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Topical Delivery of Muscarinic Receptor Antagonists Prevents and Reverses Peripheral Neuropathy in Female Diabetic Mice

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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 M₁ receptor-selective muscarinic antagonist pirenzepine when delivered by subcutaneous 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 6 hours postdelivery that approximated those previously shown to promote neurite outgrowth in vitro. Topical delivery of pirenzepine to the paw of mice with streptozotocin-induced diabetes 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, whereas 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 day/wk. Similar local effects were noted with the nonselective 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.

Introduction

Muscarinic acetylcholine receptor antagonists have been in clinical use for decades to treat such diverse conditions as Parkinson disease, peptic ulcers, overactive bladder, chronic obstructive pulmonary disease, asthma, and motion sickness, with specific drug use depending on muscarinic receptor subtype 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 M₁ subtype of the muscarinic receptor (M₁R), 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 M₁R knockout and by diverse M₁R-specific and -selective antagonists. In contrast, muscarinic receptor antagonists selective for the M_{2–4}R subtypes do not promote neurite outgrowth, whereas 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

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ABBREVIATIONS: AMPK, AMP-activated protein kinase; AUC_{0–t}, Area Under the Curve from 0 to t; IENF, intraepidermal nerve fiber; MNCV, motor nerve conduction velocity; MR, muscarinic receptor; STZ, streptozotocin; T_{max}, Time of first occurrence of C_{max}.

diabetes (Fernihough, 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 M₁R were protected from diabetes-induced neuropathy, whereas subcutaneous injection of selective M₁R 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 M₁R-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 M₁R-selective muscarinic antagonists against peripheral neuropathy.

A major concern regarding clinical use of antimuscarinic drugs is the potential for unwanted side effects due to the widespread distribution of muscarinic receptors and lack of receptor subtype specificity. Poor antibody specificity has hampered definitive distribution studies for M₁R (Jositsch et al., 2009), but studies localizing M₁R mRNA or identifying physiologic consequences of M₁R knockout have offered clues. Other than being expressed by neurons and Schwann cells of the peripheral nervous system (Tata et al., 2000a,b; Loreti et al., 2006; Calcutt et al., 2017), M₁Rs 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 M₁R-specific antagonist is muscarinic toxin 7 (Krajewski et al., 2001; Servent et al., 2011), a peptide that is not currently a viable drug candidate. Pirenzepine may serve as an alternative candidate for manipulating the cholinergic constraint of peripheral sensory nerves *in vivo*, as it is M₁R-selective relative to the M₂₋₅R subtypes (Eglen et al., 2001) and does not readily cross the blood-brain barrier, reducing the potential for disruption of central nervous system function compared with 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 antimuscarinics 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 Institutional Animal Care and Use Committee. Studies were performed in adult female C57 Bl/6J (#000664; Jackson Laboratories) or female Swiss Webster (#024; Charles River) mice maintained three to five per cage on TEK-Fresh bedding (#7099; Envigo) under a 12-hour light/dark cycle with free access to water and food (5001 diet; Purina).

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 (C57 Bl/6J mice) or 100 mg/kg (Swiss Webster mice) streptozotocin (STZ; Sigma) in sterile 0.9% saline on two consecutive days, with each injection following a 12-hour 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 and 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 (One-Touch UltraMini; Lifescan Inc.). 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) were prepared for topical application to the paw by mixing into a volume of sterile hydrogel (Intrasite #66027313; Smith & Nephew) immediately before application. Fifty microliters of pirenzepine/hydrogel was applied to the plantar surface of the hind paw of manually restrained conscious mice, and the paw was 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 min/day, 5 days/wk, unless otherwise noted, after which it was removed, and the drug preparation was cleared with a tissue. For delivery to the eye, atropine was dissolved in ophthalmic gel (Genteal; Novartis), and 20 μ l was 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 High Performance Liquid Chromatography-Mass Spectrometry with a lower detection limit of 2 nM (Drumetrix Laboratories, Greensboro, NC) following delivery of 10 mg/kg pirenzepine by oral gavage or intraperitoneal 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 (Jolivald et al., 2016). Briefly, mice were anesthetized with isoflurane, core and nerve temperatures were held at 37°C using heating pads and lamps, and fine needle stimulating electrodes were 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-millisecond square waves), and the resulting electromyogram was stored to a computer running LabChart Pro (AD Instruments). 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 three 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 (Jolivald 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 (Jolivald et al., 2016). Paw response latency to surface heat escalating at a rate of 1°C/s from a starting surface temperature of 30°C was measured using a Harreaves apparatus. Triplicate measurements were made at 5-minute intervals, and the median was used to represent 50% paw withdrawal

