RESEARCH ARTICLE

mGluR2/3 activation of the SIRT1 axis preserves mitochondrial function in diabetic neuropathy

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Abstract

Objectives: There is a critical need to develop effective treatments for diabetic neuropathy. This study determined if a selective mGluR2/3 receptor agonist prevented or treated experimental diabetic peripheral neuropathy (DPN) through glutamate recycling and improved mitochondrial function. **Methods:** Adult male streptozotocin treated Sprague-Dawley rats with features of type 1 diabetes mellitus (T1DM) or Low Capacity Running (LCR) rats with insulin resistance or glucose intolerance were treated with 3 or 10 mg/kg/day LY379268. Neuropathy end points included mechanical allodynia, nerve conduction velocities (NCV), and intraepidermal nerve fiber density (IENFD). Markers of oxidative stress, antioxidant response, glutamate recycling pathways, and mitochondrial oxidative phosphorylation (OXPHOS) associated proteins were measured in dorsal root ganglia (DRG). **Results:** In diabetic rats, NCV and IENFD were decreased. Diabetic rats treated with an mGluR2/3 agonist did not develop neuropathy despite remaining diabetic. Diabetic DRG showed increased levels of oxidized proteins, decreased levels of glutathione, decreased levels of mitochondrial DNA (mtDNA) and OXPHOS proteins. In addition, there was a 20-fold increase in levels of glial fibrillary acidic protein (GFAP) and the levels of glutamine synthetase and glutamate transporter proteins were decreased. When treated with a specific mGluR2/3 agonist, levels of glutathione, GFAP and oxidized proteins were normalized and levels of superoxide dismutase 2 (SOD2), SIRT1, PGC-1α, TFAM, glutamate transporter proteins, and glutamine synthetase were increased in DRG neurons. **Interpretation:** Activation of glutamate recycling pathways protects diabetic DRG and this is associated with activation of the SIRT1-PGC-1α–TFAM axis and preservation of mitochondrial OXPHOS function.

Introduction

There is currently no specific medication that prevents or reverses diabetic neuropathy in humans. Mitochondria (Mt) are critical regulators of neuronal survival. Reduced Mt bioenergetic function and increased oxidative stress are significant contributors in diabetic neuropathy.[1–7] Key transcription factors, for example, the silent information regulator protein 1 (SIRT 1) - Peroxisome proliferator-activated receptor-gamma coactivator 1α (PGC-1α) - mitochondrial transcription factor A (TFAM) axis, are critical in regulating Mt function in response to oxidative stress in experimental diabetic neuropathy.[1,4,8] SIRT1 in neurons is neuroprotective,[9,10] is important in axonal regeneration,[11] and deacetylates PGC-1α. The 35-kDa PGC-1α isoform is critical for reducing oxidative stress and regulation of key Mt β-oxidation enzymes.[4,12,13] The transcription factor activated by SIRT1- PGC-1α is TFAM, which promotes replication and transcription of mitochondrial DNA (mtDNA), wraps Mt DNA to protect it from oxidative stress and reduces experimental diabetes-induced neuropathy.[8,14–16]

Oxidative stress, resulting from diabetes, can also result in di-sulfide bridges in the cysteine residues of the glutamate transporters causing an inhibition of glutamate flux that results in elevated levels of extracellular glutamate.[17,18] Increased extracellular glutamate, in turn, reverses the cysteine/glutamate antiporter, decreases synthesis of glutathione (GSH or gamma glutamyl-cysteine-glycine), increases mtDNA damage, and impairs Mt oxidative phosphorylation systems (OXPHOS).[19,20] In contrast, we have previously shown in cell culture that selective activation of the metabotropic glutamate 2/3 receptor (mGluR2/3) prevents dorsal root ganglion neuronal (DRG) and Schwann cell oxidative injury through regulation of oxidative stress pathways and by maintaining GSH levels.[21,22] The neuronal protection occurs only in the presence of Schwann cells or satellite glial cells (SGC) that act to mediate glutamate uptake.[23–27] Other mGluRs, for example mGluR5, may result in increased nociceptive transmission in diabetic neuropathic pain.[28] However, it is not known if mGluR2/3 agonists can prevent experimental diabetic neuropathy, if they result in glutamate recycling, and how mGluR2/3 agonists affect critical Mt regulatory transcription factors such as the SIRT1-PGC-1α-TFAM axis and components of Mt OXPHOS.

Research design and methods

Experimental animals, diabetes induction and drug treatment

The animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee and conform to U.S. National Institutes of Health guidelines. Twelve-week-old male Sprague Dawley (SD) rats (Harlan, Indianapolis, IN) were injected with intraperitoneal (i.p) saline or with a single dose of streptozotocin (STZ) at 45 mg/kg and diabetes was confirmed (glucose>300 mg/dl; =16.7 mmol/L) as previously described.[29,30] Animals were randomly divided into the following groups with eight animals in each group: (1) nondiabetic control/saline (2) diabetes (3) diabetes treated with LY 379268 (3 mg/kg) (4) diabetes + LY 379268 (10 mg/kg) (5) nondiabetic + LY 379268 (3 mg/kg). Daily treatment with intraperitoneal LY 379268 (Tocris #2453) was started immediately after animals became diabetic and continued for 8 weeks. Treated nondiabetic animals received only 3 mg/kg LY 379268 because of the cost and limited availability of the drug.

Low Capacity Running (LCR) and High Capacity Running (HCR) rats derived from genetically heterogeneous N: NIH stock rats by artificial selection for low and high treadmill running capacity were used as described.[12] Aged male LCR and HCR rats (~12 months old) from generation 21 of selection were used for this study.[31] LCR and HCR rats were treated either with vehicle (saline) or with the previously established effective dose of LY379268 (10 mg/kg) for 8 weeks, once the animals had already developed neuropathy.

Neuropathy measurements

Mechanical allodynia was assessed using Somedic von Frey monofilaments using the Semmes-Weinstein series (Somedic Sales AB), sciatic-peroneal and tail nerve conduction studies were measured as previously described.[32,33] Briefly, rats were anesthetized with ketamine 80–90 mg/kg, and Xylazine 5–10 mg/kg and the animal was provided with thermal support and warming lamps. Electrodes were placed adjacent to the nerve to obtain near nerve recordings using a 60–80 mA square wave stimulus for 0.1–0.3 msec. NCS were performed in the tail and left hindlimb using platinum electrodes adjacent to the nerve to obtain near nerve recordings. The G1 (active) and G2 (indifferent) recording electrodes were separated by a distance of 10 mm. Tail NCS were recorded over a 9 cm distance measured from the base of the tail. Proximal nerve conduction velocities were measured over the proximal 5 cm from the base of the tail, and distal nerve conductions over the terminal 4 cm. Orthodromic motor conduction velocities were obtained by recording at the tip of the tail and stimulating with the cathode 4 and 9 cm proximal to the G1 recording electrode and subtracting the corresponding latencies. Orthodromic sensory tail conduction velocities were obtained by placing G1 at the base of the tail and stimulating 4 and 9 cm distally. Sensory responses were

averaged until the sensory nerve action potential response was stable. Distal tail motor and sensory nerve conduction velocities are reported in the tables. Tail and limb temperatures were maintained at 32–33°C. The onset latency and peak amplitude were measured. Mechanical allodynia was assessed using Somedic von Frey monofilaments using the Semmes-Weinstein series (Somedic Sales AB). Ordinal numbers above 4 were applied gently on the fat part of the both plantar heels until the hair started to bend and maintained for approximately 2 sec. The threshold was defined as the minimal bending force of the thinnest filament sensed by the rat in an ascending and descending series of applications. Intraepidermal nerve fiber density (IENFD) was measured using PGP9.5 antibody staining in a blinded fashion as previously described.[4,33] IENFD was calculated (as fibers/mm) by the number of complete baseline crossings of nerve fibers at the dermo-epidermal junction divided by the calculated length of the epidermal surface.

