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Methylglyoxal and a spinal TRPA1-AC1-Epac cascade facilitate pain in the db/db mouse model of type 2 diabetes

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ABSTRACT

Painful diabetic neuropathy (PDN) is a devastating neurological complication of diabetes. Methylglyoxal (MG) is a reactive metabolite whose elevation in the plasma corresponds to PDN in patients and pain-like behavior in rodent models of type 1 and type 2 diabetes. Here, we addressed the MG-related spinal mechanisms of PDN in type 2 diabetes using db/db mice, an established model of type 2 diabetes, and intrathecal injection of MG in conventional C57BL/6J mice. Administration of either a MG scavenger (GERP10) or a vector overexpressing glyoxalase 1, the catabolic enzyme for MG, attenuated heat hypersensitivity in db/db mice. In C57BL/6J mice, intrathecal administration of MG produced signs of both evoked (heat and mechanical hypersensitivity) and affective (conditioned place avoidance) pain. MG-induced Ca^{2+} mobilization in lamina II dorsal horn neurons of C57BL/6J mice was exacerbated in db/db, suggestive of MG-evoked central sensitization. Pharmacological and/or genetic inhibition of transient receptor potential ankyrin subtype 1 (TRPA1), adenylyl cyclase type 1 (AC1), protein kinase A (PKA), or exchange protein directly activated by cyclic adenosine monophosphate (Epac) blocked MG-evoked hypersensitivity in C57BL/6J mice. Similarly, intrathecal administration of GERP10, or inhibitors of TRPA1 (HC030031), AC1 (NB001), or Epac (HJC-0197) attenuated hypersensitivity in db/db mice. We conclude that MG and sensitization of a spinal TRPA1-AC1-Epac signaling cascade facilitate PDN in db/db mice. Our results warrant clinical investigation of MG scavengers, glyoxalase inducers, and spinally-directed pharmacological inhibitors of a MG-TRPA1-AC1-Epac pathway for the treatment of PDN in type 2 diabetes.

Keywords: type 2 diabetes, neuropathic pain, methylglyoxal, TRPA1, Epac, AC1, PKA, glyoxalase, spinal, painful diabetic neuropathy

INTRODUCTION

Neuropathic pain occurs in approximately one-third of patients with diabetes and is refractory to currently available analgesic drugs (Abbott et al., 2011). This painful diabetic neuropathy (PDN) is associated with elevated levels of methylglyoxal (MG; a reactive glucose metabolite) and decreased expression and activity of glyoxalase 1 (GLO1), the major detoxification enzyme for MG (Bierhaus et al., 2012; Huang et al., 2016; Jack et al., 2012; Skapare et al., 2013; Sveen et al., 2013). Previous studies suggest MG produces PDN by contributing to the formation of advanced glycation end-products (MG-AGEs) (Skapare et al., 2013; Sveen et al., 2013), and/or by sensitizing pronociceptive ion channels in peripheral afferents (Andersson et al., 2013; Bierhaus et al., 2012; Griggs et al., 2017; Huang et al., 2016; Koivisto et al., 2012). While these studies focused on the pathophysiology of peripheral sensory nerves in the type 1 form of diabetes, type 2 diabetes accounts for 90% of patients, is more frequently associated with PDN (Abbott et al., 2011), and the mechanisms of diabetic neuropathy may differ in these two forms of diabetes (Callaghan et al., 2012a; Callaghan et al., 2012b; Feldman et al., 2017). Furthermore, very little is known regarding the spinal mechanisms that contribute to PDN. Thus, the central mechanisms that generate and maintain PDN in type 2 diabetes is a critical gap in knowledge.

The contribution of a MG-related spinal signaling cascade to PDN in type 2 diabetes remains unknown. Recent studies implicate both transient receptor potential ankyrin subtype 1 (TRPA1) (Andersson et al., 2013; Griggs et al., 2017; Huang et al., 2016) and adenylyl cyclase isoform 1 (AC1) (Griggs et al., 2017) in the pain evoked by intraplantar administration of MG. The contribution of a spinal adenylyl cyclase, cyclic adenosine monophosphate (cAMP), protein kinase A (PKA) pathway was identified in the Zucker Diabetic Fatty rat model of type 2 diabetes (Feng et al., 2017), but the specific adenylyl cyclase isoform (e.g. AC1) was not identified and antagonism of spinal PKA was not tested. Furthermore, spinal AC1-cAMP signaling could be mediated not just by PKA, but also by isoform 1 or 2 of exchange protein directly activated by cAMP (Epac1/2). Both PKA and Epac1/2 are implicated in peripheral nociceptive sensitization in various pain conditions (Aley and Levine, 1999; Eijkelkamp et al., 2013; Gu et al., 2016; Huang and Gu, 2017; Hucho et al., 2005; Matsuda et al., 2017; Wang et al., 2013). Whether spinal TRPA1, AC1, PKA, or Epac1/2 contribute to PDN in type 2 diabetes remains an important question.

To test the hypothesis that spinal MG signals through TRPA1, AC1, PKA, and Epac1/2 to cause PDN in type 2 diabetes, we used two experimental models of neuropathic pain. First, we targeted the spinal cord dorsal horn with intrathecal administration of MG in conventional C57BL/6J mice and tested both reflexive and affective pain-like behaviors. Second, we utilized db/db mice, a model of type 2 diabetes that develops heat hyperalgesia (Bierhaus et al., 2012; Xu et al., 2014) concordant with hyperglycemia and elevated MG (Bierhaus et al., 2012). We determined the behavioral effects of agents that scavenge MG or promote overexpression of GLO1, used calcium imaging to assess MG-evoked central sensitization in *ex vivo* spinal cord slices, and evaluated the effect of intrathecal administration of inhibitors of MG, TRPA1, AC1, PKA, and Epac1/2 on pain-like hypersensitivity in the intrathecal MG and db/db models.

MATERIALS AND METHODS

Animals

Male nondiabetic control BKS (C57BLKS/J; RRID:IMSR_JAX:000662) and type 2 diabetic db/db (BKS.Cg-Dock7m+/+Leprdb/J; RRID:IMSR_JAX:000642) mice aged 6-13 wks were used. Male mice aged 8-18 wks were used for studies involving intrathecal administration of methylglyoxal to wildtype C57BL/6J (RRID:IMSR_JAX:000664) or TRPA1 and AC1 knockout mice. TRPA1-/- knockout mice (RRID:IMSR_JAX:006401) and their respective B6129PF2/J controls (RRID:IMSR_JAX:100903) were used. Congenic AC1-/- knockout mice were maintained in-house using a heterozygote breeding strategy onto a C57BL/6J background with genotypes confirmed by tail-snip PCR.

Mice were housed 2-4 animals per cage in a temperature and humidity-controlled room on a 14-hour light | 10-hour dark cycle with lights on from 06:00 to 20:00. Animals were provided water and chow *ad libitum.* Behavioral pharmacology experiments were performed during the light cycle from 8:00-18:00. Experiments were carried out in accordance with the Institutional Animal Care and Use Committee at the University of Kentucky, the International Association for the Study of Pain, the National Institutes of Health Office of Laboratory Animal Welfare Guide for the Care and Use of Laboratory Animals, and the ARRIVE guidelines.

For db/db studies, the significant difference in mass between BKS and db/db mice prevented observer blinding to subject grouping; however, blinding to drug treatment between groups was maintained. All other

experiments were performed and/or quantified by an observer blinded to the subject grouping and to the drug treatment that each group received.

Drug administration and materials

Intraperitoneal (i.p.) or intrathecal (i.t.; 5-10 µL) administration of solutions was performed as previously described (Griggs et al., 2017). Methylglyoxal (MG; M0252, Sigma-Aldrich, St. Louis, MO) and GEAP10/GERP10 (Gift from Thomas Fleming and Peter Nawroth, University Hospital of Heidelberg, Heidelberg, Germany) were diluted in 0.9% saline. HC030031 (H4415, Sigma-Aldrich) was diluted in ethanol and Tween-80 before the titration of saline and adjustment to pH 7.4. NB001 (SML0060, Sigma-Aldrich) was diluted in water. H-89 (B1427, Sigma-Aldrich), ESI-09, HJC-0350 and HJC-0197 (courtesy of Jia Zhou, The University of Texas Medical Branch, Galveston, TX) were diluted in a mixture of 8:1:1 saline:ethoxylated castor oil:ethanol. In-house MG of high purity was synthesized as previously reported (McLellan et al., 1992; Rabbani and Thornalley, 2014).

A *pCMV6-Neo* overexpression vector containing mouse *Glo1* cDNA and a *pGL3-luciferase* control vector were suspended in 150 µL liposomal transfection reagent (DOTAP, Roche, Mannheim, Germany), incubated at room temperature for 15 min, and administered as multiple i.p. injections given every other day over a 9 d period to db/db or BKS mice as previously described (Bierhaus et al., 2012).