TABLE 1

Animal group data from all studies Data are group means \pm S.D.
Statistical comparisons by one-way ANOVA with Sidak's post hoc test.

| | N | Study Deaths | Loss of Diabetes | Body Weight (g) | Plasma Glucose (mg/dl) |
|---------------------------|----|----------------|------------------|-------------------|------------------------|
| Study 1 (Fig. 2) | | | | | |
| Control + vehicle | 10 | 0 | 0 | 24.3 \pm 0.6 | 159 \pm 12 |
| STZ + vehicle | 12 | 0 | 0 | 22.4 \pm 0.5** | 358 \pm 59*** |
| STZ + 0.2% PZ | 12 | 0 | 1 | 21.1 \pm 1.3** | 386 \pm 111*** |
| STZ + 1.0% PZ | 13 | 0 | 0 | 21.3 \pm 1.8** | 459 \pm 79***† |
| STZ + 2.0% PZ | 9 | 0 | 3 | 21.5 \pm 1.3** | 483 \pm 98***†† |
| Study 2 (Fig. 3A) | | | | | |
| Control + vehicle | 4 | 3 ^a | 0 | 22.8 \pm 0.7 | 139 \pm 11 |
| Control + PZ | 7 | 0 | 0 | 22.8 \pm 0.4 | 136 \pm 12 |
| STZ + PZ (withdrawal) | 9 | 3 | 0 | 20.8 \pm 0.8*** | 438 \pm 69*** |
| STZ + PZ (reversal) | 10 | 2 | 0 | 21.3 \pm 0.5** | 495 \pm 101*** |
| Study 3 (Fig. 3B) | | | | | |
| Control + vehicle | 10 | 0 | 0 | 27.6 \pm 2.1 | 153 \pm 28 |
| STZ + vehicle | 10 | 0 | 0 | 23.4 \pm 2.2*** | 560 \pm 18*** |
| STZ + PZ (1/wk) | 10 | 0 | 0 | 22.9 \pm 2.2*** | 587 \pm 28*** |
| STZ + PZ (3/wk) | 7 | 3 | 0 | 23.3 \pm 2.8** | 593 \pm 19*** |
| STZ + PZ (5/wk) | 11 | 0 | 0 | 22.5 \pm 1.4*** | 589 \pm 34*** |
| Study 4 (Fig. 4) | | | | | |
| Control + vehicle | 10 | 0 | 0 | 20.3 \pm 1.1 | 131 \pm 15 |
| STZ + vehicle | 8 | 0 | 0 | 20.0 \pm 1.7 | 437 \pm 92*** |
| STZ + 2.0% PZ | 6 | 3 | 0 | 20.0 \pm 0.5 | 434 \pm 132*** |
| STZ + 10.0% PZ | 9 | 1 | 0 | 20.5 \pm 0.7 | 415 \pm 149*** |
| Study 5 (Fig. 5) | | | | | |
| Control + vehicle | 10 | 0 | 0 | 28.9 \pm 1.3 | 127 \pm 13 |
| STZ + vehicle | 9 | 1 | 0 | 26.9 \pm 1.8* | 525 \pm 107*** |
| STZ + 2.0% atropine (paw) | 8 | 2 | 0 | 25.7 \pm 1.3*** | 542 \pm 100*** |
| STZ + 2.0% atropine (eye) | 9 | 0 | 1 | 27.1 \pm 1.3 | 524 \pm 33*** |

PZ, pirenzepine.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. control + vehicle; † $P < 0.05$; †† $P < 0.01$ vs. STZ + vehicle.

^aAnimals died during an equipment malfunction.

threshold (50% paw withdrawal threshold in grams) 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 8 \times 8 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 6- μ m sections. Intraepidermal 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 regimen. Data are shown as group mean \pm S.D. Statistical comparison of experimental groups against the control + vehicle group and against the STZ + vehicle group at a single, predefined 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 repeated 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) by subcutaneous injection (10 mg/kg), oral gavage (10 mg/kg), or via topical application to

the right hind paw (50 μ l of 2% in hydrogel for 20 minutes). Mice were exsanguinated at 1, 3, 6, or 24 hours after drug delivery and plasma was stored at -70°C until assay of pirenzepine. The highest plasma concentrations were achieved following subcutaneous delivery of pirenzepine ($\text{AUC}_{0-t} = 4052 \text{ hour} \cdot \text{nM}$, $C_{\text{max}} = 2600 \text{ nM}$, $T_{\text{max}} = 1 \text{ hour}$), followed by oral administration ($\text{AUC}_{0-t} = 899 \text{ hour} \cdot \text{nM}$, $C_{\text{max}} = 137 \text{ nM}$, $T_{\text{max}} = 1 \text{ hour}$) and then topical administration ($\text{AUC}_{0-t} = 136 \text{ hour} \cdot \text{nM}$, $C_{\text{max}} = 15 \text{ nM}$, $T_{\text{max}} = 6 \text{ hour}$; Fig. 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

