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Topical delivery of muscarinic receptor antagonists prevents and reverses peripheral neuropathy in female diabetic mice

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Abbreviations:

AMPK: AMP-activated protein kinase

IENF: Intra-epidermal nerve fiber

MNCV: Motor nerve conduction velocity

MR: Muscarinic Receptor

MT7: Muscarinic toxin 7

PWT: Paw withdrawal threshold

STZ: Streptozotocin

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ABSTRACT

Muscarinic antagonists promote sensory neurite outgrowth in vitro and prevent and/or reverse multiple indices of peripheral neuropathy in rodent models of diabetes, chemotherapy-induced peripheral neuropathy and HIV protein-induced neuropathy when delivered systemically. We measured plasma concentrations of the M_1 receptor selective muscarinic antagonist pirenzepine when delivered by sub-cutaneous injection, oral gavage or topical application to the skin and investigated efficacy of topically delivered pirenzepine against indices of peripheral neuropathy in diabetic mice. Topical application of 2% pirenzepine to the paw resulted in plasma concentrations 6hr post-delivery that approximated those previously shown to promote neurite outgrowth in vitro. Topical delivery of pirenzepine to the paw of streptozotocin-diabetic mice dose-dependently (0.1-10.0%) prevented tactile allodynia, thermal hypoalgesia and loss of epidermal nerve fibers in the treated paw and attenuated large fiber motor nerve conduction slowing in the ipsilateral limb. Efficacy against some indices of neuropathy was also noted in the contralateral limb, indicating systemic effects following local treatment. Topical pirenzepine also reversed established paw heat hypoalgesia while withdrawal of treatment resulted in a gradual decline in efficacy over 2-4 weeks. Efficacy of topical pirenzepine was muted when treatment was reduced from 5 to 3 or 1 days per week. Similar local effects were noted with the non-selective muscarinic receptor antagonist atropine when applied either to the paw or to the eye. Topical delivery of muscarinic antagonists may serve as a practical therapeutic approach to treating diabetic and other peripheral neuropathies.

SIGNIFICANCE STATEMENT: Muscarinic antagonist pirenzepine alleviates diabetic peripheral neuropathy when applied topically in mice.

KEYWORDS: peripheral neuropathy, diabetic neuropathy, muscarinic antagonist, pirenzepine, atropine, M_1 receptor, corneal confocal microscopy, streptozotocin-diabetes

INTRODUCTION

Muscarinic acetylcholine receptor antagonists have been in clinical use for decades to treat such diverse conditions as Parkinson's disease, peptic ulcers, overactive bladder, chronic obstructive pulmonary disease, asthma and motion sickness, with specific drug use depending on muscarinic receptor sub-type selectivity and tissue accessibility (Kruse et al., 2014). Despite the widely studied and pharmacologically manipulated role of muscarinic receptors in modulating synaptic function in the central (Erskine et al., 2019; Moran et al., 2019) and autonomic (Giglio and Tobin, 2009; Tobin et al., 2009) nervous systems, it has only recently become apparent that peripheral sensory neurons maintained in vitro after extraction from adult rodents experience tonic cholinergic suppression of maximal mitochondrial respiratory capacity and neurite outgrowth (Calcutt et al., 2017). Inhibition of the M_1 sub-type of the muscarinic receptor (M_1R), which is expressed by adult sensory neurons, is critical to these effects as neurite outgrowth from sensory neuron cultures derived from adult rodents is increased by M_1R knockout and by diverse M_1R specific and selective antagonists. In contrast, muscarinic receptor antagonists selective for the $M_{2\text{-}4}R$ sub-types do not promote neurite outgrowth while the muscarinic receptor agonist muscarine suppressed neurite outgrowth (Calcutt et al., 2017).

The therapeutic implications of cholinergic constraint of sensory neurons include the potential for muscarinic antagonists to promote nerve regeneration after injury and to enhance mitochondrial activity in neurons under metabolic stress. It is becoming

increasingly apparent that a number of peripheral neuropathies, including those associated with diabetes (Fernyhough, 2015; Chandrasekaran et al., 2019), chemotherapy (Trecarichi and Flatters, 2019) and HIV infection (Roda and Hoke, 2019) share a common pathogenesis centered on impaired mitochondrial activity. In a recent study, mice lacking the M_1R were protected from diabetes-induced neuropathy while subcutaneous injection of selective M_1R antagonists prevented and reversed multiple functional and structural indices of peripheral neuropathy in rodent models of diabetes, chemotherapy-induced neuropathy and neuropathy caused by HIV-associated proteins (Calcutt et al., 2017). Efficacy of the M_1R selective antagonists pirenzepine and VU0255035 was associated with activation of AMP-activated protein kinase (AMPK), augmentation of mitochondrial Complex activity and elevated expression of components of the respiratory electron transport chain (Calcutt et al., 2017). These studies highlight the therapeutic potential of M_1R selective muscarinic antagonists against peripheral neuropathy.

A major concern regarding clinical use of anti-muscarinic drugs is the potential for unwanted side effects due to the widespread distribution of muscarinic receptors and lack of receptor sub-type specificity. Poor antibody specificity has hampered definitive distribution studies for M_1R (Jositsch et al., 2009) but studies localizing M_1R mRNA or identifying physiological consequences of M_1R knockout have offered clues. Other than being expressed by neurons and Schwann cells of the peripheral nervous system (PNS) (Tata et al., 2000a; Tata et al., 2000b; Loreti et al., 2006; Calcutt et al., 2017), M_1R are also found in the alimentary tract and associated enteric nervous system and exocrine

pancreas (Gautam et al., 2005; Tobin et al., 2009; Harrington et al., 2010), the cardiovascular system (Saternos et al., 2018) and epidermis (Ndoye et al., 1998; Kurzen et al., 2004). Moreover, the only known M_1R specific antagonist is muscarinic toxin 7 (MT7: (Krajewski et al., 2001; Servent et al., 2011), a peptide that is not currently a viable drug candidate. Pirenzepine may serve as alternative candidate for manipulating the cholinergic constraint of peripheral sensory nerves in vivo, as it is M_1R selective relative to the $M_{2\text{-}5}$R subtypes (Eglen et al., 2001) and does not readily cross the blood brain barrier, reducing the potential for disruption of central nervous system (CNS) function compared to other muscarinic antagonists (Jaup and Blomstrand, 1980; Sethy and Francis, 1990). Pirenzepine was originally developed as an orally-delivered drug to treat ulcers, acting locally in the stomach to reduce gastric acid secretion while having weak systemic side effects (Carmine and Brogden, 1985). Other approaches to reducing systemic side effects of anti-muscarinics have included use of topical delivery (Sand, 2009). In the present study, we have extended studies of the therapeutic potential of muscarinic antagonists against diabetic neuropathy to address the viability of delivery by topical application to the skin or eye to treat multiple indices of peripheral neuropathy in a mouse model of type 1 diabetes.