Pain-like behavior

Fluctuations in noise, vibrations, temperature, and other environmental variables in the behavioral testing room were minimized to optimize reliable measurements between cohorts of animals tested on different days. Animals were acclimated to each behavioral testing environment for 1 h at least 1 d prior to commencing studies and then again for 30-60 min on each day of testing.

Heat hypersensitivity

Animals were placed on a heated surface (52.5 ± 1 °C) within an acrylic enclosure (Hotplate; Columbus Instruments, Columbus, OH). The time until hindpaw withdraw response (e.g. jumping, licking, flinching) was

recorded as the heat response latency as previously described (Griggs et al., 2016). The animal was immediately removed after paw withdraw or a cutoff of 25 s to avoid tissue injury.

Mechanical hypersensitivity

Animals were placed within a rectangular acrylic box (15x4x4 cm; 3 white opaque walls and 1 clear wall) on a steel mesh grid. The plantar surface of the ventral-medial hindpaw was stimulated with an incremental series of 8 von Frey monofilaments (Stoelting, Inc., Wooddale, IL) of logarithmic stiffness using a modified up-down method as previously described (Chaplan et al., 1994; Griggs et al., 2015b). The calculated 50% mechanical withdraw threshold is reported.

Conditioned place avoidance testing

Conditioned place avoidance (CPA) testing in the current study was similar to our previously published methods (Griggs et al., 2015a; Griggs et al., 2017) with the following modifications. We used a three-chambered CPA apparatus consisting of an acrylic enclosure with manual guillotine doors (Place Preference, San Diego Instruments, San Diego, CA; http://www.sandiegoinstruments.com/place-preference/). Printed paper was used to cover the outside of the clear box so that the sides of the middle chamber were grey and the end chambers' walls had ¾" wide black and white stripes arranged either horizontally or vertically. The horizontally striped chamber had textured flooring, the vertically striped chamber had smooth flooring, and the center chamber flooring consisted of metal rods spaced 0.5 cm apart. Several methods were employed to reduce time spent in the middle chamber: 1) An acrylic divider (13.5cm x 10.5cm x 33cm, 0.5cm thick; custom design, Regal Plastics https://www.regal-plastics.com) was placed in the center chamber to decrease its size; 2) The guillotine doors separating the end chambers from the middle chamber were covered in white paper on the side facing the middle chamber; 3) The light intensity in the end chambers was reduced to 20% of that in the middle chamber. The time spent in each chamber was quantified using a 4 x 16 photobeam array.

The CPA experiment spanned 7 days. Mice were habituated to the experimenter and handling for intrathecal injections on Days 1-2, acclimated to the CPA apparatus on Day 3, and then averaged preconditioning preferences were assessed on Days 4-5. Mice that spent greater than 80% or less than 20% of

the time in any chamber were eliminated from further study due to pre-existing chamber bias (1 mouse). During conditioning on Day 6, pairing of intrathecal saline (5 µL) in the morning was followed four hours later by injection-pairing of MG (10 µg) to the opposite chamber. To avoid negative association with the intrathecal injection process, mice were placed back in the home cage for 5 mins after each intrathecal injection, prior to being placed in one of the isolated end chambers. As previously described (Griggs et al., 2015a), drug-chamber pairings were biased and counterbalanced. On Day 7, mice were allowed open access to the entire CPA apparatus in the absence of any injections to assess postconditioning preferences.

Ratiometric imaging of calcium responses in adult spinal cord slices

Ratiometric calcium imaging of adult mouse spinal cord slices was performed as previously described (Doolen et al., 2012) and as follows. Mice were anesthetized with isoflurane and perfused transcardially with 10 ml of ice-cold sucrose-containing artificial cerebrospinal fluid (aCSF) (sucrose-aCSF) that contained in mM: NaCl 95, KCl 1.8, KH_2PO_4 1.2, $CaCl_2$ 0.5, $MgSO_4$ 7, $NaHCO_3$ 26, glucose 15, sucrose 50 kynurenic acid 1, oxygenated with 95% O_2, 5% CO_2; pH 7.4. The lumbar spinal cord was rapidly (within 90 s) isolated by laminectomy from the cervical enlargement to the cauda equina, placed in oxygenated ice-cold sucrose-aCSF, cleaned of dura mater and ventral roots, and super-glued vertically to a block of 4% agar (Fisher Scientific, Pittsburgh, PA) on the stage of a Campden 5000mz vibratome (Lafayette, IN). Transverse slices (450 µm) from lumbar segments L3-L5 were cut in ice-cold sucrose-aCSF using minimum forward speed ranging from 0.03 to 1 mm/s and using maximum vibration. Dissection and slicing time was kept to a minimum to ensure slice viability.

Slices were incubated for 30 min at 32°C with Fura-2 AM (10 µM) in pluronic acid (0.1%) in oxygenated aCSF containing (in mM): NaCl 127, KCl 1.8, KH_2PO_4 1.2, $CaCl_2$ 2.4, $MgSO_4$ 1.3, $NaHCO_3$ 26, glucose 15, followed by a 20 min de-esterification period in oxygenated aCSF without Fura-2 AM or pluronic acid. Prior to imaging, slices were maintained at room temperature in oxygenated aCSF. Slices were then placed in a RC-25 recording chamber (Warner Instruments, Hamden, CT). The recording chamber was secured on a Nikon FN-1 upright microscope fitted with a 79000 ET FURA2 Hybrid filter set (Nikon Instruments, Melville, NY) and a Photometrics CoolSNAP HQ2 camera (Tucson, AZ) and perfused at 1–2 ml/min with oxygenated aCSF. Paired 340/380 images were collected using a 20x water emersion objective at 1-1.5 images per second. Relative

intracellular Ca^{2+} levels were determined by measuring the change in ratio of fluorescence emission at 510 nm in response to excitation at 340 and 380 nm (200 ms exposure). Calcium responses were evaluated using Nikon Elements software by creating a region of interest over the neuronal cell profiles in lamina I-II of the dorsal horn and calculating the peak magnitude of change in the ratio of fluorescence emission in response to consecutive stimulation at 340 and 380 nm. After the acclimation period, the following recording protocol was performed: 1 mM glutamate exposure for 5 s followed by at least 5 min washout, 60 s MG exposure followed by at least 5 min washout, 1 mM glutamate exposure for 5 s. In wildtype C57BL/6J mice, dose-dependent calcium responses to 0-10 mM MG are presented as the % of calcium response to 1 mM glutamate. In 6-8 wk old BKS and db/db mice, raw calcium responses after exposure to 10 mM MG are reported.

Blood glucose and HbA1c measurement

Mice were lightly restrained in a towel and the distal tail wiped with an alcohol swab. A small nick was made at the distal tip of the tail using a #11 scalpel blade. Initial bleeding was wiped clean with gauze and subsequent drops of blood were either loaded into a room temperature HbA1c cartridge and analyzed using a DCA Vantage Analyzer (Siemens, Munich, Germany), or placed on a glucose test strip in duplicate and inserted into a glucose monitor (TrueTrack, Walgreens, Deerfield, IL).

Methylglyoxal-derived advanced glycation end-products measurement

MG-AGEs in serum were measured by competitive ELISA (STA-811, Cell BioLabs, San Diego, CA) as previously described (Griggs et al., 2016). One part serum was diluted in three parts 1x PBS to obtain values within the range of the standard curve.

Data analysis and statistics

Results were analyzed using unpaired t-test, one-way ANOVA, or repeated measures two-way ANOVA with Holm-Sidak multiple comparison correction with $\alpha=0.05$. All data were analyzed using Prism 7.0 (GraphPad, La Jolla, CA) and are presented as mean ± SEM.

RESULTS

Administration of a MG scavenging peptide or GLO1 overexpression vector reduces hyperalgesia in type 2 diabetic db/db mice

The typical presentation of painful diabetic neuropathy is characterized by initial hyperalgesia and/or allodynia followed by progression to insensitivity and/or hypoalgesia (Feldman et al., 2017). Although a relatively recent review indicates mostly behavioral hypoalgesia in db/db mice (O'Brien et al., 2014), these studies were in mice aged 12 weeks or more. Our current studies focused on the earlier periods of heat hyperalgesia in db/db mice (Bierhaus et al., 2012; Xu et al., 2014). First, we tested whether the development of pain-like behavior in db/db mice is associated with signs of type 2 diabetes. **Figure 1A** illustrates that db/db mice develop heat hypersensitivity by 7 weeks of age. Heat hypersensitivity persisted until at least 13 wks of age (Supplemental Figure 1A). Therefore, subsequent experiments performed in the current study utilized db/db mice aged 8-13 wks, during the period of hyperalgesia, and presumably prior to the onset of hypoalgesia. Blood glucose, body weight, and glycated hemoglobin (HbA1c) were elevated in db/db mice compared to BKS controls (Supplemental Figure 1B-D). **Figure 1B** illustrates elevated serum MG-AGE levels in db/db mice.