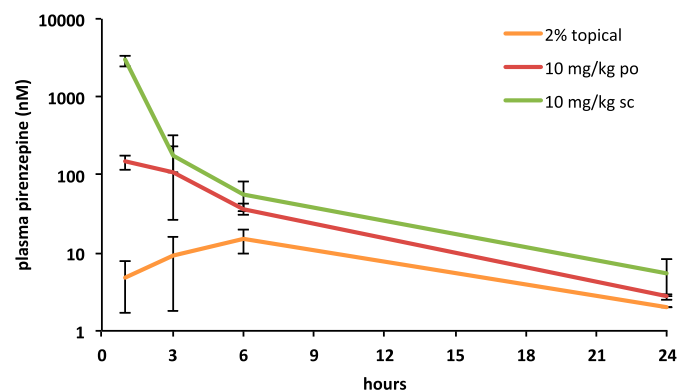


Fig. 1. Topical pirenzepine enters systemic circulation. Plasma pirenzepine concentration in mice following delivery of 10 mg/kg drug by subcutaneous injection (green line) or oral gavage (po, red line) vs. exposure of one hind paw to a 2% solution of drug in hydrogel (orange line) for 20 minutes. Data are means \pm S.D. of $N = 3$ mice per delivery route and per time point.

made diabetic with STZ, and upon confirmation of hyperglycemia, they were treated with vehicle or pirenzepine applied to the right hind paw (0.2%, 1.0%, or 2.0% in 50 μ l hydrogel, 20 min/day \times 5 days/wk) for 8 weeks. The left paw was untreated. Pirenzepine treatment did not prevent weight loss or hyperglycemia in diabetic mice, although the significantly elevated blood glucose in diabetic mice treated with high doses of pirenzepine compared with 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 (Fig. 2A). A trend toward loss of IENF in paw skin was also prevented by 2% pirenzepine but not by the 0.2% or 1.0% dose formulations (Fig. 2B). MNCV slowing (trend to decrease, Fig. 2C) and paw tactile allodynia ($P < 0.01$, Fig. 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 min/day \times 5 days/wk. The left paw was untreated. Paw response latency to heating was selected as the index of treatment efficacy (Fig. 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 (Fig. 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 (Fig. 3A). At this point, diabetic mice that had received only vehicle were transferred to topical treatment with 2.0% pirenzepine to the paw (20 min/day \times 5 days/wk). Paw heat hypoalgesia was completely reversed after 8 weeks of treatment (Fig. 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 μ l of 2.0% pirenzepine or vehicle applied to the right hind paw, 20 min/day for either 1, 3, or 5 days/wk for 10 weeks. The left paw was untreated. Pirenzepine treatment of diabetic mice had no effect on body weight or hyperglycemia compared with 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/wk completely normalizing paw heat response latency (Fig. 3B), confirming data obtained with C57 Bl/6J mice (Fig. 2A).

To determine whether higher doses of pirenzepine impacted indices of neuropathy not responsive to 2.0% topical pirenzepine (Fig. 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 50 μ l hydrogel, 20 min/day \times 5 days/wk for 8 weeks). The left paw was treated with vehicle. Pirenzepine treatment of diabetic mice had no effect on body weight or hyperglycemia compared with vehicle-treated diabetic mice (Table 1). At study end, vehicle-treated diabetic mice showed bilateral MNCV slowing (Fig. 4A), paw allodynia to von Frey filaments (Fig. 4B), paw thermal hypoalgesia (Fig. 4C), and loss of sensory nerves in paw skin (Fig. 4D). Topical pirenzepine (2.0%) prevented paw tactile allodynia, heat hypoalgesia, and loss of IENF in the treated paw but had