MATERIALS AND METHODS

Animals

All procedures on live animals were approved by the local IACUC. Studies were performed in adult female C57 Bl/6J (#000664, Jackson Laboratories, USA) or female Swiss Webster (#024, Charles River, USA) mice maintained 3-5/cage on TEK-Fresh bedding (#7099, Envigo, USA) under a 12 hr light:dark cycle with free access to water and food (5001 diet, Purina, USA). Only female mice were used to keep group variability to a minimum. We have previously reported that muscarinic antagonists are effective against indices of neuropathy in both male and female diabetic rodents (Calcutt et al. 2017). Insulin-deficient diabetes was induced by intraperitoneal injection of 90 (C57Bl/6J mice) or 100 mg/kg (Swiss Webster mice) streptozotocin (STZ: Sigma, USA) in sterile 0.9% saline on two consecutive days, with each injection following a 12hr fast. Peripheral neuropathy in this model is not the result of direct STZ-induced neurotoxicity (Davidson et al., 2009). Onset of hyperglycemia was confirmed 4-5 days after the second STZ injection and also at study end in blood obtained by tail prick from restrained but conscious animals using a strip operated glucose meter (OneTouch UltraMini, Lifescan Inc., USA). Only mice with blood glucose concentrations above 270 mg/dl at both study onset and study end were included in data analysis. Deaths during the study period and animals that reverted to normoglycemia before study end are reported in **Table 1**.

Treatment

Pirenzepine dihydrochloride or atropine (Sigma, USA) were prepared for topical application to the paw by mixing into a volume of sterile hydrogel (Intrasite #66027313, Smith & Nephew, USA) immediately before application. 50µl of pirenzepine/hydrogel was applied to the plantar surface of the hind paw of manually restrained conscious mice and the paw then enclosed in a plastic chamber that prevented the animal from ingesting or removing the drug preparation while allowing it to move freely within its cage. Vehicle treated animals received hydrogel alone. The chamber was left in place for 20 minutes per day, 5 days per week, unless otherwise noted, after which it was removed and the drug preparation cleared with a tissue. For delivery to the eye, atropine was dissolved in ophthalmic gel (Genteal, Novartis, USA) and 20µl applied to the eye of a conscious, manually restrained, mouse. The gel was left in place for 15 seconds before the animal was released. Vehicle treated animals and/or the contralateral eye received ophthalmic gel alone. Plasma pirenzepine concentrations were measured by HPLC-MS with a lower detection limit of 2nM (Drumetrix Laboratories, Greensboro, North Carolina) following delivery of 10mg/kg pirenzepine by oral gavage or ip injection to match doses previously effective against indices of neuropathy in diabetic rodents (Calcutt et al. 2017) or topical application of 2% gel based on unpublished preliminary data.

Electrophysiology

Conduction velocity of large myelinated motor fibers was measured in the sciatic nerve as described in detail elsewhere (Jolivalt et al., 2016). Briefly, mice were anesthetized with isoflurane, core and nerve temperature held at 37^{o}C using heating pads and lamps

and fine needle stimulating electrodes placed at the sciatic notch and Achilles tendon. Recording electrodes were inserted into the interosseus muscles of the ipsilateral paw. The sciatic nerve was stimulated using a PowerLab 4/30 to achieve maximal M wave amplitude (0.2-1.0 mV, 0.05 ms square waves) and the resulting electromyogram stored to a computer running LabChart Pro (AD Instruments, USA). Motor nerve conduction velocity (MNCV) was calculated as the distance between stimulation sites divided by the latency of M wave peaks produced by stimulation at the two sites. The median of 3 separate measurements was used to represent MNCV for each animal.

Response to pressure and heat stimuli

Hind limb withdrawal reaction to light touch and escalating heat stimuli were measured in conscious, unrestrained mice, as described in detail elsewhere (Jolivalt et al., 2016). Briefly, mice were allowed to acclimate to an observation chamber for 30 minutes prior to testing. Paw withdrawal to pressure applied at the plantar surface was measured using von Frey filaments applied sequentially in an up-down protocol as originally described for rats (Chaplan et al., 1994) and subsequently modified for use in mice (Jolivalt et al., 2016). Paw response latency to surface heat escalating at a rate of 1^{o}C/second from a starting surface temperature of 30^{o}C was measured using a Hargreaves apparatus. Triplicate measurements were made at 5-minute intervals and the median used to represent 50% paw withdrawal threshold (50% PWT in g) and paw heat response latency (seconds).

Nerve density in the cornea and paw

The density of sensory nerves in the corneal sub-basal nerve plexus of anesthetized and restrained mice was measured by corneal confocal microscopy, as described in detail elsewhere (Jolivalt et al., 2016). Five sequential images of the corneal sub-basal nerve plexus were obtained from isoflurane-anesthetized mice using a corneal confocal microscope (HRT3 with Rostock corneal module, Heidelberg Engineering Inc. Germany). Nerve occupancy was measured using an 8x8 grid overlaid on the images (Chen et al., 2013). Density of sensory nerve profiles in the epidermis of plantar hind paw skin was measured as described in detail elsewhere (Jolivalt et al., 2016). Briefly, plantar paw skin was removed at autopsy, immersion fixed in 4% paraformaldehyde, dehydrated in ethanol and embedded in paraffin prior to cutting 6µm sections. Intra-epidermal nerve fibers (IENF) were identified by immunostaining with anti-PGP9.5 antibody (#7863-0504, AbD Serotec, UK) and viewed by light microscopy. Nerve profiles present in the epidermis were quantified relative to length of dermal:epidermal border examined.

Analysis

All measurements were made on coded animals by observers who were unaware of the study group or treatment regime. Data are shown as group mean ± SD. Statistical comparison of experimental groups against the control+vehicle group and against the STZ+vehicle group at a single, pre-defined, time point was made by one-way ANOVA followed by Sidak's *post hoc* test. Statistical comparisons of groups across time were made by two-way repeat measures ANOVA with between group-differences identified by Dunnett's *post-hoc* test.