Previous studies indicate that elevated MG in diabetes might contribute to PDN (Andersen et al., 2018; Bierhaus et al., 2012; Griggs et al., 2017; Huang et al., 2016). To determine whether endogenous MG promotes hyperalgesia in db/db mice, we used two interventions that are validated to concurrently reduce MG levels and pain-like behavior in streptozotocin mice: a MG-scavenging peptide GERP10 (Bierhaus et al., 2012; Brings et al., 2017), or overexpression of GLO1 (Bierhaus et al., 2012). **Figure 1C** illustrates that a single intraperitoneal injection of GERP10, but not its control peptide GEAP10, increased heat response latencies in db/db but not in non-diabetic BKS mice. Compared to GEAP10, GERP10 did not alter blood glucose or HbA1c levels (Supplemental Figure 2). **Figure 1D** illustrates that multiple intraperitoneal injections of the GLO1 overexpression vector, but not control vector, increased heat response latencies in db/db without changing heat response latencies in BKS controls. This protocol of GLO1 overexpression vector administration doubled Glo1 activity in sciatic nerves of streptozotocin mice (Bierhaus et al., 2012).

FIGURE 1

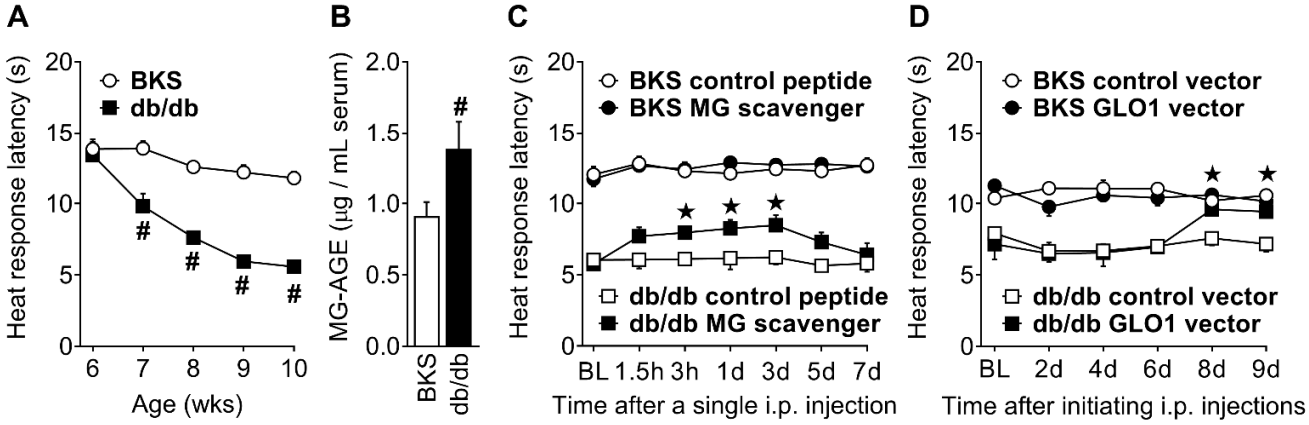

Figure 1. Methylglyoxal in type 2 diabetic db/db mice contributes to hyperalgesia. **(A)** Compared to BKS controls, heat response latencies were reduced in db/db mice [strain x time; $F(4,112)=11.5$; $p<0.0001$] at 7-10 wks of age [$p<0.0001$; two-way ANOVA] (n=15). **(B)** Serum levels of MG-derived advanced glycation end-products (MG-AGE) were elevated in db/db (1.38 ± 0.2 μg/mL serum) compared to BKS (0.91 ± 0.1 μg/mL serum) at 10-11 wks of age [$p = 0.03$; unpaired t-test] (n=8-12). **(C)** The peptide MG scavenger GERP10 (1 mg) or its control peptide GEAP10 (1 mg) were administered as a single intraperitoneal (i.p.) injection to db/db mice or BKS controls at 11 wks of age. GERP10 but not GEAP10 increased heat response latencies in db/db but not BKS controls [peptide in db/db; $F(1,13)=9.272$; $p=0.0094$] at 3 h to 3 d after administration [$p<0.05$; two-way ANOVA]. Neither GERP10 nor GEAP10 altered heat response latencies in BKS controls [peptide in BKS; $F(1,13)=0.291$; $p=0.60$] (n=7-8). **(D)** Repeated administration of GLO1 overexpression vector but not control vector increased heat response latencies in db/db [vector in db/db at 6-9 d; $F(1,7)=8.312$; $p=0.008$] at d 8-9 [$p<0.01$; two-way ANOVA]. There was no effect of GLO1 vector administration in BKS [vector in BKS at 6-9 d; $F(1,8)=0.60$; $p=0.46$] (n=4-5). **#** $p<0.05$ vs. BKS. ★ $p<0.05$ vs. db/db control peptide/vector. BL = baseline behavior prior to drug administration.

Acute intrathecal administration of methylglyoxal produces behavioral signs of PDN in conventional C57BL/6J mice

Previous studies indicate that acute intraplantar (Andersson et al., 2013; Barragan-Iglesias et al., 2018; Griggs et al., 2017; Huang et al., 2016), acute intravenous (Bierhaus et al., 2012; Brings et al., 2017), or repeated intraperitoneal (Barragan-Iglesias et al., 2018; Liu et al., 2017) administration of MG are sufficient to produce pain-like behavior in wildtype mice or rats. Two studies report that repeated intrathecal administration of MG produces mechanical hypersensitivity in rats (Liu et al., 2017; Wei et al., 2017), but whether a single intrathecal application of MG produces transient evoked or non-evoked (ongoing) pain is unknown. Therefore, we characterized the effect of intrathecal MG on heat and mechanical sensitivity, and tested whether this produces conditioned place avoidance (CPA), a measure of ongoing affective pain, in conventional C57BL/6J mice. **Figure 2A-C** illustrate that intrathecal MG produced both heat and mechanical hypersensitivity, as well as CPA. Although commercially available MG may contain impurities, comparison of the effect of Sigma MG and MG synthesized in-house via established methods (McLellan et al., 1992; Rabbani and Thornalley, 2014) on pain-like behavior (Supplemental Figure 3) or CNS neuron electrophysiology (18) yielded indistinguishable results. Thus, our results indicate that spinal application of MG, and not the potential impurities contained in commercial solutions, recapitulates key features of PDN observed in patients such as evoked hypersensitivity and stimulus-independent signs of affective pain that are reported as burning or dysesthesia.

FIGURE 2

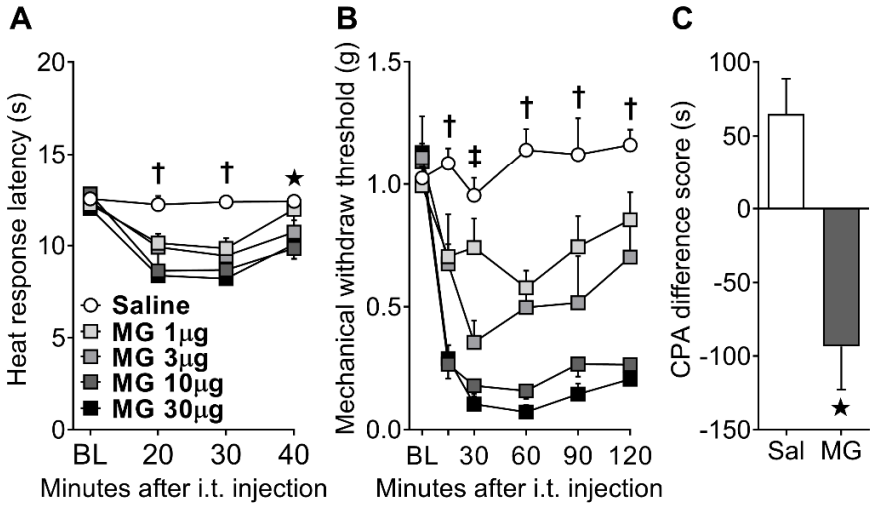

Figure 2. Acute intrathecal administration of methylglyoxal is sufficient to recapitulate multiple behavioral signs of PDN in wildtype mice. A single intrathecal (i.t.) administration of MG but not Saline (Sal) to conventional wildtype C57BL/6J mice dose-dependently decreased **(A)** heat response latencies [MG dose; $F(4,19)=5.29$; $p=0.0049$] with a significant overall effect of 3-30 μg MG [$p<0.05$; two-way ANOVA] (n=4-8) and **(B)** mechanical withdraw thresholds [MG dose; $F(4,19)=52.69$; $p<0.0001$] with a significant overall effect of 1-30 μg MG [$p<0.001$; two-way ANOVA] (n=4-8). **(C)** A single day of conditioning to i.t. MG (10 μg) produced conditioned place avoidance (CPA) as evidenced by a significant difference score (postconditioning minus preconditioning) [$p=0.0009$; unpaired t-test] (n=8). † $p<0.05$ MG 1-30 μg vs. Saline. ‡ $p<0.05$ MG 3-30 μg vs. Saline. ★ $p<0.05$ MG 10μg vs. Saline. BL = baseline behavior prior to drug administration.