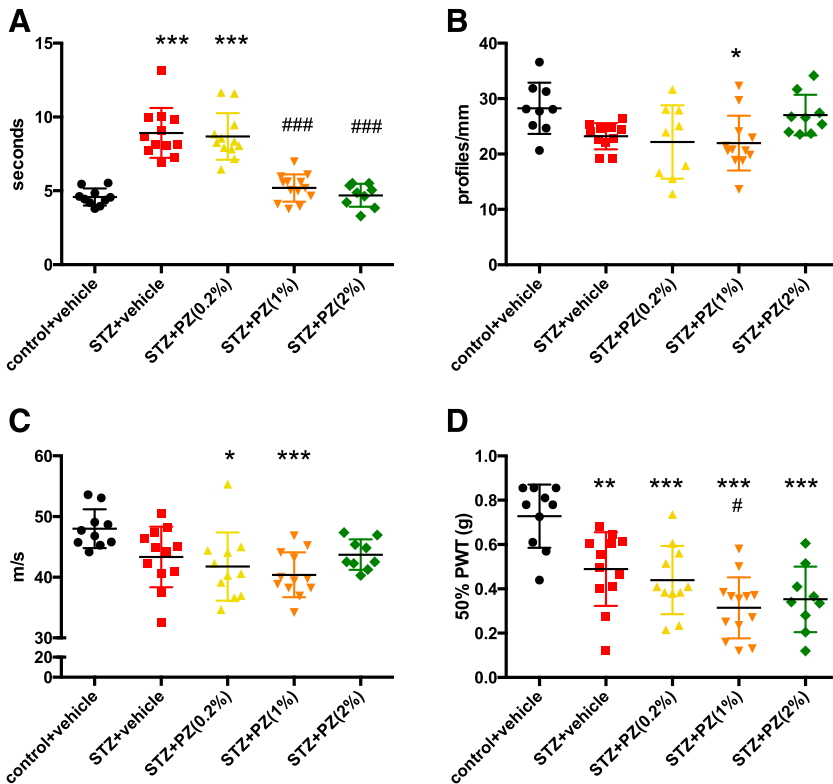


Fig. 2. Topical pirenzepine (PZ) dose-dependently prevents small fiber neuropathy in diabetic mice. Paw heat response latency (A), IENF profile density (B), motor nerve conduction velocity (C), and paw response to von Frey filaments (D) in control and STZ-diabetic mice treated with vehicle or 0.2%–2.0%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

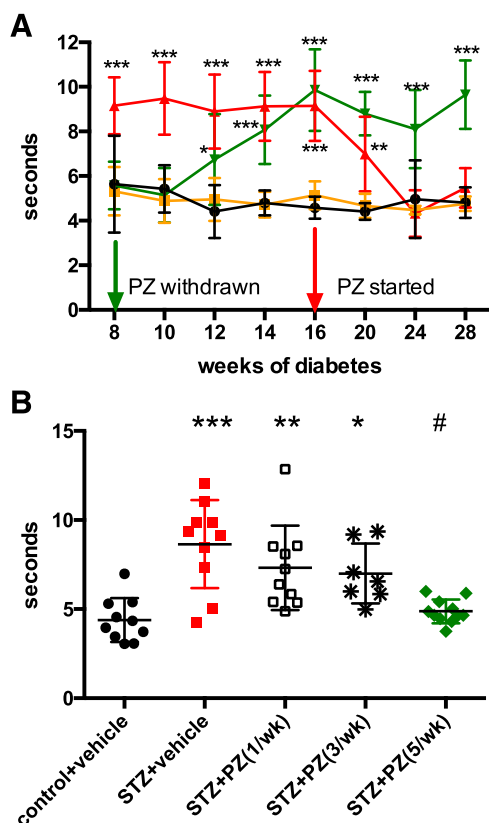


Fig. 3. Effects of topical pirenzepine dose regimen on paw heat hypoalgesia. (A) Paw heat response latency in control + vehicle (black line), control + 2.0% pirenzepine (gold line), and STZ-diabetic mice \pm 2.0% pirenzepine (PZ), either applied topically to the hind paw for 20 min/day, 5 days/wk, for 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 means \pm S.D. of $N = 4-8$ /group. $**P < 0.01$; $***P < 0.001$ vs. control + vehicle by two-way repeated measures ANOVA with Dunnett's post hoc test. (B) Paw heat response latency in control mice and STZ-diabetic mice \pm 2.0% pirenzepine applied topically to the hind paw for 20 min/day, 1, 3, or 5 days/wk, for 10 weeks from onset of hyperglycemia. Data are group means \pm S.D. of $N = 7-11$ /group. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ vs. control + vehicle, $\#P < 0.05$ vs. STZ + vehicle by one-way ANOVA with Sidak's post hoc test.