Tissue collection

At experiment termination, blood was collected; plasma glycosylated hemoglobin (GHb), insulin, and lipid profiles were measured. The rats were euthanized and DRG, sciatic nerve (SN), and hind paw skin were collected as described.[32,34] We used the whole DRG, which includes DRG neurons with surrounding satellite cells. Western blot analysis of proteins was performed as previously described.[4,8,12] L3 to L5 DRG obtained from the experimental groups were processed with a tissue lyser (Qiagen Inc.). DRG tissues were homogenized in a buffer containing 50 mmol/L Tris-HCl with 1% Triton X-100 and protease inhibitors followed by centrifugation at 13,000g. Protein samples (50 μg) were separated on a 4–20% gradient gel and transferred to PVDF membranes. The membranes were incubated overnight at 4°C. The source and dilution of the various antibodies used in this study are shown (Table S1). The membranes were then treated with the peroxidase-conjugated secondary antibody (Calbiochem). Immunostained proteins were detected by using a SuperSignal chemiluminescence kit (Pierce) and an Alpha Innotech imaging system. Protein intensity was normalized to beta actin.

Mitochondrial DNA Quantification

The ratio of mitochondrial DNA (mtDNA to nuclear DNA (nDNA) was determined by copy number using a quantitative PCR method.[4,12] Genomic DNA was isolated from the L5 DRG neurons. Mitochondrial DNA copy number was determined by PCR of two mtDNA targets: a 197-bp ND1 gene and a 199-bp Cyt B gene and the Ct

values were compared with a standard plasmid carrying ND1 and CytB mtDNA fragments. Nuclear DNA copy number was determined by PCR for a nuclear DNA 175-bp B2M gene target and the Ct values were compared with a plasmid carrying a B2M nuclear gene fragment. The ratio of mtDNA/nDNA was calculated by 2X (ND1 Copies/20 ng DNA)/(B2M Copies/20 ng DNA); and by 2 X (Cyt B Copies/20 ng DNA)/(B2M Copies/20 ng DNA).

Glutathione measurement

DRG samples were treated with 5% (v/v) sulfosalicylic acid and centrifuged at 7800g for 10 min to remove denatured proteins, and reduced glutathione (GSH) and oxidized glutathione (GSSG) were determined by an enzymatic method. The total glutathione (GSH + GSSG) content was assayed in a cuvette containing 90 μL of the supernatant in 0.1 mol/L sodium phosphate buffer (pH 7.5), 3 mmol/L 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) and 0.115 unit/mL glutathione reductase in a final volume of 1 mL. After 5 min of incubation at room temperature, 2 mmol/L NADPH was added and the kinetics of the reaction was monitored for 5 min. The increment in absorbance at 412 nm was converted to GSH concentration using a standard curve with known amounts of GSH.

Statistical analysis

Comparison of dependent variables was performed on transformed data using factorial analysis of variance (ANOVA) with post hoc Tukey test to determine the significance among the groups and individual comparisons were made using students T-test, assuming unequal variances as previously described.[32] The associations between the index of oxidative stress (DNP immunoreactivity, SOD2, glutathione levels) and measures of neuropathy (NCV and mechanical allodynia) were evaluated using Spearman correlation statistics.

Results

Blood parameters in nondiabetic and diabetic animals with and without LY379268 treatment

At the termination of the STZ diabetes experiment, plasma glucose, glycosylated hemoglobin (GHb), insulin, and lipid values were measured (Table 1). The mean plasma glucose, glycosylated hemoglobin, and lipid profile were significantly increased and insulin level significantly decreased in diabetic compared to non-diabetic control animals. The plasma glucose and glycosylated hemoglobin

were not significantly altered with LY379268 treatment. However, in diabetic rats treated with a high dose of 10 mg/kg/day LY379268, lipid measurements were decreased compared to nontreated diabetic rats. Although total cholesterol and HDL decreased, the most significant relative decrease was in triglycerides [diabetic: 636 ± 74.3 mg/dL *versus* Diabetic + LY (10): 311 ± 52.4 mg/dL ($P < 0.05$)]. LY379268 treated diabetic rats still had significantly higher lipid levels than nondiabetic animals. We did not observe overt changes in behavior and visual appearance in rats treated with LY379268 up to 10 mg/kg/day.

Aged LCR rats were significantly more obese than HCR rats.[12] LCR rats had impaired glucose tolerance (IGT), higher fasting blood glucose levels, increased cholesterol and LDL levels compared to HCR rats (Figure S1, Table 2). Treatment with LY379268 (10 mg/kg) improved the metabolic profile of LCR rats. Rats showed decreased body weight, increased insulin levels and decreased levels of triglycerides and LDL (Table 2).

Nerve conduction, sensory testing, and intraepidermal nerve fiber density measurements to evaluate neuropathy in diabetic rats with and without LY379268 treatment

The following studies determined the effect of the mGluR2/3 agonist on neuropathy. After 8 weeks of diabetes, STZ treated rats showed a significant slowing of tail motor and sensory and sciatic nerve (SN) motor nerve conduction velocities (NCVs) compared with nondiabetic control rats (Table 1) consistent with the development of

diabetic peripheral neuropathy DPN. In contrast, the diabetic animals treated with LY 379268 (3 & 10 mg/kg/day) had preserved NCVs and the velocities were comparable to nondiabetic rats consistent with protection against DPN by an mGluR2/3 agonist. LY 379268 (10 mg/kg/day) more effectively prevented neuropathy than 3 mg/kg/day and was used in subsequent studies. In the case of LCR rats, treatment with LY379268 for 8 weeks improved the metabolic profile and nerve conduction velocities (Table 2).

After 4 weeks of STZ-induced diabetes, there was a significant decrease in withdrawal threshold as compared to control rats (Fig. 1A; $P < 0.001$) that remained the same to 8 weeks, consistent with tactile allodynia. Diabetic animals treated with LY 379268 (3 & 10 mg/kg) had significantly reduced tactile allodynia ($P < 0.001$) at both 4 and 8 weeks after initiation of treatment. With high dose LY379268 (10 mg/kg/day), the diabetic rats showed an increased threshold to mechanical allodynia possibly due to the identified analgesic effect of LY379268 on nociception via TRP channels in the hind paw.[35] LCR rats showed evidence of mechanical allodynia at the start of the study (prior to giving LY 379268) consistent with the presence of neuropathy (Fig. 1B). When they were treated with high dose LY379268 (10 mg/kg/day), their tactile allodynia threshold improved to almost the level of HCR rats.

Examination of nerve fibers in skin biopsies from STZ-induced diabetic rats showed a significant decrease in the IENFD (Table 1 and Fig. 2A) compared to nondiabetic rats. Intra-epidermal fiber density was lower in diabetic than in control rats (9 ± 0.6/mm vs.17 ± 0.6/mm; $P < 0.001$). The IENFD was 15.8 ± 0.7/mm in treated animals ($P < 0.01$ compared to diabetic).