Calcium imaging and intrathecal GERP10 administration reveal MG-related spinal sensitization in db/db.

Central sensitization, defined as increased responsiveness of nociceptive neurons in the central nervous system to their normal or subthreshold afferent input, is a critical component of neuropathic pain conditions (Woolf, 2011) and is implicated in PDN (Greig et al., 2014). For example, the somatosensory cortex is altered in patients with diabetic neuropathy (Selvarajah et al., 2019), and spinal mechanisms contribute to PDN in

patients (Selvarajah et al., 2011) and type 2 diabetic rats (Feng et al., 2017; Griggs et al., 2016). Based on the premise that chronically elevated MG in the blood of mice and patients with PDN (Andersen et al., 2018; Bierhaus et al., 2012) could act at central sites of action because MG disrupts the blood-brain barrier (Toth et al., 2014a; Toth et al., 2014b) and is cell-permeable (Allaman et al., 2015), we hypothesized that MG drives spinal sensitization to produce PDN in type 2 diabetes.

To test this hypothesis, we first determined whether MG is sufficient to evoke not only pain-like behavior (Figure 2), but also to activate neurons in pain-processing areas of the central nervous system (e.g. spinal dorsal horn). **Figures 3A-C** illustrate that MG produces intracellular calcium mobilization in the dorsal horn of *ex vivo* spinal cord slices.

Second, we determined whether MG-evoked calcium mobilization in spinal dorsal horn neurons is sensitized in the setting of type 2 diabetes. **Figure 3D** illustrates greater MG-evoked calcium mobilization in *ex vivo* spinal cord slices from db/db mice as compared to BKS controls. In contrast to these studies applying MG to the spinal cord (Figures 3A-D), intraplantar administration of MG did not exacerbate heat hyperalgesia in db/db mice (Supplemental Figure 4A) nor produce a greater magnitude of nociceptive response compared to BKS controls (Supplemental Figure 4B). These results uncover a MG-evoked spinal (but not peripheral) sensitization that contributes to the pain of type 2 diabetes.

Third, we tested the hypothesis that MG in the spinal cord is necessary for painful diabetic neuropathy. **Figures 3E-F** illustrate that intrathecal injection of the MG scavenger GERP10 attenuated established heat hypersensitivity in db/db mice.

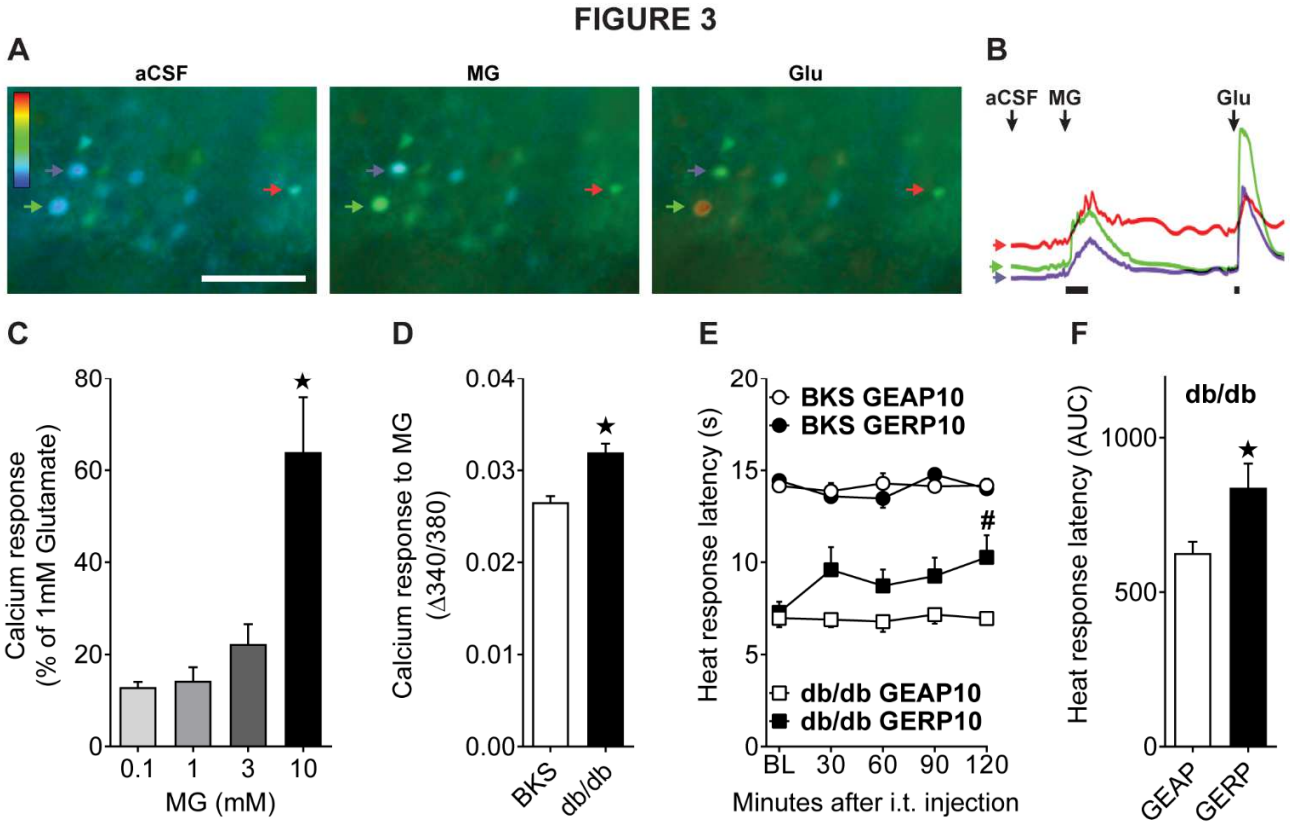

Figure 3. Ex vivo spinal application of methylglyoxal or intrathecal administration of GERP10 reveals MG-related spinal sensitization in db/db. Representative **(A)** images and **(B)** 340/380 signal traces depicting the calcium response to superfusion of artificial cerebrospinal fluid (aCSF) during acclimation, MG for 60 s (long black bar), a 5 min washout period, and then glutamate (Glu; 1 mM) for 5 s (short black bar) in wildtype C57BL/6J mice. Colored arrowheads indicate three cell profiles and their corresponding calcium response traces. **(C)** MG produced a dose-dependent increase in calcium mobilization [MG dose; $F(3,15) = 12.98$; $p = 0.0002$] (n=4-6 mice with calcium responses recorded from multiple neuronal profiles from 1-2 slices per mouse; one-way ANOVA). **(D)** MG-evoked calcium responses were exacerbated in db/db mice compared to BKS controls (n=4 mice; unpaired t-test). **(E)** Intrathecal (i.t.) administration of the peptide MG scavenger GERP10 (100 µg) but not its control peptide GEAP10 (100 µg) increased heat response latencies in db/db mice [peptide in db/db; $F(1,13) = 6.35$; $p=0.0256$] at 120 min [$p<0.05$; two-way ANOVA]. Neither GERP10 nor GEAP10 altered heat response latencies in BKS controls [peptide in BKS; $F(1,14)=1.07$; $p=0.32$] (n=7-8) **(F)** Area under-the-curve analysis of heat response latencies (30-120 min) in panel E indicates an overall anti-

hyperalgesic effect of intrathecal MG in db/db mice [p<0.05; unpaired t-test]. ★ p<0.05 vs. all other groups. **#** p<0.05 db/db GERP10 vs. db/db GEAP10. BL = baseline behavior prior to drug administration.

Genetic or pharmacological inhibition of spinal TRPA1 reduces MG-evoked hypersensitivity

We next interrogated the signaling cascade(s) involved in spinal MG-evoked pain in non-diabetic mice, and then determined the contribution of this signaling to spinally-mediated PDN in type 2 diabetic db/db mice.

Numerous studies implicate peripheral TRPA1 channels in the mechanism of several forms of MG-related neuropathic pain, including PDN in streptozotocin-treated type 1 diabetic rats (Huang et al., 2016; Koivisto et al., 2012), PDN in type 2 diabetic db/db mice (Wang et al., 2018), and MG-evoked nociception and activation of peripheral sensory neurons (Andersson et al., 2013; Eberhardt et al., 2012; Griggs et al., 2017; Ohkawara et al., 2012). But whether central TRPA1 in the spinal cord contributes to PDN in type 2 diabetes remains unclear. To address this question, we used TRPA1 knockout mice and intrathecal administration of the TRPA1 antagonist, HC030031, in the intrathecal MG model. As illustrated in **Figure 4A,** intrathecal administration of MG reduced mechanical withdraw thresholds in TRPA1+/+ but not TRPA1-/- mice. As illustrated in **Figures 4B**, intrathecal administration of HC030031 attenuated MG-evoked decreases in mechanical withdraw thresholds in C57BL/6J mice.

