Ganglioside GM3 synthase depletion reverses neuropathic pain and small fiber neuropathy in diet-induced diabetic mice

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Abstract

Background: Small fiber neuropathy is a well-recognized complication of type 2 diabetes and has been shown to be responsible for both neuropathic pain and impaired wound healing. In previous studies, we have demonstrated that ganglioside GM3 depletion by knockdown of GM3 synthase fully reverses impaired wound healing in diabetic mice. However, the role of GM3 in neuropathic pain and small fiber neuropathy in diabetes is unknown.

Purpose: Determine whether GM3 depletion is able to reverse neuropathic pain and small fibers neuropathy and the mechanism of the reversal.

Results: We demonstrate that GM3 synthase knockout and the resultant GM3 depletion rescues the denervation in mouse footpad skin and fully reverses the neuropathic pain in diet-induced obese diabetic mice. In cultured dorsal root ganglia from diet-induced diabetic mice, GM3 depletion protects against increased intracellular calcium influx in vitro.

Conclusions: These studies establish ganglioside GM3 as a new candidate responsible for neuropathic pain and small fiber neuropathy in diabetes. Moreover, these observations indicate that systemic or topically applied interventions aimed at depleting GM3 may improve both the painful neuropathy and the wound healing impairment in diabetes by protecting against nerve end terminal degeneration, providing a disease-modifying approach to this common, currently intractable medical issue.

Keywords

Neuropathic pain, small fiber neuropathy, GM3 ganglioside, obesity, glucose intolerance, diabetes

Background

Painful diabetic neuropathy (PDN) affects 25% of diabetic patients[1–3] and has a substantial impact on quality of life,[4] in addition to a high risk of pedal ulceration and amputation. Current therapies for diabetic neuropathic pain are only partially effective, despite the huge need for effective intervention. Moreover, the molecular and electrophysiological mechanisms underlying PDN are not well understood.

Pain is a physiological response to potentially dangerous noxious stimuli. However, pathological or ''neuropathic pain'' is associated with sustained excitability and pathological sustained activity of nociceptive primary

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afferent neurons, so that pain is produced in the absence of appropriate stimuli.[5–7] The molecular mechanisms leading to neuropathic pain in diabetes are poorly understood.

Neuropathic pain is known to be associated with both pathological spontaneous ectopic activity and degeneration of unmyelinated C-fibers. For many painful neuropathies, such as PDN, loss of the distal intraepidermal free-end nerve terminals manifests in a reduction in the intraepidermal nerve fiber density.[8,9] Indeed, degeneration of free-end nerve terminals, also called small fiber neuropathy (SFN), is a well-recognized complication of T2D.[5,9–11] Intraepidermal nerve fiber densities are reduced in patients with impaired glucose tolerance and clinically overt diabetes,[12] as well as in animal models of T2D, including the diet-induced obese (DIO) diabetic model.[11] Although there is substantial interest in axonal degeneration in PDN, the mechanistic details of the molecular cascade linking diabetes and neuropathic pain to nerve fiber degeneration are incompletely understood.

Gangliosides are sialylated glycosphingolipids found in high concentrations in the nervous system and known to play an important role in lipid raft-based signaling. The precursor ganglioside, GM3, and its synthesizing enzyme, GM3 synthase (GM3S), are increased in expression in canonical insulin-responsive tissues[13] and, as we have shown, in the skin of diabetic mouse models and human diabetic feet.[14,15] Increasing evidence suggests that GM3 accumulation is a mediator of Tumor Necrosis Factor (TNF)-induced insulin resistance.[13,16] We have recently shown that ganglioside GM3 plays a role in the impaired wound healing of diabetes, at least in part through its suppression of insulin/insulin-like growth factor-1 receptor signaling in epidermal cells.[14,15] Depletion of ganglioside GM3 by GM3S knockout or topically applied spherical nucleic acid (SNA) nanoconjugate-mediated knockdown of GM3S fully reverses diabetic wound healing impairment.[14,15]

Nerve fiber degeneration has been shown to be associated with the poor wound healing in diabetes, but the impact of ganglioside alterations on diabetic nerve function has not been explored. We now show that GM3S depletion fully reverses neuropathic pain and small fibers degeneration in DIO diabetic mice by preventing increased calcium influx in diabetic dorsal root ganglia (DRG) sensory neurons. These studies establish ganglioside GM3 as a new therapeutic target for neuropathic pain and small fibers neuropathy in diabetes. Moreover, these observations suggest that systemic or regional interventions to deplete GM3 prevent nerve end terminal degeneration, thereby improving both the painful neuropathy and the wound healing impairment in diabetes.