Table 1. Metabolic and neuropathy end-points in nondiabetic, diabetic and LY379268-treated diabetic rats at 2 months

Parameters	Control	Diabetic	Diabetic +LY (3)	Diabetic +LY (10)	Control +LY (3)
Body weight (g)	534 ± 9.7	396 ± 29.2***	366 ± 16.8***	352 ± 14.4***	504 ± 10.5
Plasma glucose (mg/dL)	88 ± 4	535 ± 22***	495 ± 11.4***	544 ± 23.2***	80 ± 2.4
% GHb	9.8 ± 0.25	14 ± 0.35***	14 ± 0.82***	14.1 ± 0.6***	8.8 ± 0.74
Insulin (μU/mL)	8 ± 1.45	2 ± 0.02**	2 ± 0.14**	2 ± 0.29**	7 ± 1.28
Total cholesterol (mg/dL)	64 ± 4.39	166 ± 27.5**	162 ± 24.7**	120 ± 14.6**	72 ± 2.7
Triglycerides(mg/dL)	71 ± 15	636 ± 74***	700 ± 48***	$311 \pm 52.4^{+}$	117 ± 12.6
HDL (mg/dL)	23 ± 1.25	43 ± 2.43***	$28 \pm 3.39^{\#\#}$	$32 \pm 2.15^{\#\#}$	25 ± 0.87
LDL (mg/dL)	7 ± 0.16	35 ± 4.9***	27 ± 4.3***	29 ± 6.6***	8 ± 10.4
Tail MNV (m/s)	39 ± 1	35 ± 0.8*	$39 \pm 0.5^{\#}$	$39 \pm 1^{\#}$	37 ± 1
Tail SNV (m/s)	55 ± 2	44 ± 1*	$55 \pm 1^{\#}$	$61 \pm 2^{\#}$	53 ± 2
Sciatic MCV (m/s)	57 ± 2	46 ± 2***	50 ± 3	$56 \pm 1^{\#\#\#}$	54 ± 3
IENFD (mm)	17 ± 0.6	6 ± 0.2***	$9 \pm 0.6^{\#}$	$15.8 \pm 0.7^{\#\#}$	18 ± 0.8

LDL, Low Density Lipoprotein; HDL, High Density Lipoprotein; MNV, Motor Nerve Conduction Velocity; SNV, Sensory Nerve Conduction Velocity; IENFD, Intraepidermal Nerve Fiber Density.
*$P < 0.05$, **$P < 0.01$, & ***$P < 0.001$ diabetic compared to nondiabetic rats, $^{+}P < 0.05$, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ & $^{\#\#\#}P < 0.001$ LY379268-treated diabetic as compared to untreated diabetic animals.

Table 2. Metabolic and neuropathy end-points in LCR, HCR and LY379268-treated LCR and HCR rats at 2 months.

Parameters	LCR	HCR	LCR+LY (10)	HCR+LY (10)
Body weight (g)	636 ± 1.3***	406 ± 13	550 ± 32#	387 ± 29
Fasting glucose (mg/dL)	84 ± 6.8***	60 ± 3	79 ± 4	60 ± 3
Insulin (ng/mL)	0.93 ± 0.4**	0.62 ± 0.19	0.71 ± 0.1##	0.57 ± 0.12
Total cholesterol (mg/dL)	143 ± 8**	60 ± 7	109 ± 12	62 ± 7
Triglycerides (mg/dL)	98 ± 11	85 ± 9	83 ± 7#	83 ± 6
HDL (mg/dL)	32.4 ± 1.8	30.6 ± 3.6	39.87 ± 14.4	32 ± 4.9
LDL (mg/dL)	104 ± 29.4	29.34 ± 1.6	84.19 ± 3.8	28.33 ± 3.2
Tail MNV (m/s)	34 ± 1**	42 ± 1	39 ± 1#	40 ± 1
Tail SNV (m/s)	44 ± 3**	57 ± 1	53 ± 2#	58 ± 2
Sciatic MNV (m/s)	49 ± 2**	59 ± 1	55 ± 1#	59 ± 1
IENFD (mm)	9 ± 0.7***	16.7 ± 1	15.7 ± 1##	17 ± 1

LDL, Low Density Lipoprotein; HDL, High Density Lipoprotein; MNV, Motor Nerve Conduction Velocity; SNV, Sensory Nerve Conduction Velocity; IENFD, Intraepidermal Nerve Fiber Density.

*$P < 0.05$, **$P < 0.01$ & ***$P < 0.001$ LCR compared to HCR; +$P < 0.05$, #$P, 0.05$ & ##$P < 0.01$ LY379268-treated LCR as compared to untreated LCR animals.

In aged LCR rats there was a significant decrease in the IENFD in LCR rat paw skin compared to HCR rats (Table 2 and Fig. 2B) and treatment with LY379268 (10 mg/kg) for 2 months increased the IENFD from 9/mm to 16/mm ($P < 0.01$). Thus, LY379268 prevented neuropathy in an STZ model of T1DM experimental neuropathy and treated neuropathy in a LCR IGT/hyperinsulinemic model.

Oxidative stress and oxidative stress regulatory proteins (SIRT1, PGC1-α, and TFAM) with and without LY379268 treatment

To study the mechanism of protection by LY379268 treatment, we prepared DRG protein extracts and DNA from non-diabetic, STZ diabetic and LY379268-treated diabetic rats. The presence of protein carbonyls measured by 2, 4-dinitrophenylhydrazine (DNPH) reactivity was used as a marker of oxidative stress.[36] Western blot revealed a significantly increased DNP immunoreactivity in DRG from diabetic rats as compared with nondiabetic animals (Fig. 3A and B, ***$P < 0.001$). Treatment with LY379268 (10 mg/kg) in diabetic animals significantly decreased DNP to control levels (Fig. 3B ###$P < 0.001$). Western blot analysis for the mitochondrial antioxidant protein SOD2 is shown in Figure 3C. SOD2 levels were not decreased in diabetic animals, but the level was increased in LY379268 (10 mg/kg) treated DRG (Fig. 3C, ##$P < 0.01$) compared to diabetic animals. The levels of SOD2 also increased in LY379268 (3 mg/kg) treated control rats compared to untreated control animals (#$P < 0.05$). Measurement of total glutathione levels (Fig. 3D) showed a significant decrease in diabetic DRG (***$P < 0.001$) and the levels were normalized in LY379268 treated diabetic DRG. After
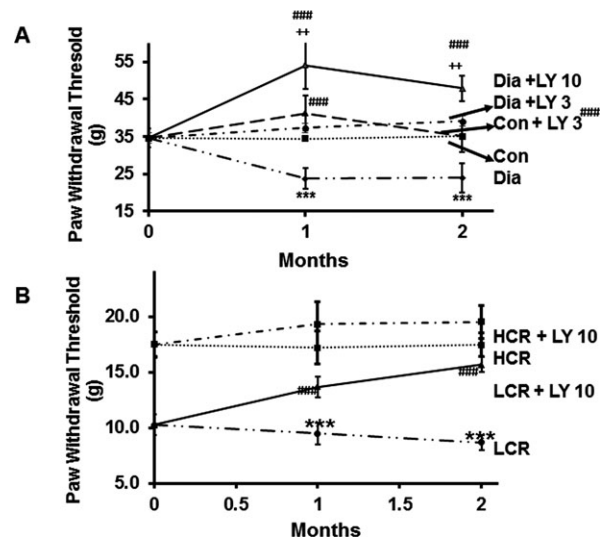

Figure 1. Von-Frey paw withdrawal threshold testing shows improved mechanical allodynia in LY379268 treated STZ diabetic and LCR rats. Mechanical allodynia (Von-Frey) testing was performed as described in the Materials and Methods. Diabetic rats showed a significant decrease in the paw withdrawal threshold. LY 379268 (10 mg/kg/day/i.p) treatment for 2 months significantly prevented mechanical allodynia. The line represents the mean value and data for individual animals are also given. Aged LCR rats showed increased sensitivity to mechanical allodynia and LCR rats treated with LY379268 at the high dose of 10 mg/kg/day improved their tactile allodynia threshold to the level of HCR rats. ***$P < 0.001$ diabetic compared to nondiabetic control rats and LCR compared to HCR; ###$P < 0.001$ in diabetic + LY 379268 (10 mg/kg) compared to diabetic rats and LCR+LY379268 compared to LCR (10 mg/kg).