FIGURE 4

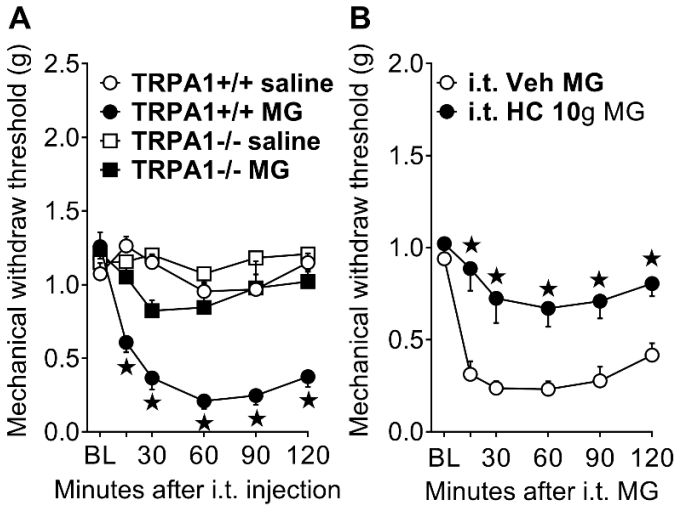

Figure 4. Spinal TRPA1 facilitates methylglyoxal-induced hypersensitivity. (A) Intrathecal administration of MG (10 µg) reduced mechanical withdraw thresholds in control TRPA1+/+ mice [drug in TRPA1+/+; $F(1,13) = 117.3$; $p < 0.0001$] from 15-120 min [$p < 0.0001$]. After intrathecal MG administration, mechanical withdraw thresholds were greater in TRPA1-/- compared to TRPA1+/+ [TRPA1 genotype with MG; $F(1,14) = 97.97$; $p < 0.0001$] at all timepoints tested [$p < 0.0001$] (n=7-8; two-way ANOVA). **(B)** Intrathecal administration of HC030031 (10 µg) 15 min prior to intrathecal MG (10 µg) attenuated MG-evoked decreases in mechanical withdraw thresholds in C57BL/6J mice [TRPA1 antagonist; $F(1,10) = 28.28$; $p = 0.0003$] at all timepoints tested [$p < 0.01$] (n=5-7; two-way ANOVA). ★ $p<0.05$ vs. all other groups. BL = baseline behavior prior to drug administration.

Genetic or pharmacological inhibition of spinal AC1 reduces MG-evoked hypersensitivity

Intrathecal administration of the pan adenylyl cyclase (AC) inhibitor SQ22536 attenuates mechanical and heat hypersensitivity in type 2 diabetic Zucker Diabetic Fatty rats (Feng et al., 2017), suggesting that AC contributes to PDN, but this study did not address which of the 9 adenylyl cyclase isoforms (AC1-9) was involved. Our previous study indicates that pain evoked by intraplantar MG requires AC1 (Griggs et al., 2017), and AC1 in the spinal cord contributes to inflammatory and neuropathic pain (Corder et al., 2013; Wang et al., 2011). Therefore, we tested the hypotheses that spinal AC1 mediates MG-evoked pain by using AC1 knockout (AC1-/-) mice and an AC1 selective antagonist, NB001, in the intrathecal MG model. As illustrated in **Figure 5A,** intrathecal injection of MG decreased mechanical withdraw thresholds in AC1+/+ but not AC1-/- mice. As illustrated in **Figure 5B**, intrathecal injection of NB001 prevented mechanical hypersensitivity induced by intrathecal MG in C57BL/6J mice.

FIGURE 5

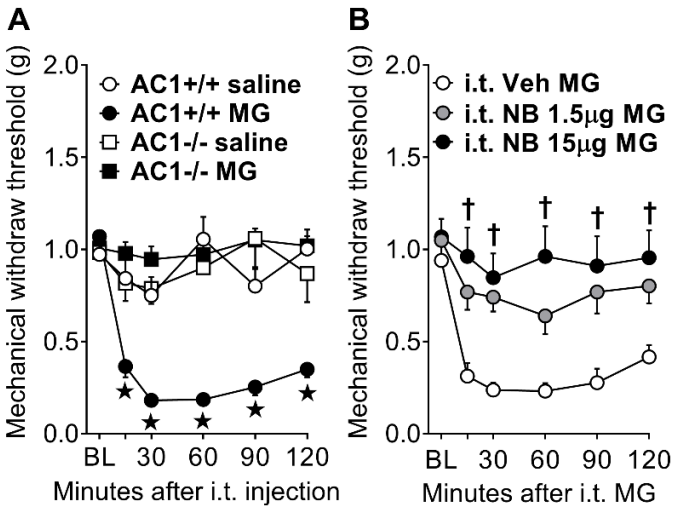

Figure 5. Spinal AC1 facilitates methylglyoxal-induced hypersensitivity. (A) Intrathecal injection of MG (10 µg) decreased mechanical withdraw thresholds in AC1+/+ but not AC1-/- mice [AC1 genotype with i.t. MG; $F(1,18) = 462$; $p < 0.0001$] at 15-120 min [$p < 0.0001$] (n=5-10; two-way ANOVA). **(B)** Intrathecal administration of NB001 (NB 1.5-15 µg) 15 min prior to intrathecal MG (10 µg) attenuated MG-evoked decreases in mechanical withdraw thresholds in C57BL/6J mice [NB001 dose; $F(2,11) = 13.11$; $p = 0.0012$] at 15-120 min [$p < 0.05$] (n=4-5; two-way ANOVA). ★ $p<0.05$ vs. all other groups. † $p<0.05$ i.t. NB 1.5-15µg MG vs. i.t. Veh MG. BL = baseline behavior prior to drug administration.

Intrathecal administration of inhibitors of PKA or Epac1/2 attenuates MG-evoked hypersensitivity

AC1-derived cAMP drives secondary messenger signaling via PKA or Epac. Previous studies indicate that PKA contributes to PDN in Zucker Diabetic Fatty rats (Feng et al., 2017) or streptozotocin mice (Tsantoulas et al., 2017), while Epacs contribute to other types of inflammatory or neuropathic pain (Eijkelkamp et al., 2013; Gu et al., 2016; Huang and Gu, 2017; Hucho et al., 2005; Wang et al., 2013). To test the hypotheses that spinal PKA or Epac1/2 mediate MG-induced nociception, we determined whether intrathecal administration of antagonists of PKA, Epac2, or Epac1/2 would attenuate pain-like behavior in the intrathecal MG model. **Figure 6A** illustrates that the PKA inhibitor H-89 attenuated MG-evoked decreases in mechanical withdraw thresholds.

Figure 6B illustrates that the Epac2 selective inhibitor HJC-0350 (Chen et al., 2013b) attenuated MG-evoked decreases in mechanical withdraw thresholds. **Figures 6C-D** illustrate that the Epac1/2 nonselective inhibitors ESI-09 (Chen et al., 2013a; Zhu et al., 2015) or HJC-0197 (Chen et al., 2012) attenuated MG-evoked decreases in mechanical withdraw thresholds.