Materials and methods

Animals

All animal experiments were approved by the Institutional Animal Care and Use Committee at Northwestern University. Animals were housed with food and water ad libitum and kept on 12-h light cycle. Wild-type (WT) C57BL/6 (Jackson Laboratories) and GM3 synthase knockout (GM3S KO) (backcrossed to C57BL/6 background) male mice were studied. Mice were weighed twice weekly.

High fat diet-induced (DIO) diabetes as a model of T2D

High fat diet (HFD) induces insulin resistance and is therefore used to create rodent models of T2D.[11] WT and GM3S KO mice were fed with a 60% fat diet for 10 weeks beginning at 6 weeks of age. Control mice were fed a regular (11% fat) diet (RD). Glucose tolerance testing (GTT) was performed as previously described.[11] Briefly, mice were fasted for 12 h, injected with a 45% D-glucose solution (2 mg glucose/kg body weight), and blood glucose was measured before and at 30, 60, and 120 min after glucose injection using an OneTouch UltraMini glucose meter and OneTouch Ultra test strips. The cutoff for diabetes was determined based on $\geqslant 2$ SD above the mean for WT RD mice.

Statistical analysis. Data were analyzed by two-tailed unpaired Student's *t*-test, with $p < 0.05$ considered to be significant.

Functional studies of sensation

For *Von Frey behavioral studies*, mice were habituated to the testing apparatus for 15 min/day beginning two days before behavioral testing.[7,17–20] After this acclimation, each filament was applied to six spots spaced across the glabrous side of the hind paw. Mechanical stimuli were applied with seven filaments, each differing in the bending force delivered (10, 20, 40, 60, 80, 100, and 120 mN; 100 mN = 10.197 g force equivalence), but each fitted with a flat tip and a fixed diameter of 0.2 mm. The filaments were tested in order of ascending force, with each filament delivered for 1 s in sequence from the first to the sixth spot, alternating from one paw to the other. The interstimulus interval was 10–15 s.[20] For the thermal studies (Ugo Basile), the presence of thermal hyperalgesia was determined by measuring foot withdrawal latency to heat stimulation (thermal paw withdrawal) using the Hargreaves' plantar test apparatus.[6,7] Briefly, each mouse was placed in a box with a temperature-maintained glass floor and, after habituation, an infrared

heat generator was aimed at the mouse's hind paw under the floor. The stimulus shuts off automatically when the hind paw moves or after 20 s. Reaction time was recorded, with a shortening of the withdrawal latency indicating thermal hyperalgesia. Measurements were repeated four times on each paw at 5 min intervals, with the latter three pairs of measurements used as data. Mice were tested on each of three successive days.

Statistical analysis for functional studies. The incidence of foot withdrawal was expressed as a percentage of six applications of each filament as a function of force. A Hill equation was fitted to the function (Origin version 6.0, Microcal Software) relating the percentage of indentations eliciting a withdrawal to the force of indentation. From this equation, the threshold force was obtained and defined as the force corresponding to a 50% withdrawal rate. The GB-Stat School Pack software (Dynamic Microsystem) was used to statistically evaluate all data. The one-way analysis of variance with a Dunnett's Multiple Comparison test was used to analyze the difference between multiple experimental groups, with $p < 0.05$ considered to be significant.

Assessment of GM3S expression in DRG and sciatic nerve

DRG sensory neurons and sciatic nerves from WT RD and HFD mice were removed after 10–11 weeks on the selected diet. Total protein was then extracted from DRG and sciatic nerves from three mice in each group using cell lysis buffer (Cell Signaling Tech), and 30 g total protein from each group (WT RD and WT HFD) was loaded onto 10% SDS-PAGE gel. After separation, protein was transferred onto nitrocellulose membrane and the GM3S was determined by Western blotting using anti-GM3S antibody (Santa Cruz).

Detection of cutaneous innervation

Harvested feet were fixed in Bouin's fixative for 12 h, and then the overlying footpad skin was dissected, submerged in 15% sucrose solution for 12 h, and embedded in Optimal Cutting Temperature Compound (Tissue-Tek). Cryostat sections (80 m) were blocked for 1 h at room temperature (RT) in 5% normal goat serum, 0.3% Triton X-100, and 1% Bovine Serum Albumin (BSA) diluted. Samples were incubated with anti-PGP 9.5 antibody (Millipore #AB1761-I, 1:850 dilution) at 4°C for 12 h, followed by incubation with Alexa Fluor 488-conjugated goat anti-rabbit antibody (1:1000 dilution) for 1 h at RT. DAPI 1:10 (400 mM stock) was applied for 10 min at RT to counterstain cell nuclei.