2 months of diabetes, DNP immunoreactivity and glutathione levels significantly correlated with the sciatic-peroneal NCV and paw withdrawal thresholds ($R^2 = 0.64$;
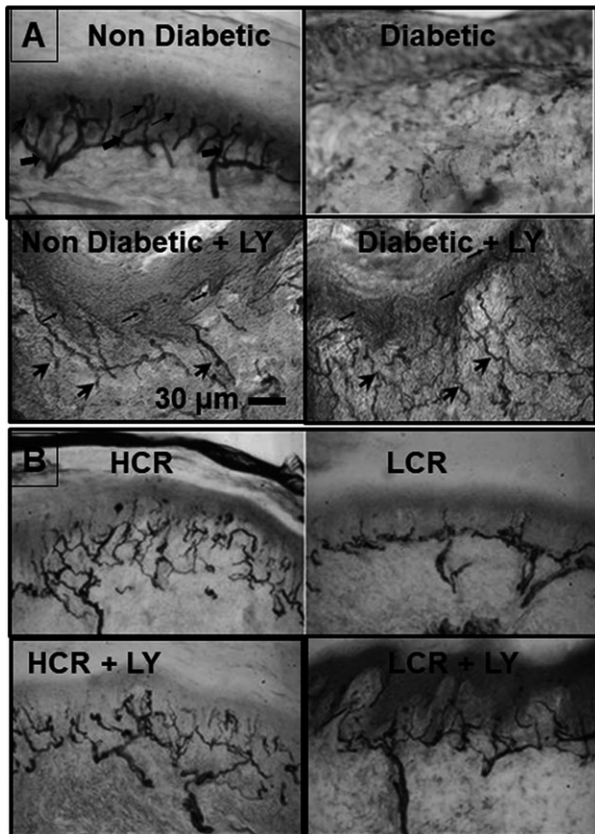
Figure 2. Intraepidermal nerve fiber immunohistochemistry and fiber counts in paw skin biopsy are improved in LY379268 treated STZ diabetic and LCR rats. Images show 50 μm paw skin sections immune-stained with anti-PGP 9.5 antibody. Small arrows indicate intra-epidermal nerve fibers; large arrows indicate dermal nerve fibers (scale bar 30 μm). There is a reduction in IENFD in STZ diabetic rats as compared with nondiabetic animals (A). Treatment with 10 mg/kg LY379268 in diabetic animals prevented the loss of epidermal fibers. Similar improvement in IENFD was seen in LCR rats treated with LY379268 (B).

$P < 0.001$), whereas the association was not significant between SOD2 levels and NCV or paw withdrawal thresholds ($R^2 = 0.06$; NS).

The effect of diabetes and LY379268 treatment on key mitochondrial transcription factors, for example SIRT1, PGC-1α, TFAM (Fig. 4A) was determined using western blotting in DRG from STZ diabetic rats (antibodies listed in Table S1). SIRT1, PGC-1α and TFAM were all increased in diabetic DRG (Fig. 4A to D; *$P < 0.05$) with a further very significant increase in protein levels in LY379268 treated DRG (Fig. 4A to D; ##$P < 0.01$) compared to diabetic DRG. The ratio of mitochondrial DNA (mtDNA) to nuclear DNA (nDNA) was determined to measure the effect of diabetes and LY379168 treatment on mtDNA (Table 3). The relative ratio of NADH dehydrogenase subunit 1 (ND1, encoded by mtDNA) over

beta-2-microglobulin (B2M, encoded by nDNA) showed a significant decrease in diabetic DRG (**$P < 0.01$). LY379268 (10 mg/kg) normalized mtDNA in diabetic DRG. To determine changes in mitochondrial complex levels, an OXPHOS antibody cocktail that detects subunits from 4 OXPHOS complexes was used (Fig. 5A; quantification of the individual complexes is shown in Figure 5B to D). The results showed a significant decrease in the levels of subunits from OXPHOS complexes that contain subunits encoded by mtDNA (Complex I, IV and V). In contrast, Complex II, encoded entirely by nDNA, showed no significant decrease.

Changes in glial fibrillary acidic protein (GFAP), glutamine synthase (GS) and glutamate transporters (GLT-1 and GLAST) in diabetic DRG with and without LY379268 Treatment

LY379268 is a potent and selective agonist for the mGluR 2/3 metabotropic glutamate receptor that has been shown in the central nervous system to regulate glutamate metabolism via glutamate recycling pathways that involve glial/neuronal interactions and mitochondrial metabolism. We tested if LY379268 also regulates glutamate recycling pathways in DRG from STZ rats by measuring the protein levels of glutamate transporter proteins [GLT-1 (EAAT2) and GLAST (EAAT2)], GS and GFAP, a marker of gliosis (Fig. 6A). The most significant result of this study was the dramatic increase in GFAP in STZ diabetic DRG and return to basal levels in LY379268 treated diabetic DRG similar to that in nondiabetic DRG (Fig. 6B; $P < 0.001$). On the other hand, Glutamine Synthetase (GS), a critical enzyme in the recycling pathway of glutamate-glutamine cycle was decreased in diabetic DRG and normalized in LY379268 treated DRG (Fig. 6C; $P < 0.001$). A similar decrease was also noted in GLT-1 and GLAST proteins in diabetic DRG and was normalized in LY379268 treated diabetic DRG (Fig. 6D).

LCR DRG are positive for GFAP (Fig. 7A), whereas HCR rats DRGs are mostly negative for GFAP. Treatment of LCR rats with LY379268 decreased the levels of GFAP (Fig. 7B, $P < 0.001$). We interpret this result to suggest that a similar mechanism of activation of glutamate recycling pathway occurs in LCR rats treated with LY379268, to that observed in STZ diabetic rats.

Discussion

This the first in vivo study to confirm that the administration of an mGluR3 agonist, LY379268, prevented neuropathy in a STZ T1D model of diabetic neuropathy or treated a LCR model of insulin resistance and glucose intolerance with neuropathy. LY379268 [(-)-2-oxa-4-
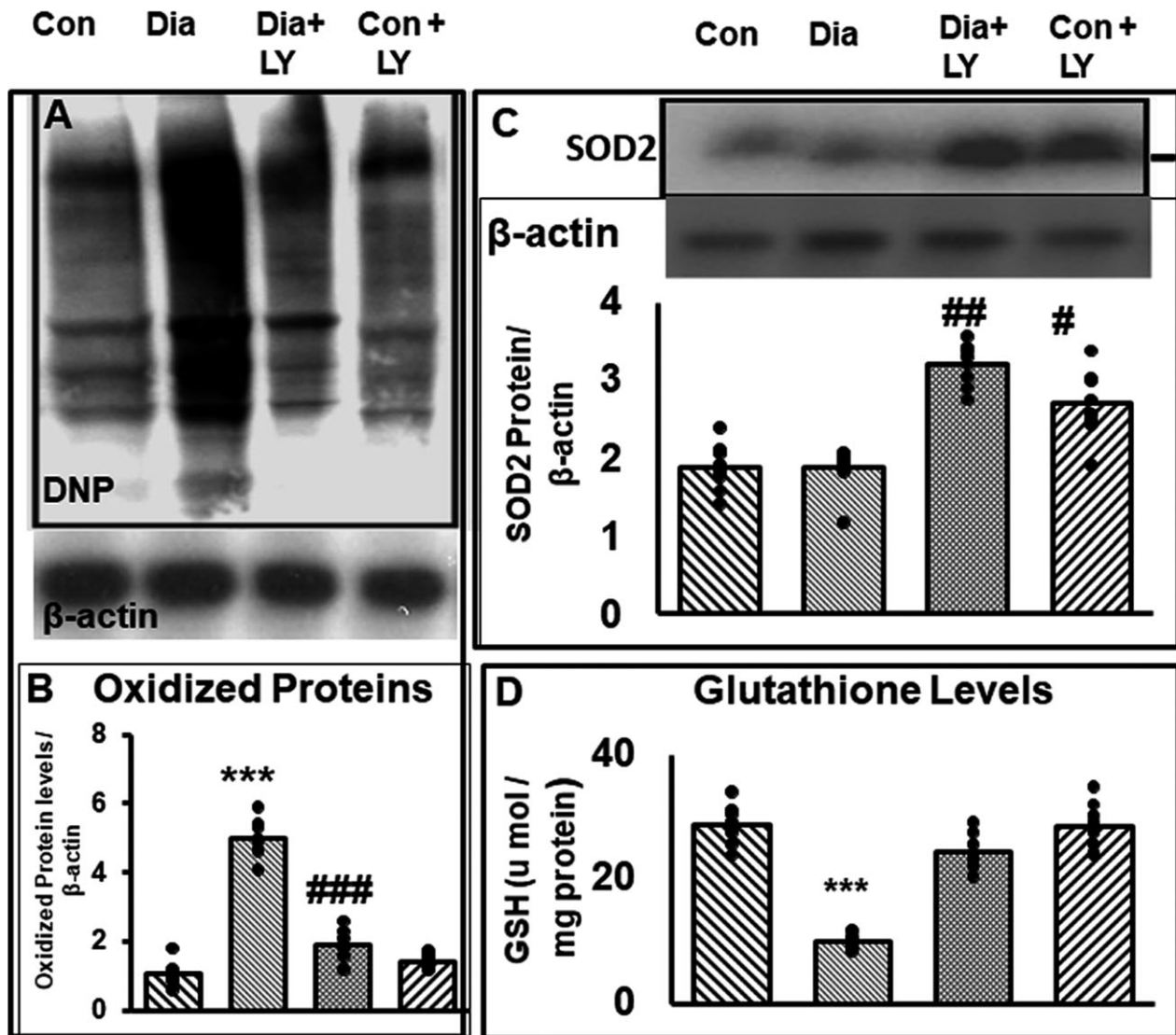
Figure 3. The mGluR2/3agonist, LY379268, reduces oxidized proteins, and increases SOD2 and glutathione levels in diabetic DRG. Proteins were extracted from DRG, run on 4 to12% SDS-PAGE, transferred to PVDF membrane and probed either with anti-DNP or with anti-SOD2 antibody. The immunoreactivity was quantified using Image J software. For glutathione enzymatic measurement, nonprotein supernatant was used. Oxidized protein measured by DNP was increased in diabetic DRG and reduced by LY379268 treatment (Figure A and B). LY379268 increased SOD2 protein levels (Figure 3C) and normalized glutathione levels (Figure 3D). Figure 3B, ***$P < 0.001$ diabetic compared to nondiabetic control rats; ###$P < 0.001$ diabetic + LY 379268 (10 mg/kg) compared to diabetic rats. In Figure 3C, #$P < 0.05$ and ##$P < 0.01$ LY 379268 treated nondiabetic as compared control or diabetic rat. In Figure 3D,*** $P < 0.001$ diabetic compared to nondiabetic control rats.