FIGURE 6

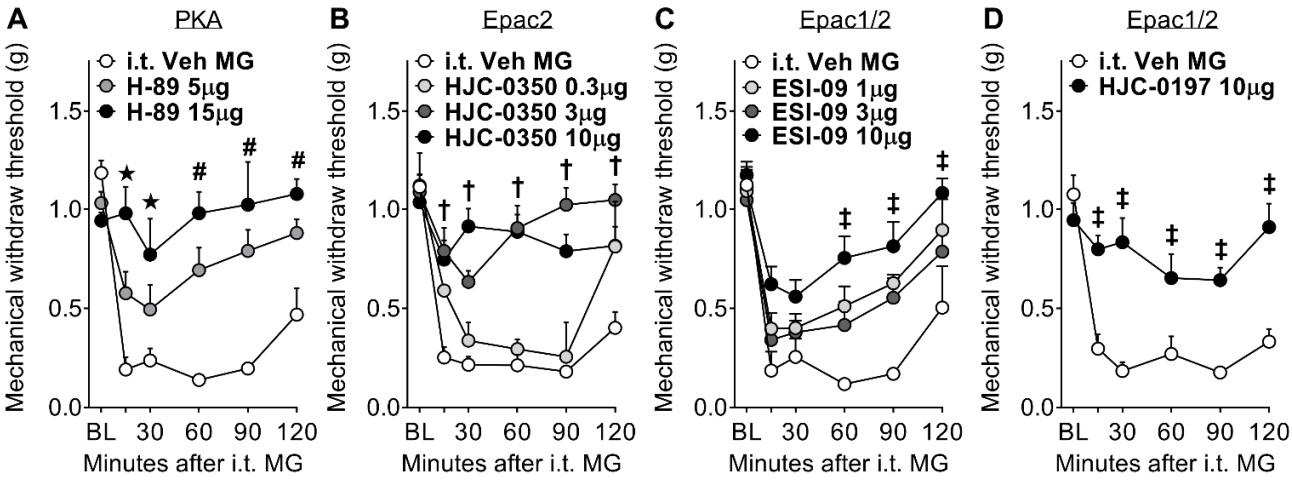

Figure 6. Spinal PKA and Epac1/2 facilitate methylglyoxal-induced hypersensitivity. (A) Intrathecal (i.t.) administration of H-89 attenuated MG-evoked decreases in mechanical withdraw thresholds in C57BL/6J mice [dose; $F(3,13) = 20.29$; $p = 0.0001$] (n=4-6; two-way ANOVA). **(B)** The Epac2 selective inhibitor HJC-0350 attenuated MG-evoked decreases in mechanical withdraw thresholds [dose; $F(3,20) = 53.14$; $p < 0.0001$] (n=2-11; two-way ANOVA). **(C)** The Epac1/2 inhibitor ESI-09 attenuated MG-evoked decreases in mechanical withdraw thresholds [dose; $F(3,18) = 8.176$; $p = 0.0012$] (n=4-9; two-way ANOVA). **(D)** The Epac1/2 inhibitor HJC-0197 attenuated MG-evoked decreases in mechanical withdraw thresholds [dose; $F(1,10) = 49.64$; $p < 0.0001$] (n=6; two-way ANOVA). ★ $p<0.05$ H-89 15 μg vs. i.t. Veh MG. **#** $p<0.05$ H-89 5-15μg vs. i.t. Veh MG. **†** $p<0.05$ HCJ-0350 3-10μg vs. i.t. Veh MG. **‡** $p<0.05$ ESI-09 10μg or HJC-0197 10μg vs. i.t. Veh MG. BL = baseline behavior prior to drug administration.

Intrathecal administration of inhibitors of TRPA1, AC1, and Epac1/2 attenuate heat hyperalgesia in db/db

The above results indicate that intrathecal MG recapitulates multiple signs of PDN (Figure 2) through a spinal signaling cascade involving TRPA1, AC1, PKA, and Epac (Figures 4-6). To address a recent study (Barragan-Iglesias et al., 2018) that questions the physiological relevance of studies administering high concentrations of exogenous MG, and to extend our results to an established model of chronic PDN in type 2 diabetes, we tested whether intrathecal administration of inhibitors of TRPA1, AC1, PKA, and Epac1/2 would also reduce pain-like behavior in db/db mice. **Figure 7A** illustrates that intrathecal administration of the TRPA1 antagonist HC030031 reversed heat hypersensitivity in db/db mice when compared to intrathecal vehicle, without altering heat response latencies in BKS controls. **Figure 7B** illustrates that intrathecal administration of the AC1 antagonist NB001 attenuated heat hypersensitivity in db/db mice, without altering heat response latencies in BKS controls. As **Figure 7C** illustrates, the PKA antagonist H-89 did not change heat response latencies in db/db mice. Heat response latencies in BKS control mice after intrathecal administration of vehicle or H-89 were also similar (Supplemental Figure 5). **Figure 7D** illustrates that the Epac1/2 inhibitor HJC-0197 but not the Epac2 selective inhibitor HJC-0350 attenuated heat hypersensitivity in db/db mice.

FIGURE 7

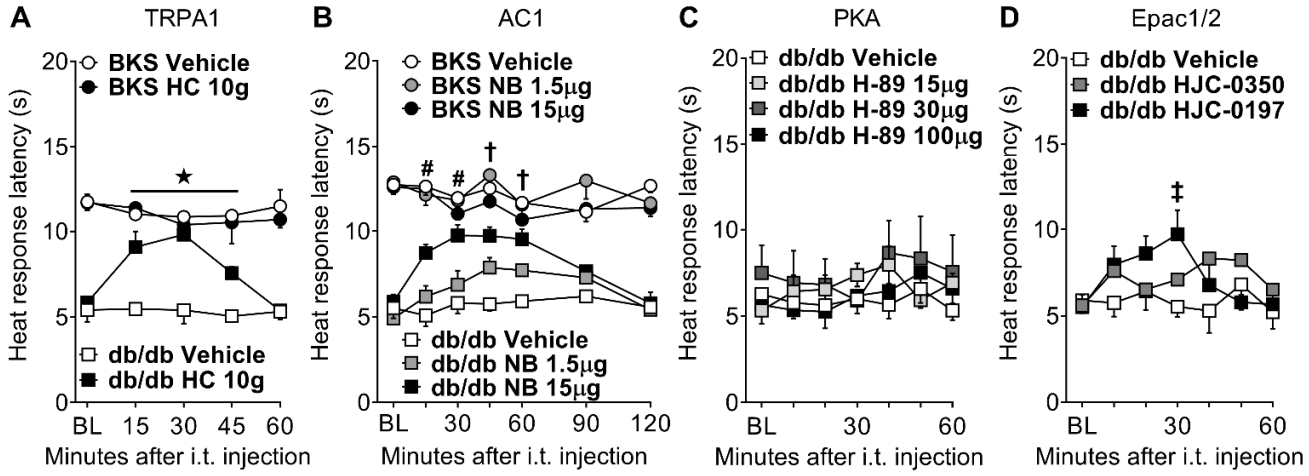

Figure 7. Intrathecal administration of inhibitors of TRPA1, AC1, Epac1/2 but not PKA attenuate heat hyperalgesia in db/db mice. (A) Intrathecal administration of HC030031 (10 μg) transiently reversed heat

hypersensitivity in db/db mice when compared to vehicle [TRPA1 antagonist in db/db; $F(1,6) = 25.29$; $p = 0.0024$] from 15-45 min [$p < 0.01$]. There was no effect of HC030031 in non-diabetic controls (BKS) mice [TRPA1 antagonist in BKS; $F(1,6) = 0.218$; $p = 0.657$] (n=4; two-way ANOVA). **(B)** Intrathecal administration of NB001 transiently attenuated heat hypersensitivity in db/db mice when compared to vehicle [NB001 dose in db/db; $F(2,9) = 21.77$; $p = 0.0004$] at 15-60 min [15 µg NB001; $p < 0.0001$]. There was no effect in BKS at any timepoint tested [NB001 dose in BKS; $F(2,9) = 0.907$; $p = 0.44$] (n=4; two-way ANOVA). **(C)** The PKA inhibitor H-89 (15-100 µg; i.t.) did not alter heat response latencies in db/db at any timepoint tested (n=6). **(D)** The Epac1/2 inhibitor HJC-0197 (10 µg) but not the Epac2 selective inhibitor HJC-0350 (10 µg) transiently increased heat response latencies at 30 min after intrathecal administration [$p=0.03$] (n=6; two-way ANOVA). ★ $p<0.05$ db/db Vehicle vs. db/db HC. **#** $p<0.05$ db/db Vehicle vs. db/db NB001 15µg. **†** $p<0.05$ db/db Vehicle vs. db/db NB001 1.5-15µg. **‡** $p<0.05$ db/db HJC-0197 vs. db/db Vehicle.

DISCUSSION

Here we sought to investigate the MG-related spinal mechanisms that generate and maintain PDN in type 2 diabetes. This is novel, since most preclinical rodent studies of PDN focus on the pathophysiology of peripheral nerve terminals or dorsal root ganglia neurons in the streptozotocin model of type 1 diabetes. We began by showing that systemic administration of either a MG scavenging peptide or a GLO1 overexpression vector reduce hyperalgesia in db/db. Next, we revealed MG-related central sensitization in the spinal cord of type 2 diabetic mice by showing both exacerbated calcium responses to MG in the dorsal horn and reduction of pain-like behavior by intrathecal GERP10 in db/db. Finally, we further addressed the MG-related central signaling pathways of PDN with a dual approach involving db/db mice and a newly-developed model of spinal-mediated hyperalgesia—intrathecal administration of MG. With calcium imaging and behavioral studies that included both pharmacological inhibition and genetic knockouts and two models of diabetes-related pain, we discovered that a novel spinal MG-TRPA1-AC1-Epac pathway mediates pain in type 2 diabetic db/db mice.