Confocal analysis. Nerves were imaged by confocal microscopy (Nikon A1R) and composite Z-stack images were obtained. The epidermal–dermal junction was outlined by a blinded observer, and three other blinded reviewers counted the number of nerves crossing this junction for the junctional length. The dermis was traced using ImageJ and, after subtracting the background, the green fluorescence was quantified as a percentage of total area using the GFP channel.

Statistical analysis. Unpaired Student's *t*-tests were used to analyze and compare the data and calculate *p*-values using Graph Pad web site, with $p < 0.05$ considered significant.

Culture and intracellular calcium imaging of DRG neurons

DRG sensory neurons from WT and GM3S KO mice (HFD and, as control RD) were removed after 10–11 weeks on the selected diet and cultured as previously described.[17,21] Briefly, DRG neurons were acutely dissociated and digested with collagenase IV (1 μg/mL) and papain (30 U/mL) (Worthington Biochemical Corp, Lakewood, NJ). Cells were plated on laminin (20 μg/mL) or, as a control, poly-L-lysine (20 μg/mL)-coated glass coverslips and cultured at 37°C with 5% CO_2 for 48 h in adult neurogenic medium: F12 with L-glutamine, 0.5% FBS, 1 × N2 (Life Technologies), penicillin (100 μg/mL), and streptomycin (100 U/mL). The response of acutely cultured DRG neurons to different concentration of potassium (K^+) (10, 25, and 50 mM) or capsaicin (cap) (1, 2.5, 5, and 10 μM) were recorded using intracellular calcium imaging as previously described.[17] Acutely cultured DRG cells were loaded with fura-2 AM (3 μM, Invitrogen, Carlsbad, CA) for 25 min at RT in balanced salt solution (BSS) (NaCl (140 mM), Hepes (10 mM), $CaCl_2$ (2 mM), $MgCl_2$ (1 mM), glucose (10 mM), and KCl (5 mM)). Cells were rinsed with BSS, mounted onto a chamber that was placed onto the inverted microscope, and continuously perfused with BSS (2 mL/min). Intracellular calcium ($[Ca^{2+}]_i$ was measured by digital video microfluorometry with an intensified CCD camera coupled to a microscope and MetaFluor software. Cells were illuminated with a 150 W xenon arc lamp, and the excitation wavelengths of the fura-2 (340/380 nm) were selected by a filter changer.

Statistical analysis. Differences between the control and various treatment groups were assessed by analysis of variance with $p < 0.05$ considered significant.

Results

GM3S depletion fully reverses neuropathic pain in DIO mice

GM3S KO mice (courtesy of Dr R. Proia, NIH)[22] have previously been described and show no detectable GM3 or downstream gangliosides (such as GM2, GM1, and GD3).[15,22,23] GM3S KOs and their WT littermate controls were fed either a RD containing 11% fat or a HFD consisting of 60% fat for 10 weeks before testing. Two hour GTTs showed higher glucose levels in the WT and GM3S KO mice on a HFD than in the WT and GM3S KO mice on a RD (WT HFD vs. RD, $p < 0.001$; GM3S KO HFD vs. RD, $p < 0.05$). GM3S

KO mice exhibited variably improved glucose tolerance, with a significantly lower 2 h level of glucose overall in GM3S KO HFD than in WT HFD mice ($p < 0.01$) (Figure 1(a)).