no impact on MNCV slowing in the ipsilateral limb. Similar efficacy was noted in the vehicle-treated contralateral limb. Increasing pirenzepine dose to 10.0% 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 antimuscarinic 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 50 μ l hydrogel, 20 min/day \times 5 days/wk for 8 weeks) or the right eye (2.0% in ophthalmic gel \times 5 days/wk 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 with vehicle-treated diabetic mice (Table 1). Diabetes induced significant MNCV slowing (Fig. 5A), tactile allodynia (Fig. 5B), heat hypoalgesia (Fig. 5C), significantly reduced IENF profile density (Fig. 5D), and corneal sub-basal nerve plexus density (Fig. 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. Ocular delivery of atropine also significantly prevented or attenuated paw heat hypoalgesia in both hind paws, but it did not prevent 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 antimuscarinics include development of receptor subtype 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 gastrointestinal 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 central nervous system 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 24 hours 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 modulating 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

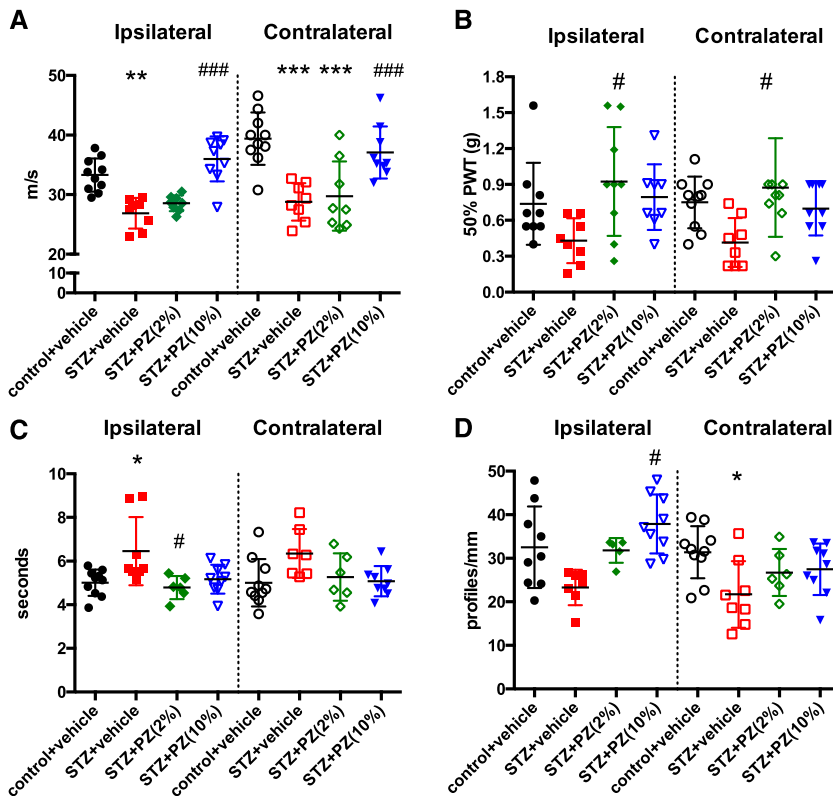


Fig. 4. Systemic effects of topical pirenzepine (PZ). STZ-diabetic mice were treated from onset of diabetes with vehicle or 2.0%–10.0% pirenzepine in hydrogel applied to the hind paw for 20 min/day, 5 days/wk. Motor nerve conduction velocity (A) and paw response to von Frey filaments (PWT: Paw withdrawal threshold) (B) were measured after 4 weeks of treatment, and paw response to heat (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 means \pm S.D. of $N = 7$ –10/group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. control + vehicle, # $P < 0.05$; ### $P < 0.001$ vs. STZ + vehicle by one-way ANOVA with Sidak's post hoc test.

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; Jolivald 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, 2000), may contribute to thermal hypoalgesia prior to detectable IENF depletion. It is also possible that there is impact of pirenzepine on select subtypes of IENF that is not discriminated when all IENFs are quantified using the pan-neuronal marker PGP9.5 (Beiswenger et al., 2008).