RESULTS

Adult female C57 Bl/6J mice received a single dose of pirenzepine dihydrochloride (Sigma, USA) by sub-cutaneous injection (10mg/kg), oral gavage (10mg/kg) or via topical application to the right hind paw (50 µl of 2% in hydrogel for 20 minutes). Mice were exsanguinated at 1, 3, 6 or 24 hours after drug delivery and plasma stored at -70^{o}C until assay of pirenzepine. The highest plasma concentrations were achieved following subcutaneous delivery of pirenzepine ($AUC_{0\text{-}t}$ = 4052 hr*nM, C_{max} = 2600 nM, T_{max} = 1 hr), followed by oral administration ($AUC_{o\text{-}t}$ = 899 hr*nM, C_{max} = 137 nM, T_{max} = 1 hr) and then topical administration ($AUC_{0\text{-}t}$ = 136 hr*nM, C_{max} = 15 nM, T_{max} = 6 hr: **Figure 1**).

To determine whether pirenzepine applied topically to the hind paw could prevent onset of indices of neuropathy in a rodent model of diabetes, adult female C57 Bl/6J mice were made diabetic with STZ and, upon confirmation of hyperglycemia, treated with vehicle or pirenzepine applied to the right hind paw (0.2, 1.0 or 2.0% in 50µl hydrogel, 20 minutes/day x 5 days/week) for 8 weeks. The left paw was untreated. Pirenzepine treatment did not prevent weight loss or hyperglycemia in diabetic mice while the significantly elevated blood glucose in diabetic mice treated with high doses of pirenzepine compared to vehicle-treated diabetic mice was not seen in later studies (**Table 1**). At study end, paw thermal hypoalgesia in STZ diabetic mice was significantly prevented by treatment with 1.0% or 2.0% pirenzepine, but not by 0.2% pirenzepine (**Figure 2A).** A trend towards loss of IENF in paw skin was also prevented by 2%

pirenzepine, but not by the 0.2% or 1.0% dose formulations (**Figure 2B**). MNCV slowing (trend to decrease, **Figure 2C**) and paw tactile allodynia ($p<0.01$, **Figure 2D**) in STZ-diabetic mice were not significantly protected at any dose of pirenzepine.

To establish the duration that efficacy persists after withdrawal of treatment and whether topical pirenzepine could reverse established neuropathy, adult female C57 Bl/6J mice were made diabetic with STZ and, upon confirmation of hyperglycemia, were treated with 2.0% pirenzepine or vehicle applied to the right hind paw, 20 minutes/day x 5 days/week. The left paw was untreated. Paw response latency to heating was selected as the index of treatment efficacy (**Figure 2A**) because it is amenable to repeated testing over time. Pirenzepine treatment of diabetic mice had no effect on body weight or blood glucose levels in either control or diabetic mice (**Table 1**). After 8 weeks of treatment, paw heat hypoalgesia present in vehicle-treated diabetic mice was prevented in diabetic mice treated with pirenzepine (**Figure 3A**). Pirenzepine treatment was then withdrawn and replaced with treatment by vehicle. Serial testing of paw heat response latency demonstrated a slowly progressing loss of heat sensation that stabilized by week 16 of diabetes (**Figure 3A**). At this point, diabetic mice that had received only vehicle were transferred to topical treatment with 2.0% pirenzepine to the paw (20 minutes/day x 5 days/week). Paw heat hypoalgesia was completely reversed after 8 weeks of treatment (**Figure 3A**). In a separate study to establish optimal dose frequency, adult female Swiss Webster mice were made diabetic with STZ and upon confirmation of hyperglycemia were treated with 50 µl of 2.0% pirenzepine or vehicle applied to the right hind paw, 20 minutes/day for either 1, 3 or 5 days/week for 10

weeks. The left paw was untreated. Pirenzepine treatment of diabetic mice had no effect on body weight or hyperglycemia compared to vehicle-treated diabetic mice (**Table 1**). Paw heat hypoalgesia present in vehicle-treated mice was prevented in a dose frequency-dependent manner, with dosing 5 days/week completely normalizing paw heat response latency (**Figure 3B**), confirming data obtained with C57 Bl/6J mice (**Figure 2A**).

To determine whether higher doses of pirenzepine impacted indices of neuropathy not responsive to 2.0% topical pirenzepine (**Figure 2**) and were capable of having systemic effects, adult female STZ-diabetic C57 Bl/6J mice were treated from onset of hyperglycemia with vehicle or pirenzepine applied to the right hind paw (2.0 or 10.0% in 50µl hydrogel, 20 minutes/day x 5 days/week for 8 weeks). The left paw was treated with vehicle. Pirenzepine treatment of diabetic mice had no effect on body weight or hyperglycemia compared to vehicle-treated diabetic mice (**Table 1**). At study end, vehicle-treated diabetic mice showed bilateral MNCV slowing (**Figure 4A**), paw allodynia to von Frey filaments (**Figure 4B**), paw thermal hypoalgesia (**Figure 4C**) and loss of sensory nerves in paw skin (**Figure 4D**). Topical pirenzepine (2.0%) prevented paw tactile allodynia, heat hypoalgesia and loss of IENF in the treated paw but had no impact on MNCV slowing in the ipsilateral limb. Similar efficacy was noted in the vehicle-treated contralateral limb. Increasing pirenzepine dose to 10% prevented all measured indices of neuropathy, including MNCV slowing, in both treated and untreated contralateral limbs.

To determine whether efficacy of pirenzepine extended to another anti-muscarinic agent and whether local and systemic efficacy could be achieved when delivery was topical to the eye, adult female Swiss Webster mice were treated from onset of hyperglycemia with atropine applied to either the right hind paw (2.0% in 50µl hydrogel, 20 minutes/day x 5 days/week for 8 weeks) or the right eye (2.0% in ophthalmic gel x 5 days/week for 8 weeks). Vehicle was applied to the contralateral eye or paw and also to groups of control and STZ-diabetic mice. Atropine treatment of diabetic mice by either route had no effect on body weight or hyperglycemia compared to vehicle-treated diabetic mice (**Table 1**). Diabetes induced significant MNCV slowing (**Figure 5A**), tactile allodynia (**Figure 5B**), heat hypoalgesia (**Figure 5C**), significantly reduced IENF profile density (**Figure 5D**) and corneal sub-basal nerve plexus density (**Figure 5E**). Atropine delivered to the paw prevented MNCV slowing, heat hypoalgesia and loss of IENF in the ipsilateral limb, but not in the contralateral, vehicle-treated limb and had no effect on paw tactile allodynia or corneal nerve density in either limb or eye. Atropine delivered to the eye prevented loss of corneal sub-basal nerve plexus density in the treated eye, but not the contralateral, vehicle-treated eye. Occular delivery of atropine also significantly prevented or attenuated paw heat hypoalgesia in both hind paws, but not MNCV slowing, tactile allodynia or loss of IENF.