As previously shown, the mean weights after 10 weeks on the HFD were higher in WT (mean ± standard error (SE), 32.5 ± 1.4 g) and GM3S KO (28.7 ± 1.0 g) mice when compared with WT (23.9 ± 0.7 g) or GM3S KO (21.5 ± 1.3 g) on the RD ($p < 0.001$) but were similar between WT and GM3S HFD mice ($p = 0.08$) (Figure 1(b)). These data suggest that GM3S depletion has no ameliorative effect on diet-induced obesity, but partially improves the glucose intolerance induced by HFD in some mice.
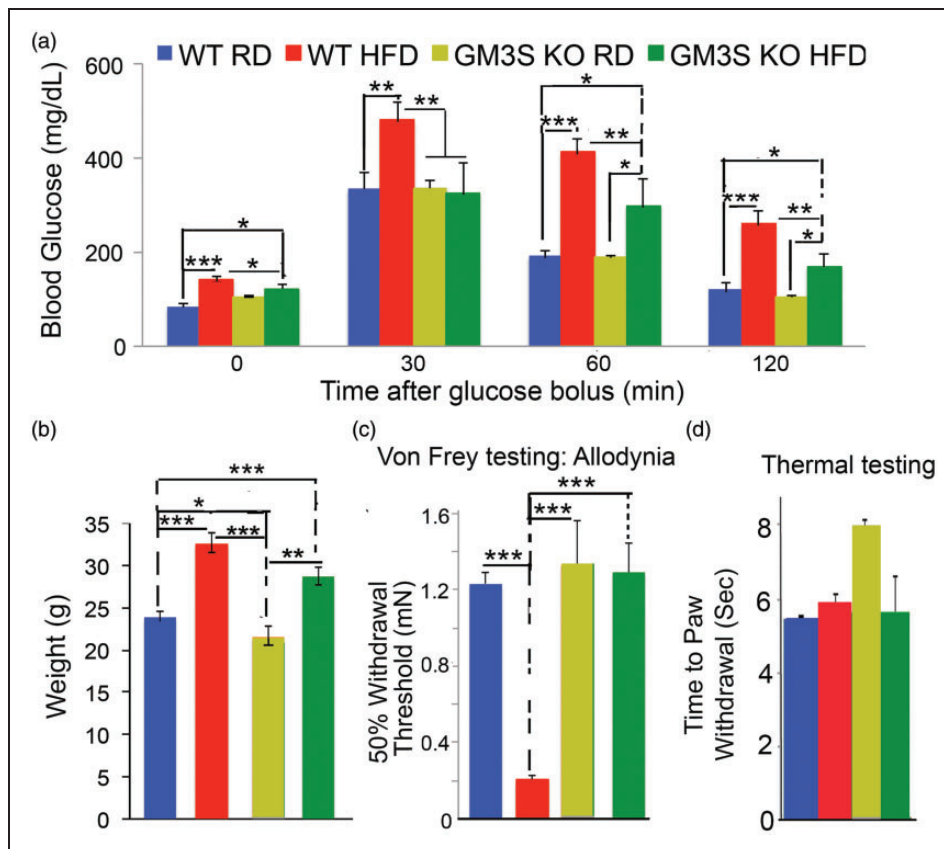

Figure 1. GM3S depletion fully reverses neuropathic pain in DIO mice. GM3 synthase knockout (GM3S KO) mice and their WT littermate controls were fed either a Regular Diet (RD) or a high fat diet (HFD) for 10 weeks. (a) GM3S knockout variably improves glucose tolerance. When compared with RD-fed mice, HFD-fed mice had significantly higher baseline fasting and 30, 60, and 120 min glucose levels (mg/dL). Overall, glucose levels in the GM3S KO HFD mice were slightly higher than those in the WT RD mice but were much lower than in WT HFD mice. However, the improvement was variable in GM3S KO HFD mice. (b) Obesity is not impacted by GM3 depletion. Mean weights were higher in WT HFD (n = 10) and GM3S KO HFD (n = 24) mice compared with WT RD (n = 8) and GM3S KO RD (n = 11) mice but not in WT HFD versus GM3S KO HFD mice ($p = 0.08$). (c) GM3S depletion fully reverses neuropathic pain in DIO mice. Pain behavior was assessed using Von Frey filaments. In WT HFD diabetic mice, the withdrawal threshold required to elicit a response was reduced compared with WT RD non-diabetic mice, demonstrating the development of neuropathic pain. No difference was noted in GM3S KO HFD mice compared with WT RD or GM3S KO RD, showing that GM3S depletion fully reverses neuropathic pain in DIO mice. (d) All mouse groups show normal mean thermal sensitivity. Values are expressed as mean ± SE.*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

GM3S depletion fully reverses neuropathic pain in DIO mice

The effect of GM3S depletion on diabetic neuropathic pain was assessed in DIO mice using Von Frey filaments. In these functional studies, results were tallied by observers who were blinded to subgroup (although obesity in HFD mice was easily noted). The withdrawal threshold required to elicit a response was significantly reduced in WT HFD diabetic mice when compared with WT RD non-diabetic mice, showing the development of neuropathic pain (Figure 1(c)). In contrast, the withdrawal threshold required to elicit a response in GM3S KO HFD mice resembled that in WT RD and GM3S KO RD non-diabetic controls, suggesting that GM3S depletion fully reverses neuropathic pain in DIO mice (Figure 1(c)). Thermal testing is normal in DIO mice, as previously noted,[11] and similarly was normal in our WT HFD, GM3 KO HFD, and GM3 KO RD mice (Figure 1(d)).