aminobicyclo(3.1.0)hexane-4,6-dicarboxylic acid] is a highly potent and systemically available mGlu2/3 receptor agonist.[37,38] We propose a novel mechanism of protection that involves activation of glutamate recycling pathways to normalize mitochondrial OXPHOS metabolism.

Decreased sensitivity to pain with LY379268 treatment has been noted following the injection of the inflammatory agent carrageenan into the footpad of mice or with the TRPV1 activator capsaicin.[37] A similar analgesic effect is also observed with (2R, 4R)-4-aminopyrrolidin-2, 4-

dicarboxylate, another Group II mGluR3 agonist.[35] It was supposed that these mGluR2/3 agonists modulate the sensory receptors in the peripheral tissue and produce the analgesic effect.[35] The results presented here suggest that activation of the group II mGlu2/3 receptor also occurs at the level of DRG. LY379268 prevented diabetes-induced nerve conduction slowing in both motor and sensory axons, prevented somatosensory deficits as measured by mechanical allodynia in distal nerve fibers in the hindlimb and prevented diabetes-induced reduction in the most
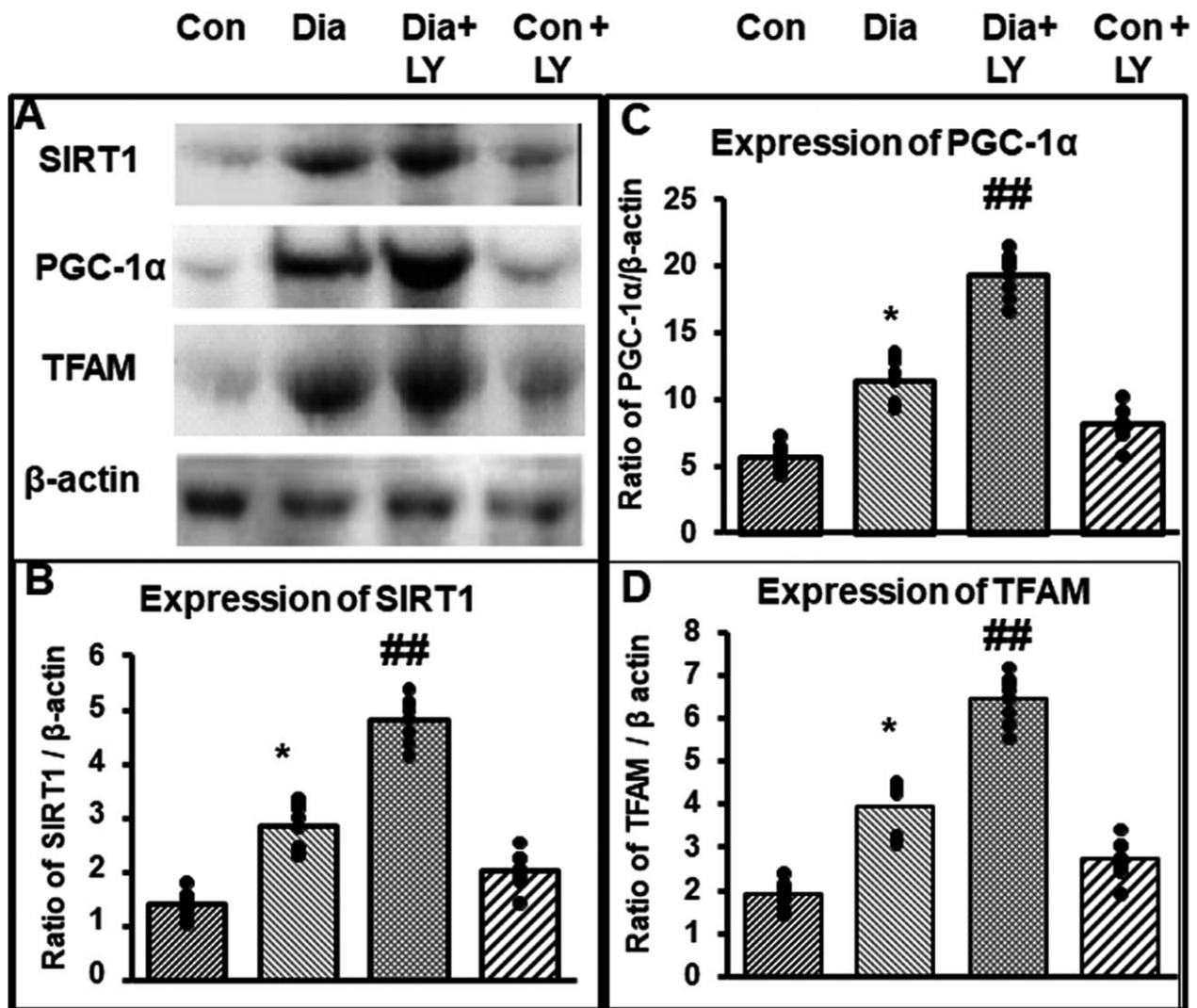
Figure 4. Western blot analysis of mitochondrial regulatory protein shows an increase in diabetic and a further increase in LY379268 treated STZ diabetic DRG. The source and the dilution of the antibodies used are summarized in Table S1. (A–D) Western blots show that SIRT1, PGC-1α and TFAM proteins were increased in diabetic DRG and treatment with LY379268 showed a further significant increase in SIRT1, PGC-1α and TFAM. *$P < 0.05$ diabetic compared to nondiabetic rats; ##$P < 0.01$ diabetic + LY 379268 (10 mg/kg) compared to diabetic rats.

distal unmyelinated nerve fibers in the hindlimb paw epidermis and dermis (Fig. 2).