Endogenous MG is necessary for painful diabetic neuropathy in type 2 diabetes

Elevated methylglyoxal is a risk factor for developing diabetic polyneuropathy (Andersen et al., 2018) and is correlated with PDN in patients (Bierhaus et al., 2012). We report elevated MG-AGE in serum of db/db mice that exhibit pain-like behavior. This extends previous studies describing elevation of free MG or MG-AGE in serum of patients with diabetes (Andersen et al., 2018; Sveen et al., 2013), the streptozotocin mouse model of type 1 diabetes (Bierhaus et al., 2012), the Zucker Diabetic Fatty rat model of type 2 diabetes (Griggs et al., 2016), and db/db mice (Bierhaus et al., 2012). In non-diabetic subjects, experimental elevation of MG via repeated systemic administration of either a GLO1 inhibitor in mice (Andersson et al., 2013), MG in rats (Liu et al., 2017), or MG in mice (Barragan-Iglesias et al., 2018) produces pain-like hypersensitivity. This suggests that MG is sufficient to cause PDN. A likely source of elevated MG and the corresponding PDN in db/db mice is reduced GLO1 activity. Indeed, reduced GLO1 activity is reported in type 2 diabetic rats (Skapare et al., 2012) and db/db mice (Bierhaus et al., 2012), and is associated with PDN in type 1 and type 2 diabetic patients (Skapare et al., 2013). Interestingly, a recent study reports that upregulation of MG recapitulates the development of type 2 diabetes (Moraru et al., 2018), supporting the idea that MG causes type 2 diabetes. Thus, pharmacotherapeutic strategies to reduce or sequester MG have the potential to mitigate the pathological complications of diabetes, including PDN.

To determine whether endogenous MG is necessary for painful diabetic neuropathy in type 2 diabetes, we used two interventions that have been validated to reduce MG and pain-like behavior in the streptozotocin model of type 1 diabetes (Bierhaus et al., 2012). First, MG scavenging with GERP10 reduced behavioral signs of PDN in db/db mice (Figures 1, 3), consistent with studies showing that GERP10 or other MG scavenging molecules such as aminoguanidine, metformin, or D-arginine reduce hypersensitivity in the streptozotocin model (Bierhaus et al., 2012; Huang et al., 2016). Since our results cannot definitively distinguish a peripheral versus central site of action, further studies are needed to determine whether GERP10 crosses the blood-brain barrier in db/db mice. Nevertheless, these results provide support for the development of novel MG scavenging molecules as a pharmacotherapy for PDN that do not produce the side effects that derailed clinical trials of aminoguanidine (Thornalley, 2003). For example, CycK(Myr)R4E exhibits a long circulatory half-life, rapid scavenging, and reduces MG-AGE and MG-evoked heat hypersensitivity in mice without noticeable side effects (Brings et al., 2017).

Our second intervention to reduce MG, administration of a GLO1 overexpression vector, also attenuated pain-like behavior in db/db mice. This extends a previous study showing that administration of the same GLO1 overexpression vector reduced MG, increased activity of GLO1 in sciatic nerve, and attenuated hyperalgesia in the streptozotocin model (Bierhaus et al., 2012). Other studies indicate a critical contribution of GLO1 activity in PDN. For example, copy number of the *GLO1* gene, elevated in BALB/cByJ mice as compared to closely related BALB/cJ mice, is positively correlated with both GLO1 expression and reduction of streptozotocin-induced peripheral neuropathy (Jack et al., 2012). Similarly in diabetic patients, minor allele single nucleotide polymorphisms in *GLO1* are linked to decreased GLO1 activity (Peculis et al., 2013) and reduced GLO1 activity is associated with PDN (Skapare et al., 2013), suggesting that decreased GLO1 in a subset of diabetic patients could be a risk factor for developing PDN. This work provides a strong rationale for ongoing research aiming to develop new strategies that promote GLO1 activity so as to alleviate PDN in type 2 diabetes. For example, activators of the transcription factor Nrf2 promotes expression of GLO1, protecting against MG-mediated DNA and protein damage in cultured Hep2G cells (Xue et al., 2012). In patients, co-administration of trans-resveratrol and hesperatin increased GLO1 expression and decreased MG by activating Nrf2. (Xue et al., 2016). In db/db mice, the Nrf2 activator sulforaphane alleviated pain-like mechanical hypersensitivity (McDonnell et al., 2017), further supporting Nrf2 as a target for treating PDN in type 2 diabetes. Taken together, these results suggest that endogenous GLO1 is important for protecting against MG-associated PDN.

MG-evoked spinal nociceptive transmission is increased in type 2 diabetes

Neuropathic pain associated with type 2 diabetes remains a difficult condition to treat, in part because the central mechanisms that generate and maintain PDN require further study. To this end, we show that intrathecal administration of MG is sufficient to produce hyperalgesia. This extends previous reports that intraplantar, systemic, and intravenous administration of MG evokes hyperalgesia on a similar time scale (Andersson et al., 2013; Bierhaus et al., 2012; Griggs et al., 2017; Huang et al., 2016; Liu et al., 2017), and is consistent with pain-like behavior evoked by intrathecal MG administration in rats (Liu et al., 2017; Wei et al., 2017). We go beyond these previous studies by showing that intrathecal MG induces affective pain, as evidenced by the production of conditioned place avoidance. In addition, we report that MG increases calcium mobilization in neurons within lamina II of the dorsal horn. This is consistent with MG-evoked calcium

mobilization in peripheral sensory neurons isolated from the dorsal root ganglia (Andersson et al., 2013; Eberhardt et al., 2012). These results suggest that MG activates nociceptive neurons at both spinal and peripheral sites to drive nociceptive transmission in diabetes.

The current results support the hypothesis that MG facilitates central sensitization in the spinal cord to produce pain in type 2 diabetic mice. Compared to control BKS mice, MG-evoked calcium responses were exacerbated in db/db, indicating sensitization of a spinal signaling mechanism that is responsive to MG. This is consistent with the induction of long-term potential of C-fiber-evoked field potentials by local application of MG to the rat spinal dorsal horn (Wei et al., 2017). Further supporting a spinal site of action for endogenous MG-evoked pain, intrathecal administration of: 1) aminoguanidine reduced elevated spinal MG and pain in a rat model of lumbar disk herniation (Liu et al., 2017); 2) metformin, a first line treatment for type 2 diabetes and a putative MG scavenger (Kender et al., 2014), reduced bortezomib-induced increases in spinal MG levels (Wei et al., 2017); and 3) GERP10 attenuated pain-like behavior in db/db (current results). Since GLO1 activity is reduced in the spinal cord of type 2 diabetic rats (Skapare et al., 2012), and circulating levels of MG are increased in diabetes, we speculate that elevated levels of spinal MG contribute to the maintenance of PDN in type 2 diabetes. However, further studies in db/db mice are needed to determine: i) MG levels in spinal cord tissue and cerebrospinal fluid, and ii) the effect of both systemic and intrathecal delivery of GLO1 inducers (e.g. resveratrol/hesparatin, sulphoraphane) on pain-like behavior and spinal MG and GLO1 activity levels.

The millimolar (mM) concentrations of MG injected intrathecally or applied to spinal cord slices in the current results are supraphysiological when compared to the micromolar (μM) concentrations of MG reported in human blood or tissues. Perhaps these high concentrations of exogenous MG are needed to overcome intact glyoxalase catabolism in non-diabetic mice to achieve the 5-10 fold increase of MG reported in the plasma of patients with PDN. Supporting this idea, intravenous administration of MG (0.5-4.6 mM) increased the concentration of MG in the plasma of non-diabetic wildtype mice by only 2-fold (Bierhaus et al., 2012), suggesting that intrathecal administration of 14 mM MG (Figure 2) or superfusion of 10 mM MG (Figure 3) could recapitulate a physiologically relevant increase (5-10 fold) in plasma MG reported in PDN patients (Bierhaus et al., 2012). Nevertheless, to address this limitation of our intrathecal MG model, we conducted corollary studies in the db/db model of PDN in type 2 diabetes.

A spinal TRPA1-AC1-PKA/Epac cascade contributes to MG-evoked pain during type 2 diabetes

TRPA1

Previous studies suggest that peripheral TRPA1 channels mediate streptozotocin-induced hyperalgesia (Wei et al., 2009), but this interpretation was confounded by recent evidence that streptozotocin directly activates TRPA1 (Andersson et al., 2015), and thus required validation in a model of type 2 diabetes (Feldman et al., 2017). Our approach using intrathecal MG and db/db models avoided these pitfalls and demonstrated that pharmacological or genetic inhibition of *spinal* TRPA1 reduced pain-like behavior. This indicates that spinal MG-TRPA1 activity contributes to neuropathic pain associated with type 2 diabetes. The spinal TRPA1 site of action is unclear, but could be at the central terminals of the primary afferent neuron or involve astrocytes. Astrocytic TRPA1 was previously shown to regulate basal calcium in astrocytes (Shigetomi et al., 2011) and contribute to long-term potentiation in the hippocampus (Shigetomi et al., 2013). TRPA1-immunoreactivity is present in the spinal dorsal root, suggesting transport from the DRG to central terminals (Anand et al., 2008). Furthermore, activity-dependent release of the reactive metabolites 5,6-EET (Sisignano et al., 2012) or HxA3 (Gregus et al., 2012) or superfusion of AITC (Sisignano et al., 2012) activates presynaptic TRPA1 at the central terminals of primary afferents, increasing glutamatergic input and the frequency of spontaneous excitatory post-synaptic potentials in lamina I-II neurons of the spinal cord dorsal. Whether TRPA1 mediates the exacerbated calcium response evoked by MG in the db/db spinal dorsal horn (Figure 3) remains an interesting future direction. Nevertheless, reduction of pain evoked by intrathecal MG or in the db/db model by spinal application of HC030031 supports further investigation of PDN therapies targeting the inhibition of spinal TRPA1.