DISCUSSION

Clinical use of muscarinic antagonists is associated with side effects due to the widespread distribution of MR subtypes across organ systems, lack of receptor subtype

specificity of antagonists and, in some cases, production of toxic metabolites. Strategies to mitigate side effects of anti-muscarinics include development of receptor sub-type specific (or highly selective) compounds that have limited distribution and tissue penetration profiles. For example, oral delivery of the hydrophilic M_1R selective antagonist pirenzepine was developed to allow local delivery to the alimentary tract for treatment of ulcers (Carmine and Brogden, 1985; Stockbrugger, 1988). Similarly, the development history of oxybutynin to treat overactive bladder shows progression from oral to topical transdermal gel and patch formulations to avoid first pass metabolism in the upper GI tract and liver, thereby reducing production of metabolites associated with side effects while maintaining systemic therapeutic levels of the parent compound (Sand, 2009). Our discovery studies that demonstrated the therapeutic potential of muscarinic antagonists in rodent models of peripheral neuropathy focused on subcutaneous delivery (Calcutt et al., 2017) to obtain comprehensive systemic exposure of pirenzepine, a M_1R selective muscarinic antagonist with low CNS penetrance (Jaup and Blomstrand, 1980; Sethy and Francis, 1990). We have extended these discovery studies by investigating the efficacy of topically delivered pirenzepine against multiple indices of neuropathy in a mouse model of diabetes. There is precedence for the clinical viability of this approach as topical delivery of antimuscarinics is currently used to treat overactive bladder (oxybutynin) and hyperhidrosis (glycopyrrolate).

Topical delivery of pirenzepine to the paw dose-dependently prevented multiple indices of peripheral neuropathy in the ipsilateral limb of diabetic mice. Assays were performed 24hr after the last delivery of pirenzepine, a time when drug has cleared from

circulation, suggesting long-acting effects. This is consistent with recent studies demonstrating that M_1R antagonists activate an AMPK-dependent energy sensing signaling cascade driving mitochondrial function in sensory neurons and modulate microtubule polymerization in these cells (Calcutt et al., 2017; Sabbir et al., 2018; Sabbir and Fernyhough, 2018). There were some notable differences in the dose required to protect against specific indices of diabetic neuropathy. For example, paw heat hypoalgesia was prevented at topical pirenzepine doses of 1.0% or higher, whereas preventing loss of the epidermal small sensory fibers that transduce heat pain sensation required 2.0% pirenzepine or higher. This dissociates impact of pirenzepine on nerve density and function at the 1.0% dose and suggests that factors other than global IENF depletion contribute to loss of heat sensation in diabetic mice. There is precedence for this disassociation, as paw thermal hypoalgesia occurs in short-term diabetic mice in the absence of concurrent IENF loss (Beiswenger et al., 2008; Jolivalt et al., 2015). Neurochemical consequences of diabetes, such as impaired synthesis, axonal transport and release of neuropeptides associated with thermal pain transmission (Diemel et al., 1994; Fernyhough et al., 1994; Calcutt et al., 1998; Calcutt et al., 2000), may contribute to thermal hypoalgesia prior to detectable IENF depletion. It is also possible that there is impact of pirenzepine on select sub-types of IENF that is not discriminated when all IENF are quantified using the pan-neuronal marker PGP9.5 (Beiswenger et al., 2008).

Diabetic neuropathy is frequently described as a small fiber neuropathy due to the early appearance of positive and negative symptoms of small fiber dysfunction (Oaklander

and Nolano, 2019). However, indices of large myelinated fiber dysfunction, such as sensory and motor nerve conduction slowing, are also early features of human diabetic neuropathy (Bril, 2016), albeit of less immediate concern to the patient, while large fiber demyelination, axonal degeneration and loss are established pathological features (Malik, 2014). Topical pirenzepine therapy exhibited a dose-dependent capacity to prevent allodynia to von Frey filaments in STZ-diabetic mice, with no effect of doses at or below 1.0% and efficacy in both treated and untreated contralateral limbs at higher doses. The intermediate dose of 2.0% showed efficacy in only one of two replicate experiments, a finding for which we currently have no explanation. As allodynia persists in C-fiber deficient diabetic rodents (Khan et al., 2002) this disorder likely involves large sensory fibers. We have previously demonstrated that prevention of allodynia in STZ-diabetic rats by pirenzepine is not an acute phenomenon (Calcutt et al., 2017). The concept of gradual regulation of neuronal phenotype is also supported in small sensory fibers where reversal of paw thermal hypoalgesia took 4-8 weeks while withdrawal of pirenzepine precipitated onset of hypoalgesia over a similar time course. Our prior studies aligned neuroprotection by anti-muscarinics with upregulation of AMPK signaling and preservation of mitochondrial bioenergetics (Calcutt et al. 2017). M_1 antagonism also removes a constraint on microtubule polymerization and mitochondrial trafficking (Sabbir et al. 2018) and promotes a biased β-arrestin dependent ERK-CREB signal (Sabbir and Fernyhough 2018) so that both blocking growth-impeding signals and initiating other signaling cascades may contribute to neuroprotection. Whether autonomic neuropathy (Schmidt, 2014) is afforded similar protection remains to be determined.

The neuroprotective properties of pirenzepine extended to dose-dependent preservation of large fiber MNCV, albeit requiring doses higher than those for protection of tactile allodynia and thermal hypoalgesia. It is notable that MNCV was preserved only at topical doses of 10.0% pirenzepine, whereas small fiber function (heat sensation) and structure (IENF) were normalized at doses as low as 1.0-2.0%. Whether this is an intrinsic feature of the different components of the PNS or simply reflects the relative ease of access of pirenzepine to sensory neuronal terminals in the epidermis and/or cell bodies in the dorsal root ganglia versus motor nerve terminals in muscle and/or their cell bodies in the ventral spinal cord, remains to be established. Our finding that topical delivery of 10.0% pirenzepine to one hind paw was neuroprotective in the contralateral paw certainly suggests that there is a systemic component to the effects of topical pirenzepine. Topical 2.0% pirenzepine had a C_{max} of 15 nM, matching concentrations that promote increased neurite outgrowth from adult sensory neurons in vitro (Calcutt et al., 2017). This may therefore represent a target therapeutic concentration for actions on small sensory neurons, although we cannot exclude indirect actions of pirenzepine in vivo.