Diabetic neuropathy is frequently described as a small fiber neuropathy because of 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, although large fiber demyelination and axonal degeneration and loss are established pathologic 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 in which reversal of paw thermal hypoalgesia took 4–8 weeks; withdrawal of pirenzepine precipitated onset of hypoalgesia over a similar time course. Our prior studies aligned neuroprotection by antimuscarinics with upregulation of AMPK signaling and preservation of mitochondrial bioenergetics (Calcutt et al., 2017). M_1R antagonism also removes a constraint on microtubule polymerization and mitochondrial trafficking (Sabbir et al., 2018) and promotes a biased β -arrestin-dependent extracellular signal-regulated kinase-cAMP-responsive element binding protein (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%. It remains to be established whether this is an intrinsic feature of the different components of the peripheral nervous system 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. 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

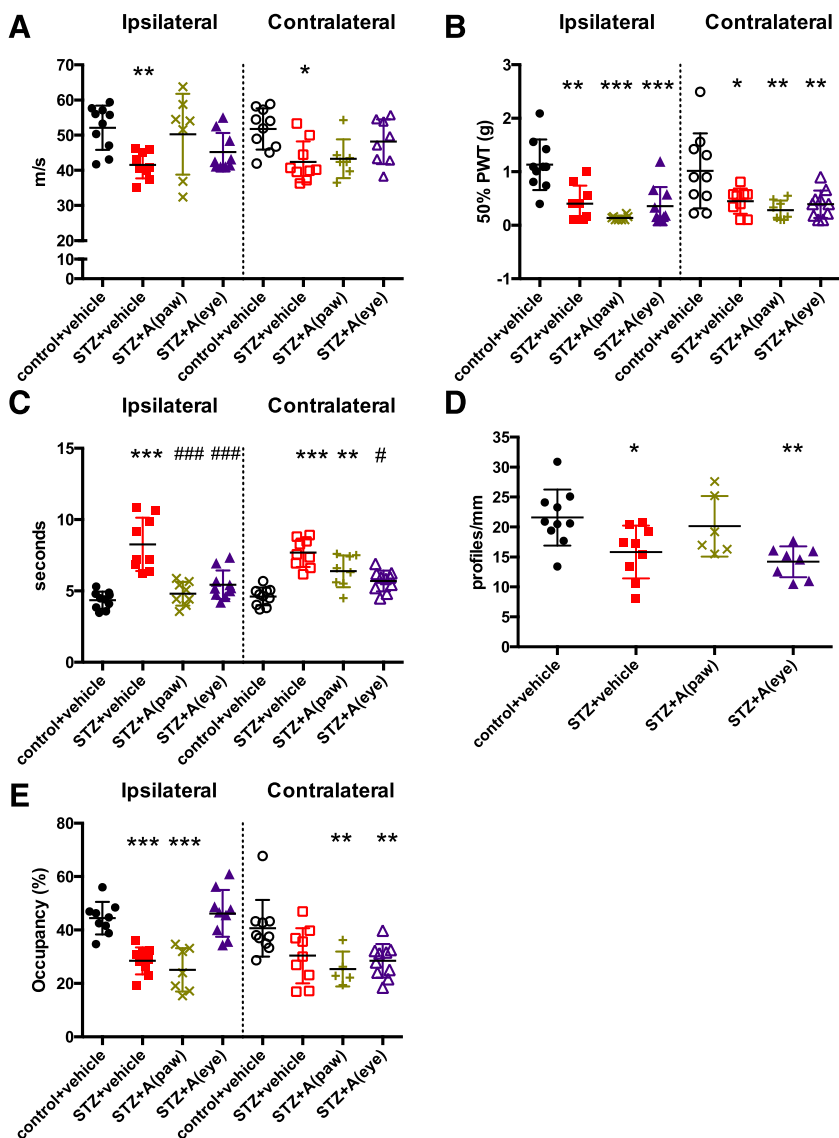


Fig. 5. Systemic effects of topical atropine (A). STZ-diabetic mice were treated from onset of diabetes with vehicle or 2.0% atropine (A) in hydrogel applied to the hind paw for 20 min/day, 5 days/wk, or to the eye (50 μ l in saline) 5 days/wk. Motor nerve conduction velocity (A), paw response to von 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 means \pm S.D. of $N = 6-10$ /group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ 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

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.

Although 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 five muscarinic receptor subtypes, 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 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 antimuscarinics to treat diabetic neuropathy. The availability of topical formulations of antimuscarinics may facilitate evaluation of the translational potential of this therapeutic approach for a condition that has no Food and Drug Administration–approved medication.

Authorship Contributions

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

Conducted experiments: Frizzi, Han, Mota, Guernsey.

Performed data analysis: Jolivalt, Kotra, Calcutt.

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

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