AC1

Spinal AC1 contributes to latent pain sensitization and the spinal mechanisms of chronic pain (Corder et al., 2013; Wang et al., 2011). We now report that either intrathecal administration of the AC1 inhibitor NB001 or genetic deletion of AC1 prevented mechanical hypersensitivity induced by intrathecal MG. Furthermore,

intrathecal NB001 attenuated pain-like behavior in db/db mice, implicating AC1 in the maintenance of PDN in type 2 diabetes. NB001 also inhibits pain associated with nerve injury (Wang et al., 2011) or inflammation (Corder et al., 2013), probably through reducing the AC1-mediated overproduction of cAMP and its downstream signaling in response to painful stimulation (Corder et al., 2013). Although we did not measure spinal cAMP in db/db or after intrathecal MG, intrathecal administration of a pan AC inhibitor attenuated elevated cAMP in the spinal cord and pain-like behavior in Zucker Diabetic Fatty rats (Feng et al., 2017). Taken together, these results suggest that spinal AC1 superactivity contributes to PDN in type 2 diabetes.

PKA/Epac

PKA and Epac1/2 are two potential effectors of AC1-derived cAMP production and thus hypersensitivity in PDN. Increased activation of PKA in the spinal cord is reported in Zucker Diabetic Fatty rats (Feng et al., 2017), suggesting that spinal PKA contributes to PDN in type 2 diabetes. We found that pretreatment with intrathecal H-89 alleviated mechanical hypersensitivity evoked by intrathecal MG. However, intrathecal administration of H-89 did not alter already-established hyperalgesia in db/db mice. These seemingly discrepant results might be explained by the timing of PKA antagonism: preventing MG-evoked pain sensitization with pretreatment of H-89 in the intrathecal MG model versus targeting ongoing, established sensitization in db/db. This suggests that spinal PKA is important for the initiation of painful sensitization, but not maintenance. Alternatively, it may be that chronic suppression of PKA is needed to reduce PDN. To test these alternative explanations, a future study could determine whether chronic inhibition of PKA during the early stages of hyperglycemia (e.g. circa 6 weeks of age) alters the progression of hyperalgesia in db/db.

Despite studies implicating Epacs in various pain conditions (Aley and Levine, 1999; Eijkelkamp et al., 2013; Gu et al., 2016; Huang and Gu, 2017; Hucho et al., 2005; Matsuda et al., 2017; Wang et al., 2013), including hyperalgesic priming in the DRG and peripheral nerve (Huang and Gu, 2017; Wang et al., 2013), the contribution of spinal Epac to pain and/or whether Epacs mediate PDN remained unclear. The current findings address these gaps with the intrathecal administration of newly-developed inhibitors of Epac isoforms (Chen et al., 2013a; Chen et al., 2013b; Chen et al., 2012; Chen et al., 2014; Wang et al., 2017; Zhu et al., 2015). Our data demonstrates inhibition of MG-evoked hypersensitivity by intrathecal administration of HJC-0350, ESI-09, and HJC-0197, extending the therapeutic targeting of Epac1/2 isoforms to the dorsal horn of the spinal cord.

Because HJC-0197 (nonselective Epac1/2 inhibitor) but not HJC-0350 (Epac2 selective inhibitor) attenuated hyperalgesia in db/db, we conclude that Epac1 is a critical isoform that contributes to spinally-mediated PDN in type 2 diabetes. This is consistent with previous studies in pain models of inflammation, nerve-injury, or plantar incision indicating that pharmacological inhibition, genetic knockout, or antisense oligodeoxynucleotide knockdown of Epac1 reduced peripherally-mediated pain-like behavior (Eijkelkamp et al., 2013; Gu et al., 2016; Matsuda et al., 2017; Wang et al., 2013). A caveat is that knockout of Epac1 in mice and embryonic stem cells disrupts pancreatic β-cells and glucose-stimulated insulin secretion, exacerbating streptozotocin-induced type 1 diabetes (Kai et al., 2013). Perhaps new Epac inhibitors could be designed to target Epac1 at locations specific to the nervous system to avoid off-target effects within the pancreas. Nevertheless, our findings provide support for further investigation of the contribution of spinal Epac1 to PDN in type 2 diabetes.

TRPA1-AC1-PKA/Epac signaling cascade.

Our current results suggest that TRPA1 activity initiates AC1-PKA and/or AC1-Epac signaling cascades that maintain MG-evoked pain. In support of our proposed linkage between TRPA1-derived calcium, AC1, and PKA, either removal of extracellular calcium, application of an AC antagonist, or application of H-89 each blocked the potentiation of capsaicin-evoked DRG neuron activity mediated by TRPA1 (Spahn et al., 2014). In support of Epacs as a downstream effector, AC1-derived cAMP drives inflammatory pain via peripheral Epac1/2 (Hucho et al., 2005). We suggest that complex protein-protein interactions between intracellular effector molecules (e.g. AC, PKA, Epac) and their putative extracellular signal transduction elements (e.g. MG-TRPA1 activity) promotes hyperexcitability and pain. Interestingly, a recent study reported that the scaffolding protein A-kinase anchoring protein (AKAP) mediates phosphorylation of TRPA1 by PKA (Brackley et al., 2017). Furthermore, the generation of hyperalgesic priming required both TRPA1 and AKAP (Brackley et al., 2017). This suggests that TRPA1, AKAPs, and PKA work together to contribute to the initiation of persistent painful sensitization. AKAPs may also mediate subcellular colocalization of AC and Epacs to produce pain (Huang and Gu, 2017). Whether the maintenance of complex protein-protein interactions by AKAPs contributes to PDN remains an interesting future direction.

Conclusions

Despite decades of research, diabetic neuropathy continues to be complicated by neuropathic pain in up to 50% of patients (Abbott et al., 2011). The current study aimed to establish novel targets for the development of new therapies for PDN. First, we establish the therapeutic utility of MG scavengers and GLO1 enhancers in a mouse model of type 2 diabetes, extending a study in streptozotocin-evoked type 1 diabetes (Bierhaus et al., 2012). This is important because prior preclinical studies of PDN tended not to distinguish between type 1 and type 2 patient classes (Callaghan et al., 2012a; Feldman et al., 2017), despite the fact that rigorous glycemic control reduces neuropathy in type 1 but not type 2 diabetic patients (Callaghan et al., 2012b). Second, our results are the first to identify multiple targets within the spinal cord (e.g. the inhibition of MG, TRPA1, AC1, Epac) as potential therapies for PDN associated with type 2 diabetes. This is in line with a recent report highlighting the need to identify the central mechanisms that drive neuropathic pain in diabetes (Feldman et al., 2017).

Our results indirectly support the combination of resveratrol and hesperatin treatment for PDN, as this induces GLO1 activity in patients (Xue et al., 2016), as well as metformin, an FDA-approved anti-diabetic treatment, which acts as an MG scavenger to reduce MG levels in type 2 diabetic patients (Beisswenger et al., 1999; Kender et al., 2014) and attenuates pain-like behavior in streptozotocin rats (Ma et al., 2015). Interestingly, a recent study indicates that metformin ameliorates both the upregulation of DRG membrane TRPA1 and hypersensitivity in db/db (Wang et al., 2018), adding support to targeting TRPA1 inhibition as a pharmacotherapy for PDN in type 2 diabetes. We conclude that MG produces evoked hypersensitivity and affective pain, and sensitization of a spinal TRPA1-AC1-Epac signaling cascade facilitates hyperalgesia in db/db mice, supporting further clinical study of agents targeting this pathway in type 2 diabetic patients with pain.

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AUTHOR CONTRIBUTIONS

RBG designed experiments, collected data, analyzed the data, and wrote the manuscript. DEL, SD, RRD, WF, CW, DFS, GPS contributed to the data collection. PW and JZ contributed the Epac inhibitors ESI-09, HJC-0350, and HJC-0197 and JZ reviewed the manuscript. SB, TF, and PPN contributed GERP10, GEAP10, in-house synthesized MG, and the GLO1 overexpression and control vectors and reviewed the manuscript. KS and BKT designed experiments and revised the manuscript.

DECLARATION OF INTEREST

TF and PPN are inventors on patent WO2010136182 entitled “Methylglyoxal-scavenging compounds and their use for the prevention and treatment of pain and/or hyperalgesia.” JZ is a co-inventor on patent EP2811990B1 entitled “Modulators of exchange proteins directly activated by cAMP (Epacs)”. RBG, DEL, SD, RRD, CW, WF, DFS, GPS, PW, KS, and BKT declare no competing financial interests.

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