While M_1R specific and selective antagonists have therapeutic potential, there are a number of less selective muscarinic antagonists in current clinical use. Atropine antagonizes all 5 muscarinic receptor sub-types and topical atropine, acting via M_3R, is used as a mydriatic and cycloplegic during ophthalmic procedures. Atropine delivered to the paw of diabetic mice replicated the effects of pirenzepine, in that it prevented

MNCV slowing, heat hypoalgesia and loss of IENF in the ipsilateral limb. However, there was not complete concordance as atropine was effective against MNCV slowing at a lower dose (2.0%) than pirenzepine (10.0%), but did not prevent paw tactile allodynia at any dose. Further, the effects of 2.0% pirenzepine on indices of neuropathy in the contralateral limb were not observed when using 2.0% atropine. The extent to which these differences reflect chemical and/or pharmacological differences in the two muscarinic antagonists requires investigation. Given the widespread ophthalmic use of atropine in humans, we also investigated the consequences of delivery to the eye on both local corneal sensory nerves and more distant sensory and motor nerves. Systemic efficacy of drugs delivered to the eye has precedence, as topical delivery of insulin has systemic effects on glucose metabolism (Liu and Chiou, 1994). Loss of corneal nerve density in diabetic mice was completely prevented by atropine, but only in the treated eye. Delivery of atropine to the eye also prevented paw heat hypoalgesia and had minor effects on MNCV slowing but without effect on paw IENF density. These data reinforce the disassociation of paw thermal sensation and IENF density discussed above and also demonstrate that topical delivery of atropine to the eye can produce effects on systemic indices of neuropathy that are most sensitive to muscarinic antagonists. Measuring corneal nerve density has been championed as a biomarker for generalized small sensory fiber neuropathy (Petropoulos et al., 2019) and there is good evidence that corneal nerve density recovers in advance of other indices of neuropathy in patients cured of diabetes following pancreatic transplantation (Azmi et al., 2019). However, as topical atropine to the paw prevented multiple indices of neuropathy in the treated limb, but did not prevent corneal nerve loss, caution should be exercised when

considering using corneal nerve density as the sole arbiter of therapeutic efficacy against small fiber neuropathies.

The muscarinic antagonists pirenzepine and atropine exhibited broad-spectrum neuroprotective properties against multiple indices of peripheral neuropathy when applied to the paw of diabetic mice. Efficacy was notable in the treated limb but, for pirenzepine in particular, was also evident in the untreated contralateral limb. Discord in the therapeutic profiles of pirenzepine and atropine may be the result of their distinct chemical and pharmacological properties. These data offer a plausible therapeutic approach for the use of anti-muscarinics to treat diabetic neuropathy. The availability of topical formulations of anti-muscarinics may facilitate evaluation of the translational potential of this therapeutic approach for a condition that has no FDA-approved medication.

Authorship Contributions:

Participated in research design: Frizzi, Kotra, Fernyhough and Calcutt

Conducted experiments: Frizzi, Han, Mota and Guernsey

Performed data analysis: Kotra, Jolivalt and Calcutt

Wrote or contributed to the writing of the manuscript: Jolivalt, Fernyhough and Calcutt

Conflict of Interest

PF and LK are co-founders, shareholders and serve on the Board of Directors of Winsantor Inc. NAC is a co-founder and shareholder in Winsantor Inc. KF is an employee of Winsantor Inc.

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Footnotes

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Legends for Figures

FIGURE 1: Topical pirenzepine enters systemic circulation. Plasma pirenzepine concentration in mice following delivery of 10mg/kg drug by sub-cutaneous injection or oral gavage versus exposure of 1 hind paw to a 2% solution of drug in hydrogel for 20 minutes. Data are mean±SD of N=3 mice per delivery route and per time point.

FIGURE 2: Topical pirenzepine dose-dependently prevents small fiber neuropathy in diabetic mice. Paw heat response latency (A), IENF profile density (B), motor nerve conduction velocity (C) and paw response to von Frey filaments (D) in control and STZ-diabetic mice treated with vehicle or 0.2% - 2.0% pirenzepine in hydrogel applied to the hind paw for 20/min/day, 5 days/week for 12 weeks. Data are group mean ± SD of N=9-13/group. *p<0.05, **p<0.01 and ***p<0.001 vs control+ vehicle, #p<0.05, ###p<0.001 vs STZ+vehicle by one way ANOVA with Sidak's post-hoc test.

FIGURE 3: Effects of topical pirenzepine dose regime on paw heat hypoalgesia. **(A):** Paw heat response latency in control+vehicle (black line), control+2% pirenzepine (gold line) and STZ-diabetic mice ± 2.0% pirenzepine (PZ) either applied topically to the hind paw for 20/min/day, 5 days/week for 8 weeks from onset of hyperglycemia before stopping treatment (withdrawal group, green line) or beginning at week 16 of diabetes for a further 12 weeks (reversal group, red line). Data are group mean ± SD of N=4-8/group. **p<0.01 and ***p<0.001 vs control+vehicle by two way repeat measures ANOVA with Dunnett's post-hoc test. **(B)**: Paw heat response latency in control mice

and STZ-diabetic mice ± 2.0% pirenzepine applied topically to the hind paw for 20/min/day, 1, 3 or 5 days/week for 10 weeks from onset of hyperglycemia. Data are group mean ± SD of N=7-11/group. *p<0.05, **p<0.01 and ***p<0.001 vs contro+vehicle, #p<0.05 vas STZ+vehicle by one way ANOVA with Sidak's post-hoc test.

FIGURE 4: Systemic effects of topical pirenzepine. STZ-diabetic mice were treated from onset of diabetes with vehicle or 2.0% - 10% pirenzepine in hydrogel applied to the hind paw for 20/min/day, 5 days/week. Motor nerve conduction velocity (A) and paw response to von Frey filaments (B) were measured after 4 weeks of treatment and paw response to heat (panel C) and IENF profile density (D) were measured after 8 weeks of treatment. Measurements were made in both the treated paw and the contralateral vehicle-treated paw. Data are group mean ± SD of N=7-10/group. *p<0.05, **p<0.01 and ***p<0.001 vs control+vehicle, #p<0.05, ###p<0.001 vs STZ+vehicle by one way ANOVA with Sidak's post-hoc test.