In diabetic neuropathy, release of glutamate is associated with increased oxidative stress and decreased mitochondrial function.[21,38] We interrogated this glutamate hypothesis of DPN by inducing the glutamate reuptake pathway with an mGluR2/3 agonist to elucidate the mechanism of protection. Glutamate is transported to the axon terminals for synaptic release and induces an ionotropic uptake mechanism involving Na^+ and Ca^{2+} ions.[39] Recent evidence from the peripheral nervous system (PNS) show that the machinery for production, paracrine release, and recycling of glutamate occurs in sensory ganglia including the enzymes amidohydrolase, glutaminase,[40,41] GLAST, GLT1,[42] as well as the recycling enzyme GS.[26,40] This local release is a nonsynaptic glutamatergic transmission that occurs within the ganglia. Nonsynaptic release of glutamate into the extracellular space within the ganglion is shown in cultured whole ganglia in response to KCl or capsaicin.[43] Knockdown of glutamate-glutamine recycle enzymes or glutamate transporters result in increased extracellular glutamate.[44] The knockdown was found to be confined to the satellite glial cells (SGC) that are found wrapping the neuronal somata, isolating the ganglion cell bodies.[44,45] SGC contain all the protein necessary for the uptake and recycling of glutamate.[26] These findings

Table 3. Mitochondrial DNA copy number in DRG.

Tissue	B2M Copies/10 ng DNA	ND1 Copies/10 ng DNA	Ratio (mtDNA/nDNA)
Control	10,600 ± 1215	12,237 K ± 30 K	1145 ± 175
Diabetic	11,350 ± 1250	7160 K ± 69.7 K	630 ± 75**
Diabetic + LY (3)	11,210 ± 1250	9120 K ± 59.6K	807 ± 75[#]
Diabetic + LY (10)	11,720 ± 1200	13, 890 K ± 179 K	1170 ± 190[##]
Control + LY (3)	11,920 ± 1200	13, 930 K ± 161 K	1160 ± 130

**P < 0.01 Diabetic versus Non-diabetic; [#]P < 0.05; [##]P < 0.01; Diabetic + LY 379268 versus Diabetic.
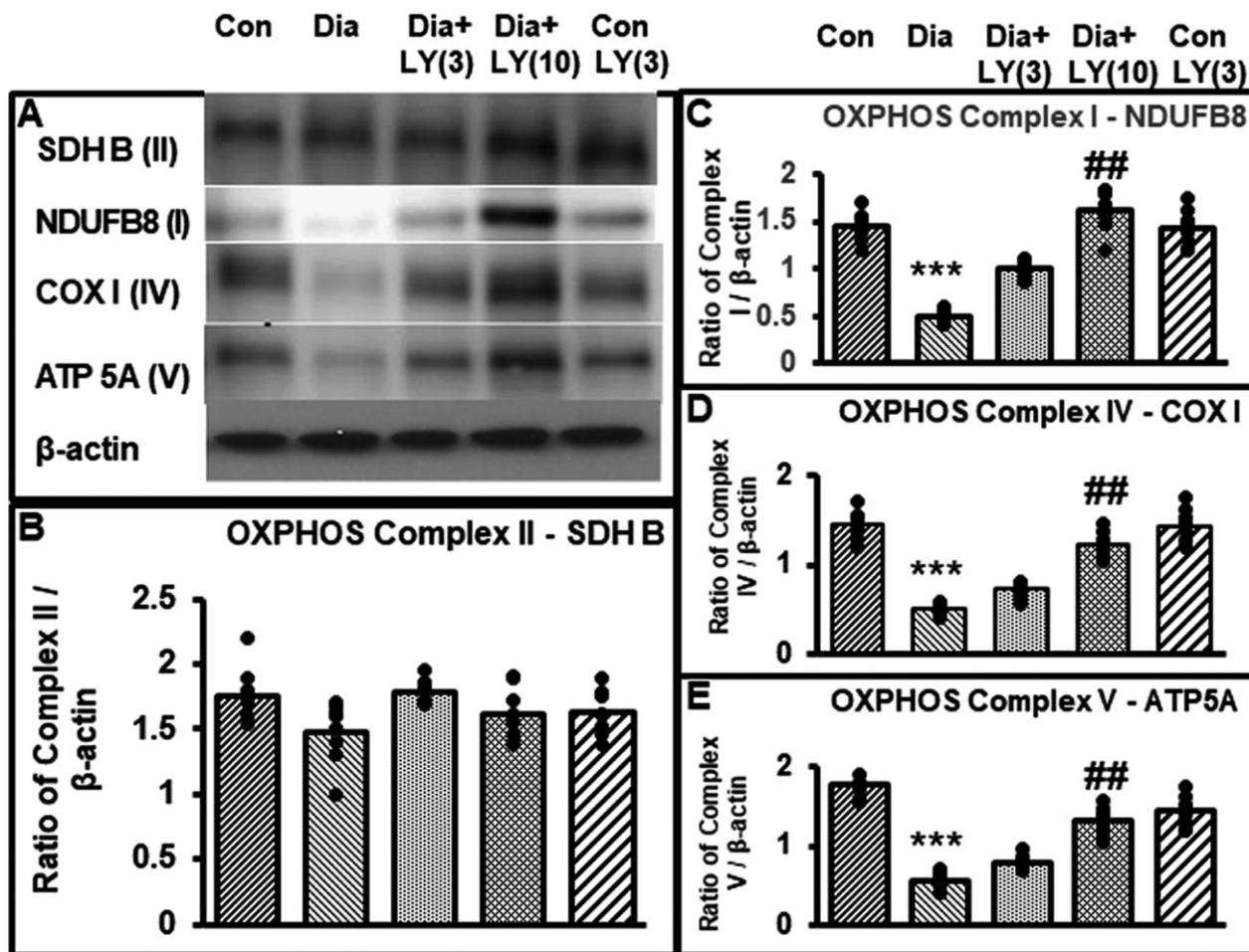

Figure 5. mtDNA encoded OXPHOS complexes are reduced in diabetic DRG and normalized by LY379268. The source and the dilution of the antibodies used are summarized in Table S1. Four monoclonal antibodies (mAbs), one each against OXOHOS complex (CI, CII, CIV and CV), CI subunit NDUFB8, CII-30 kDa, CIV subunit I and CV alpha subunit as an optimized premixed cocktail. (A) Western blots of subunit proteins from 4 of the 5 OXPHOS complexes are shown. OXPHOS complexes I, III, IV and V contain subunits that are coded by mtDNA whereas OXPHOS complex II contain subunits that are coded by nuclear DNA. Diabetic DRG showed a decrease in OXPHOS complexes that contain mtDNA-encoded subunits (C-E) but not nDNA-encoded subunits (B). ***P < 0.001 diabetic compared to nondiabetic control rats; [##]P < 0.01 diabetic + LY 379268 (10 mg/kg) compared to diabetic rats.

suggest that glutamatergic transmission within the ganglion could impact the nociceptive threshold.[46] Importantly DRG neurons do express glutamate receptors,