FIGURE 5: Systemic effects of topical atropine. STZ-diabetic mice were treated from onset of diabetes with vehicle or 2.0% atropine in hydrogel applied to the hind paw for 20/min/day, 5 days/week or to the eye (50µl in saline) 5 days/week. Motor nerve conduction velocity (A), paw response to von Frey filaments (B), paw response to heat (C), IENF profile density (D) and occupancy of corneal nerves of the sub-basal nerve plexus were measured after 12 weeks of treatment. Measurements were made in both the treated paw/eye and the contralateral vehicle-treated paw/eye, except for IENF

density, for which only tissue from the treated paw was collected. Data are group mean ± SD of N=6-10/group. *p<0.05, ** p<0.01 and *** p<0.001 vs control+vehicle, #p<0.05, ##p<0.01, ###p<0.001 vs STZ+vehicle by one way ANOVA with Sidak's post-hoc test

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TABLE 1: Animal group data from all studies. Data are group mean±SD. Statistical comparisons by one-way ANOVA with Sidak's *post-hoc* test. *p<0.05, **p<0.01 and *** p<0.001 vs control+vehicle; †p<0.05 and †† p<0.01 vs STZ+vehicle.. ⁺ animals died during an equipment malfunction.

	N	Study deaths	Loss of diabetes	Body weight (g)	Plasma glucose (mg/dl)
STUDY 1 (Figure 2)					
Control +vehicle	10	0	0	24.3±0.6	159±12
STZ +vehicle	12	0	0	22.4±0.5**	358±59***
STZ+0.2% PZ	12	0	1	21.1±1.3**	386±111***
STZ+1.0% PZ	13	0	0	21.3±1.8**	459±79***†
STZ+2.0% PZ	9	0	3	21.5±1.3**	483±98***††
STUDY 2 (Figure 3A)					
Control +vehicle	4	3⁺	0	22.8±0.7	139±11
Control +PZ	7	0	0	22.8±0.4	136±12
STZ +PZ (withdrawal)	9	3	0	20.8±0.8***	438±69***
STZ+PZ (reversal)	10	2	0	21.3±0.5**	495±101***
STUDY 3 (Figure 3B)					
Control +vehicle	10	0	0	27.6±2.1	153±28
STZ +vehicle	10	0	0	23.4±2.2***	560±18***
STZ+PZ (1/wk)	10	0	0	22.9±2.2***	587±28***
STZ+PZ (3/wk)	7	3	0	23.3±2.8**	593±19***
STZ+PZ (5/wk)	11	0	0	22.5±1.4***	589±34***
STUDY 4 (Figure 4)					
Control +vehicle	10	0	0	20.3±1.1	131±15
STZ +vehicle	8	0	0	20.0±1.7	437±92***
STZ+2% PZ	6	3	0	20.0±0.5	434±132***
STZ+10% PZ	9	1	0	20.5±0.7	415±149***
STUDY 5 (Figure 5)					
Control +vehicle	10	0	0	28.9±1.3	127±13
STZ +vehicle	9	1	0	26.9±1.8*	525±107***
STZ+2% atropine (paw)	8	2	0	25.7±1.3***	542±100***
STZ+2% atropine (eye)	9	0	1	27.1±1.3	524±33***

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Figures

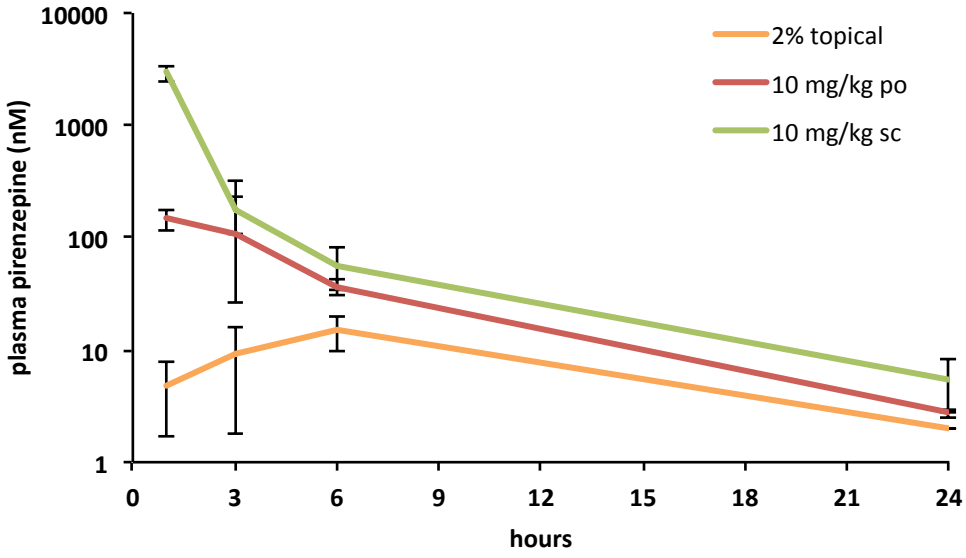

Figure 1

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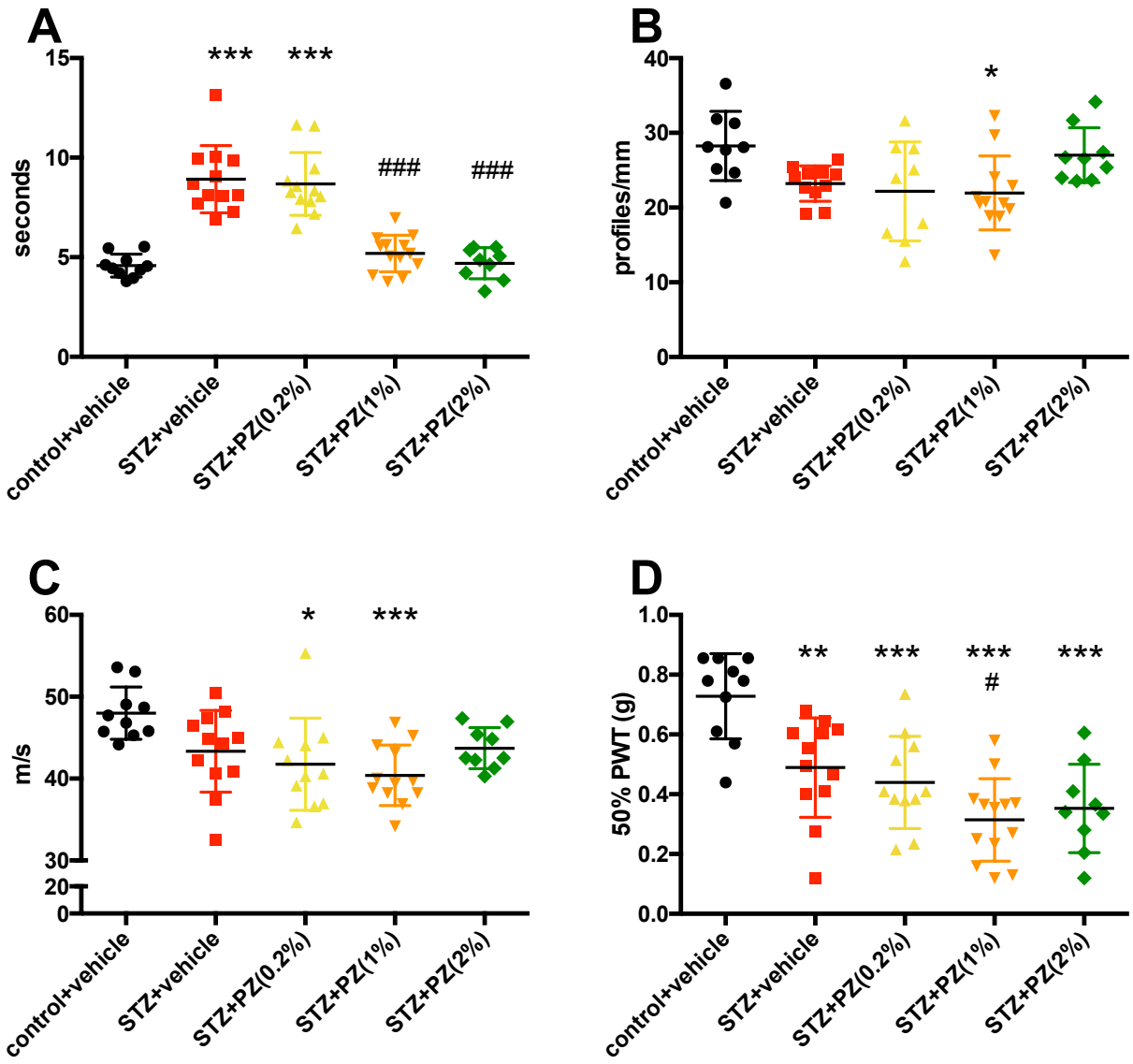

Figure 2

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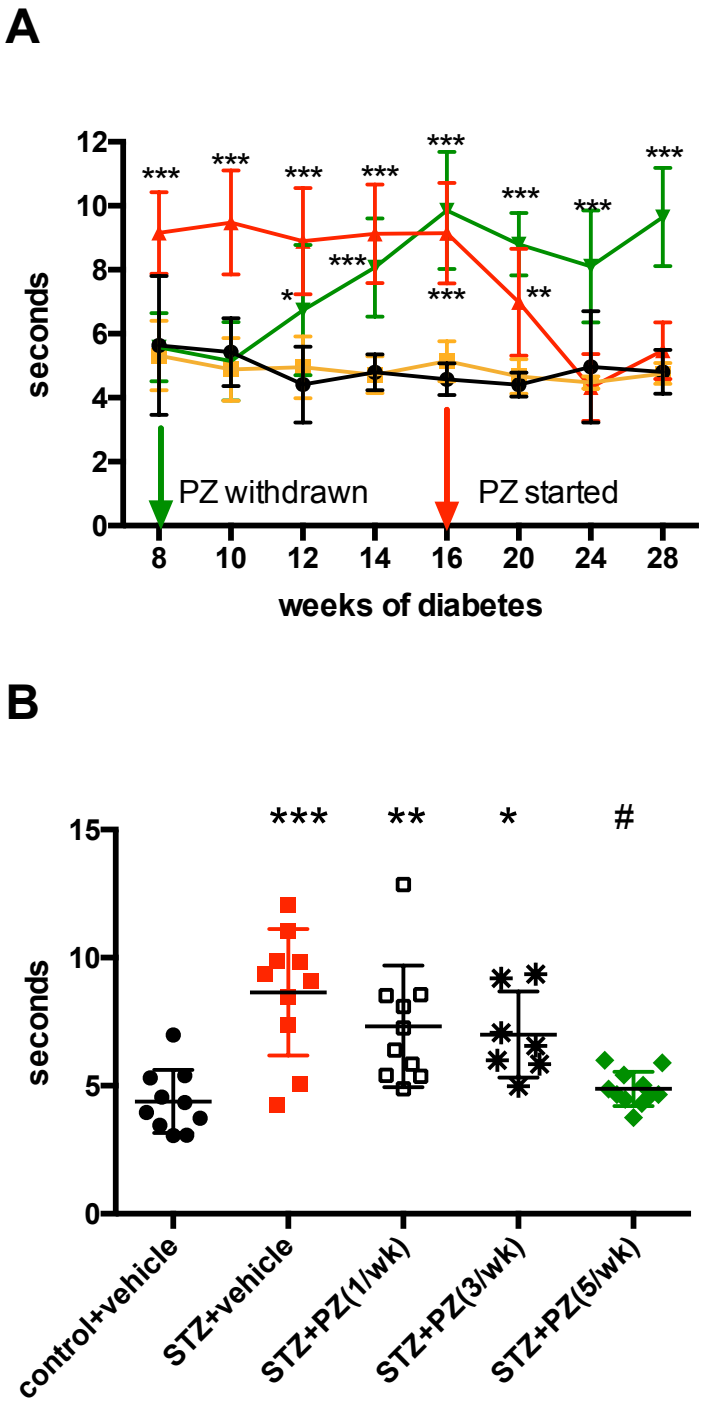