including mGluR2/3, in their soma. Thus, in addition to the vesicular transport of glutamate to axon terminals for synaptic release, the DRG neurons release and uptake
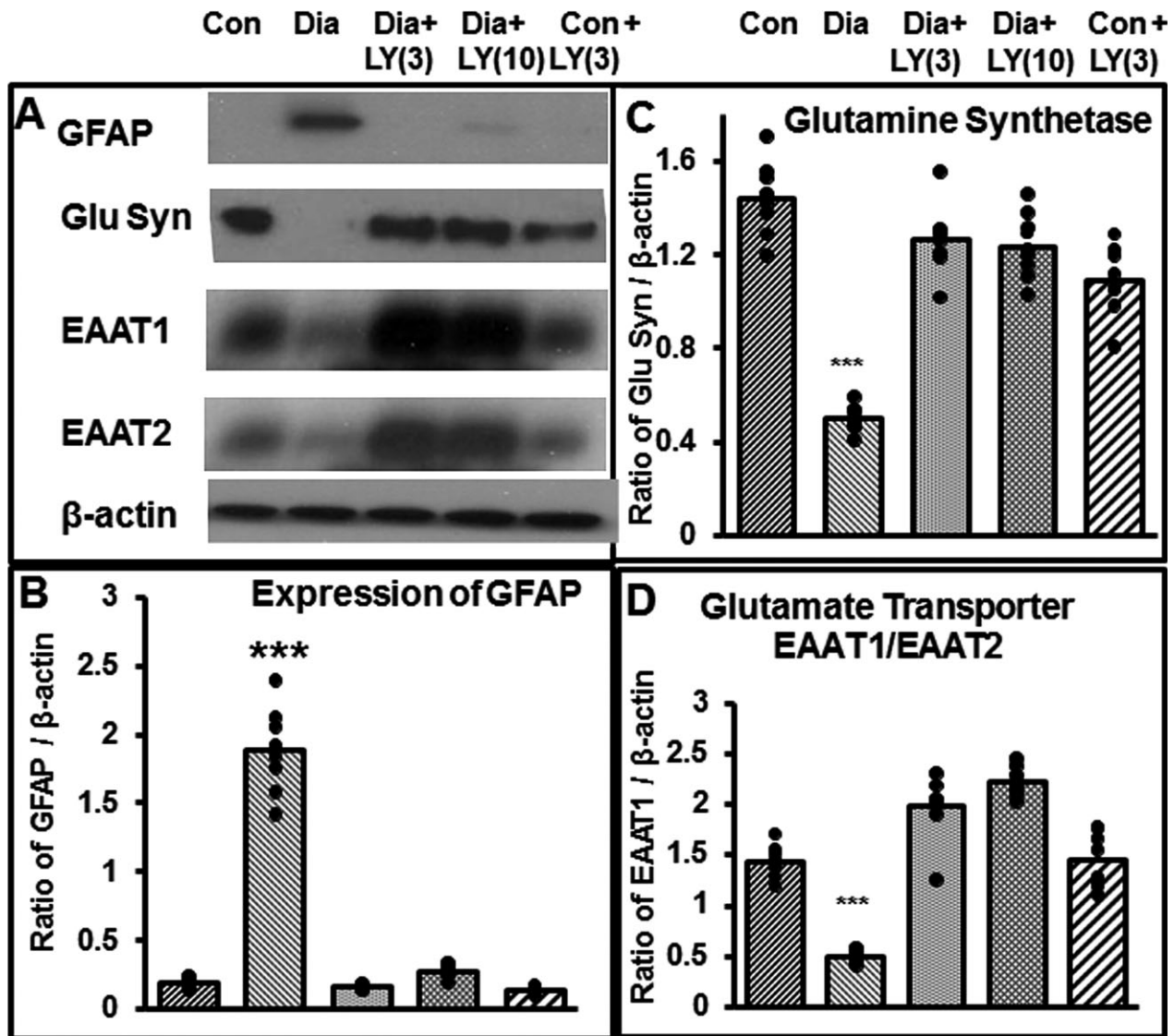
Figure 6. Western blot analysis of GFAP shows an increase in diabetic DRG whereas glutamate recycling enzymes (glutamine synthetase (GS), glutamate transporters EAAT1 (GLAST) and EAAT2 (GLT-1) are decreased in diabetic DRG. LY369268 treatment reversed the increase in GFAP and increased the glutamate recycling enzymes. The source and the dilution of the antibodies used are summarized in Table S1. (A) Western blots show that GFAP was barely detectable in nondiabetic and LY379268 treated diabetic DRG extracts but showed a striking increase in diabetic DRG extracts. GS, EAAT proteins decreased in diabetic DRG extracts but were normalized in LY379268 treated rat DRG extracts. (B) Quantification of GFAP confirmed a 10–20-fold increase in GFAP levels in diabetic DRG compared to LY379268 treated diabetic and nondiabetic rat DRG. (C) Glutamine synthetase and (D) Glutamate transporter protein levels were decreased in diabetic DRG extracts and were normalized with LY379268 treatment. ***$P < 0.001$ diabetic as compared with nondiabetic.

glutamate for interganglionic glutamatergic communication.[44, 45]

Results from several investigators have shown GFAP to be present at barely detectable levels in normal DRG. Diabetes markedly increases GFAP levels, suggesting that the increase may represent induction of gliopathic pain.[47] Treatment with the mGluR2/3 agonist, LY379268, prevented increased GFAP levels. The increase in GFAP is

likely to represent differentiation of SGC as a response to increased glutamate release in diabetes. The decrease in levels of glutamate transporters and recycling proteins are likely to exacerbate the glutamate accumulation and further increase GFAP levels. All these changes are reversed in rats treated with an mGluR2/3 agonist.

A mechanistic model (Fig. 8) is that hyperglycemia induces oxidative stress, promotes release of glutamate
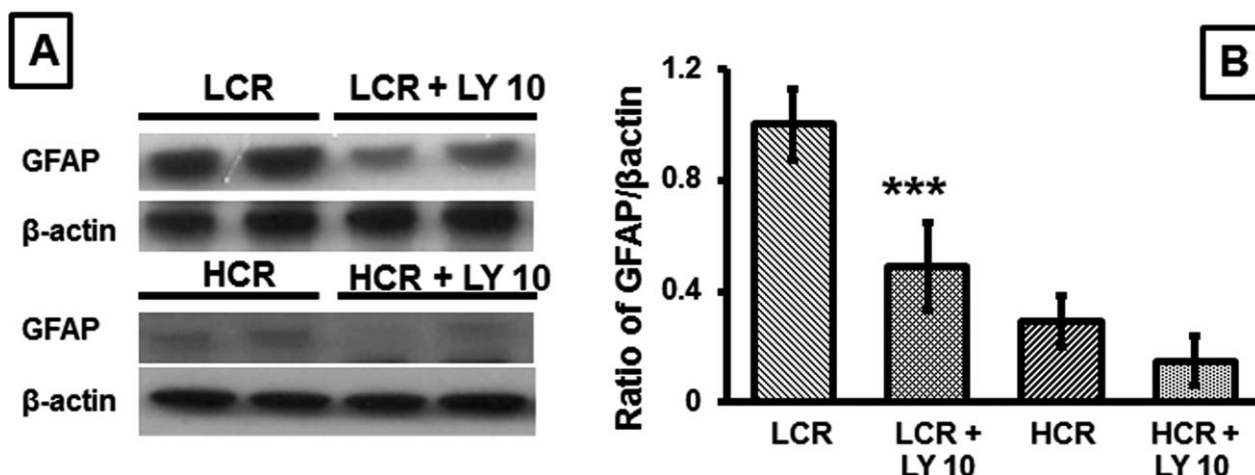
Figure 7. Western blot analysis of GFAP shows an increase in LCR DRG compared to HCR DRG neurons. LY369268 treatment reversed the increase in GFAP in LCR DRG neurons. (A) Western blots show that GFAP was barely detectable in HCR and LY379268 treated HCR DRG extracts but showed a striking increase in LCR DRG extracts and was significantly decreased in LY379268 treated LCR rat DRG extracts. (B) Quantification of GFAP confirmed a 5-10-fold decrease in GFAP levels in LY379268 treated LCR compared to LCR rat DRG (***$P < 0.001$).