Figure 3

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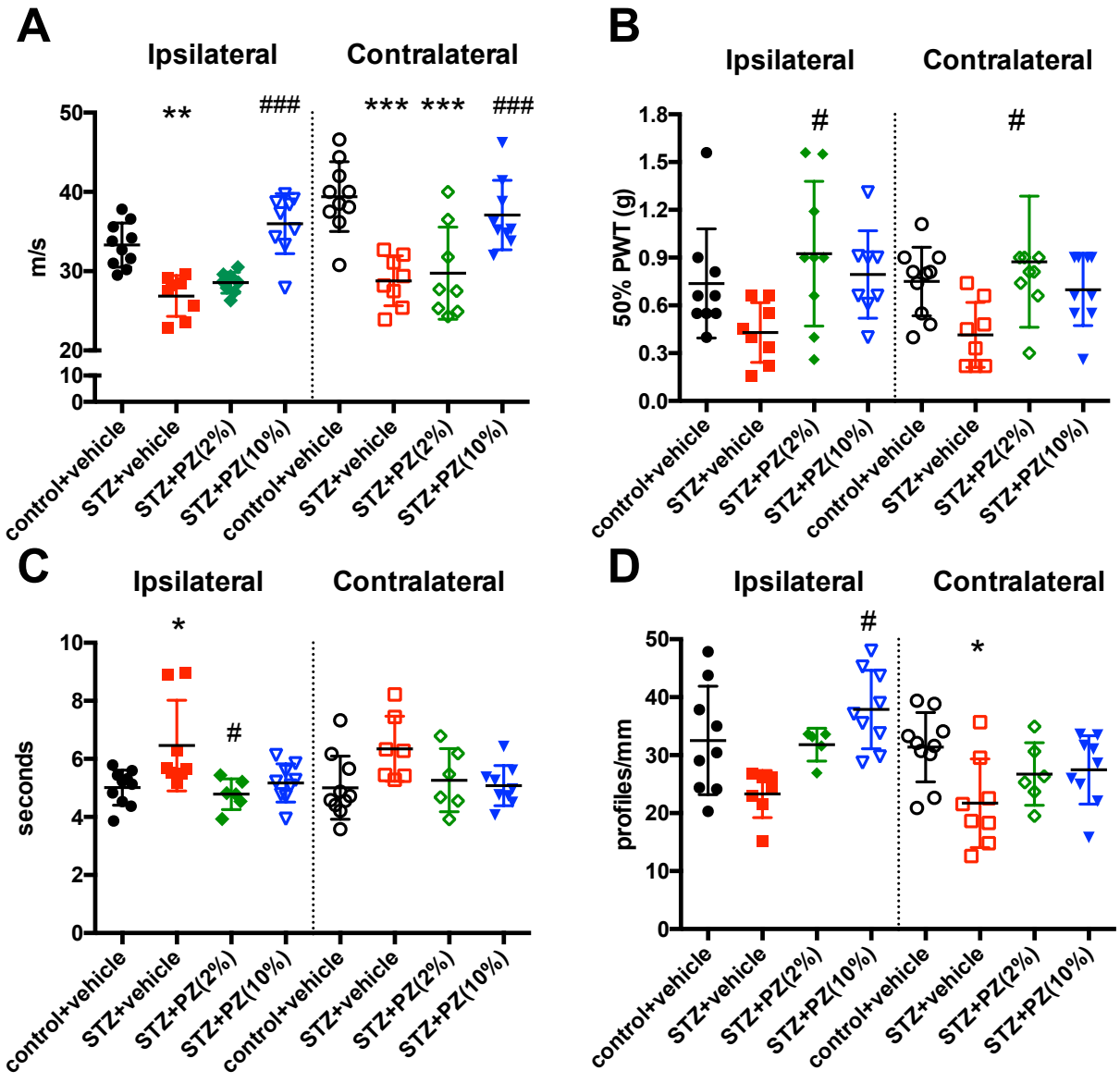

Figure 4

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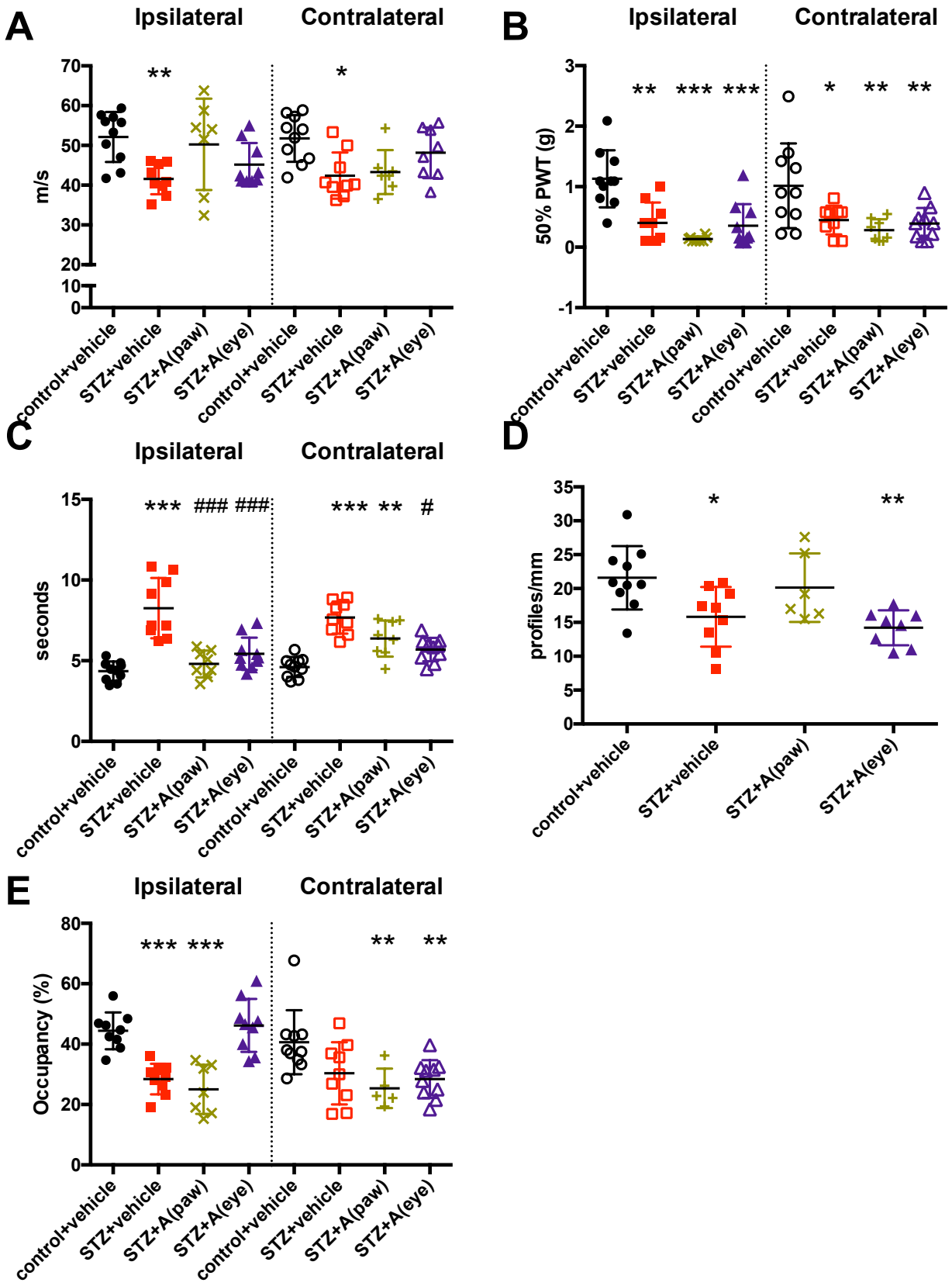

Figure 5