from DRG neurons, and that LY 379268 removes extracellular glutamate by activating the glutamate recycling pathway to decrease excitability of the neurons and promote neuroprotection.[44,45,48] The mechanism that we propose is that LY379268 activates glutamate recycling pathways in surrounding satellite glial cells (SGC), normalizes neuronal mitochondrial OXPHOS metabolism and prevents development of neuropathy. GFAP is one of

the markers that we used to advance this hypothesis of increased glutamate recycling: diabetes increases GFAP-positive SGC and LY379268 treatment decreased GFAP-positive cells. This view is supported by the following evidence: (1) There are three known glutamate transporter proteins: GLAST, GLT1, and EAAC1.[42] SGC express GLAST1 and GLT1, whereas EAAC1 is expressed in neurons.[42] (2) Sciatic nerve injury decreases expression of
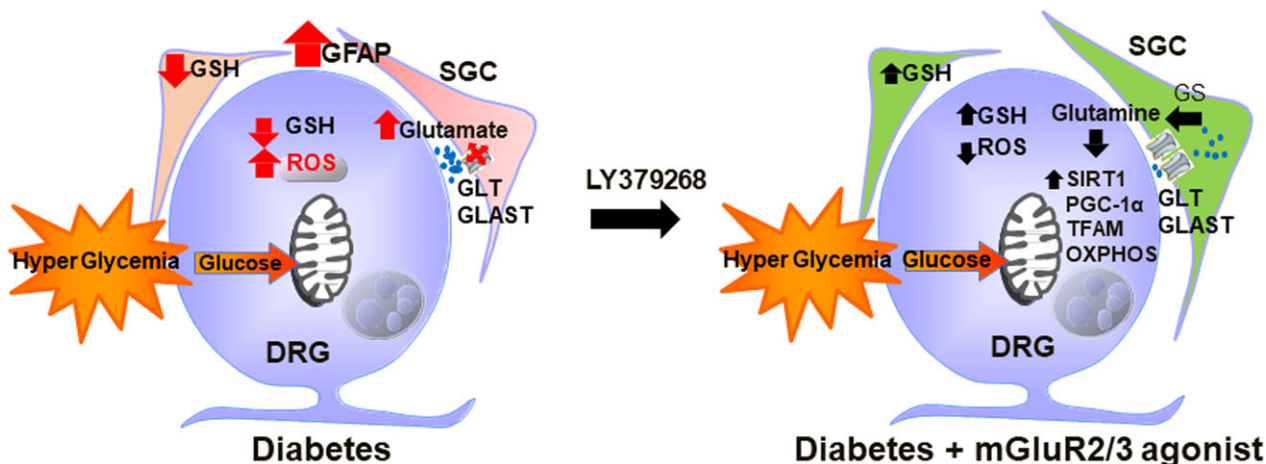

Figure 8. Proposed hypothesis: the role of satellite ganglion cells (SGC) and dorsal root ganglia (DRG) neurons in glutamate recycling. Glutamate transporters GLT-1 and GLAST are present in SGC to transport extracellular glutamate into the SGC where glutamine synthetase (GS) converts it to glutamine, which is eventually recycled to the neuron for conversion into glutamate. In diabetes, hyperglycemia-induced oxidative stress affects glutamate transport proteins, increasing extracellular glutamate, causing expansion of GFAP positive SGC. LY379268 treatment promotes glutamate uptake and is therefore likely to decrease extracellular glutamate, preventing expansion of GFAP positive SGC cells. In addition, mGluR 2/3 receptors present in DRG neurons decrease extracellular glutamate. Thus, activation of the mGluR2/3 receptor with an agonist likely constrains sensory transmission and importantly nociceptive transmission. This allows modification of glutamate-induced receptor potentials before impulses are generated and transmitted to the spinal cord.

GLAST and GLT-1.[49] This decrease in glutamate transport causes an increase in extracellular glutamate, which in turn is associated with neuropathic pain.[49] (3) Rapid removal of glutamate from the perineuronal space prevents the cytotoxic effects of glutamate.[39] (4) SGC cells convert glutamate to glutamine using the enzyme GS and glutamine is the taken up by neurons through glutamine transporters and converted back to glutamate to be reused. (5) mGluR2/3 receptors, present in DRG neurons, can further reduce the levels of extracellular glutamate. (6) Both neurons and SGC can eliminate the toxicity of extracellular glutamate. (7) The synthesis of GSH involves 2 steps, ligation of glutamate with cysteine to form gamma-glutamyl-cysteine and the addition of glycine to the c-terminus to form GSH. This pathway exists in both neurons and SGC. GSH made by SGC is secreted to the intracellular space and taken up by neurons to replenish GSH.[50] This is consistent with our previous findings that exposure to a mGluR2/3 agonist prevents glucose-induced neuronal injury in DRG neuronal cultures, only in the presence of Schwann/SGC cells, by increasing glutathione and maintaining mitochondrial function.[21,22,51] NMDA receptor antagonists are not as protective as mGluR2/3 agonists suggesting that ionotropic pathways are not involved in this pathway.[21,22,51]

Our results from western blots of mitochondrial transcription factors and OXPHOS proteins suggest that the physiological response to diabetic injury is insufficient to protect DRG neurons. Levels of SIRT1, PGC-1α, and TFAM were increased in STZ diabetic DRG after fairly acute STZ-induced diabetes. In contrast, levels of PGC-1α, and TFAM were decreased in more chronic diabetes.[4] This suggests that the mitochondrial machinery responds to hyperglycemia-induced injury to DRG, albeit the response is insufficient to protect them. LCR animals treated with LY379269, there was a slight loss of weight and improvement in LDL, triglyceride, and HDL levels. This may represent improved mitochondrial fatty acid metabolism possibly related to upregulation of the SIRT1-PGC-1α-TFAM axis (Fig. 4) but this will require further study.

Impaired lipid metabolism and accumulation of oxidized lipids has clearly been implicated in the etiology of diabetic neuropathy.[52,53] In this study, HDL was increased in the more acute STZ model of diabetes (Table 1) but reduced in the more chronic LCR obesity model (Table 2). This is similar to humans, where HDL levels are relatively preserved in young T1DM and reduced in chronic T2DM.[54] PGC-1α is increased by treatment with LY379269 (Fig. 4) and is implicated in improved mitochondrial fatty acid metabolism[13] and in the severity of diabetic neuropathy.[4] Thus, potentially LY379269 may treat diabetic neuropathy by reducing levels of certain toxic lipids and upregulating proteins associated with improving mitochondrial

function. Recycling of glutamate with LY379268 further increased levels of SIRT1, PGC-1α, and TFAM. This further increase occurred even though LY379268 diabetic rats were hyperglycemic (Table 1). TFAM regulates mitochondrial replication and transcription.[8] Even though levels of TFAM were observed to be increased, our results showed a significant decrease in overall DRG mtDNA levels (Table 3). One plausible explanation for the paradox of increased TFAM but decreased mtDNA is that although there is a physiological upregulation of TFAM due to hyperglycemia, there is also an increase in protein oxidation (Fig. 3). The result is that even though TFAM protein levels increased, TFAM was ubiquitinated causing it to be nonfunctional and thus preventing TFAM-induced mtDNA replication and transcription.[55]

Overall the present study indicates that a selective mGluR2/3 agonist is effective in reducing the severity of diabetic neuropathy. Our results provide in vivo evidence supporting the previous in vitro data obtained from DRG neuronal and Schwann cell cultures.[21,22,51] In vitro results delineated a potential mechanism of reducing oxidative stress by increasing glutathione levels that did not involve ionotropic NMDA pathways but likely involved metabolomic mGluR pathways. Our hypothesis summarized in Figure 8 suggests that one mechanism of protection is likely to involve presynaptic glutamate release, glutamate uptake, conversion of glutamate to glutamine to provide precursors for GSH synthesis, and substrate for neuronal mitochondrial oxidative metabolism. In turn, improved Mt function with LY379269 is associated with upregulation of the SIRT1-PGC-1α-TFAM axis.

Acknowledgments

by the Department of Anesthesiology at the University of Michigan, Ann Arbor.

Authors Contributions

K.C., A.M., J.W.R. conceived the design and experiments of the study. K.C., A.M., T.G.D., J. C., A.R.S., N.N., P.K., A.S., C-Y.H. collected the data. K.C., A.M., and J.W.R. analyzed the data. G.F., L.G.K., S.L.B. contributed to the materials for analysis. K.C., J.W.R. wrote the manuscript. C-Y.H., G.F., L.G.K., S.L.B., J.W.R. edited the manuscript.

Conflict of Interest

None declared.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Intraperitoneal Glucose Tolerance Test shows impaired glucose tolerance in LCR rats compared to HCR rats. Mean blood glucose after administration of intraperitoneal glucose challenge (1.5 g/kg/) in HCR and LCR rats. Data is in mean $\pm$ SEM. *$P<$0.05; **$P<$0.01 LCR compared to HCR rats.

Table S1. Source and the dilutions of the antibodies used in this study.