Given that GM3S knockout variably improves mouse glucose tolerance, we were able to divide GM3S KO mice into diabetic and non-diabetic to assess the role of improved glucose tolerance versus reduction of GM3S itself on the observed reversal of the neuropathy. Cutoff levels for diabetes in mice depend on a wide variety of factors, including strain, age, sex, and environment of the mice subjected to GTTs.[24] To compare "diabetic" versus "non-diabetic" GM3S KO HFD mice, we first established cutoff levels by assessing glucose tolerance in 80 WT littermate RD mice, setting the cutoff at 2 SDs above the mean for glucose at 2 h after glucose challenge ($\geqslant 182$ mg/dL). GM3S KO HFD mice were then divided into two groups: (i) GM3S KO HFD Group 1 with GTT 2 h glucose levels $\geqslant 182$ mg/dL (diabetic, n = 6); and (ii) GM3S KO HFD Group 2 with glucose levels < 182 mg/dL (non-diabetic, n = 17) (Figure 2(a)). Regardless of diabetic status, the withdrawal threshold required to elicit a response in GM3S KO HFD mice was significantly higher than WT HFD
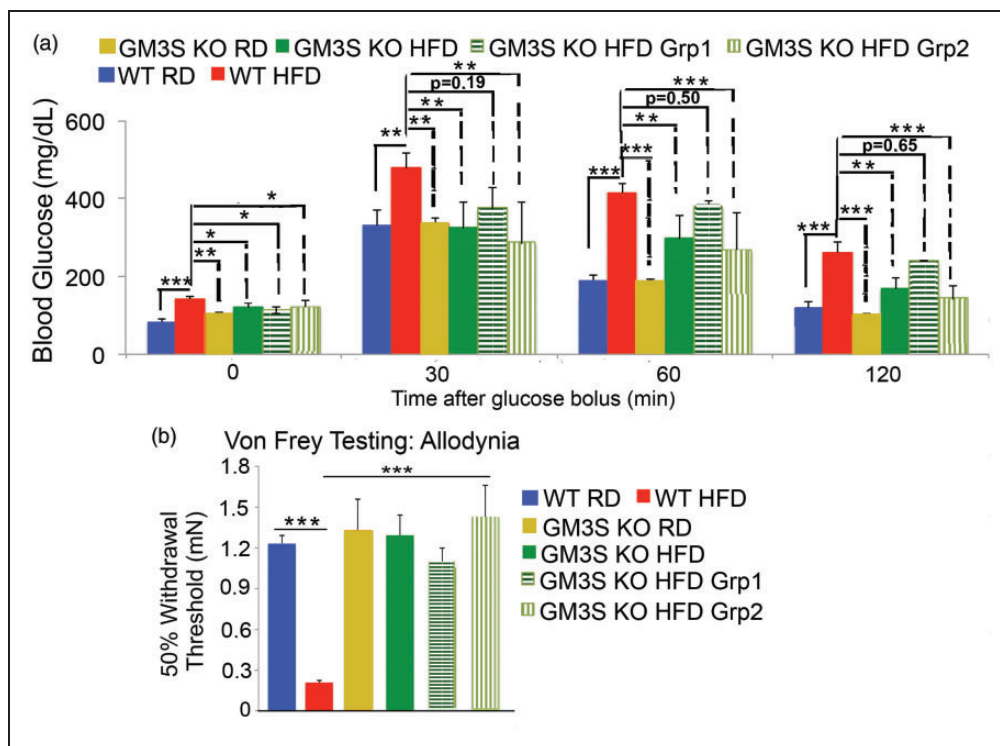

Figure 2. GM3S depletion fully reverses neuropathic pain, even in diabetic mice. GM3S KO HFD mice were divided into diabetic (Group 1; 2 h GTT glucose levels $\geqslant 182$ mg/dL) or non-diabetic (Group 2, <182 mg/dL) based on a threshold of $\geqslant 2$ SD above the mean for 2 h glucose levels in our WT RD mice (n = 80). As shown in (a), the fasting baseline levels of glucose in the two GM3S KO HFD groups remained higher than WT RD baseline levels, but the 2 h glucose levels in Group 2 were not higher than those of WT RD mice (WT RD, n = 9; WT HFD, n = 9; GM3S KO RD, n = 9; GM3S KO HFD overall, n = 23; GM3S KO HFD Group 1, n = 6; GM3S KO HFD Group 2, n = 17). (b) The Von Frey Pain Behavior Test demonstrated that the withdrawal threshold required to elicit a response in GM3S KO HFD Groups 1 and 2 was significantly higher than WT HFD, but similar to WT RD and GM3S KO RD mice. Values are expressed as mean $\pm$ SE. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
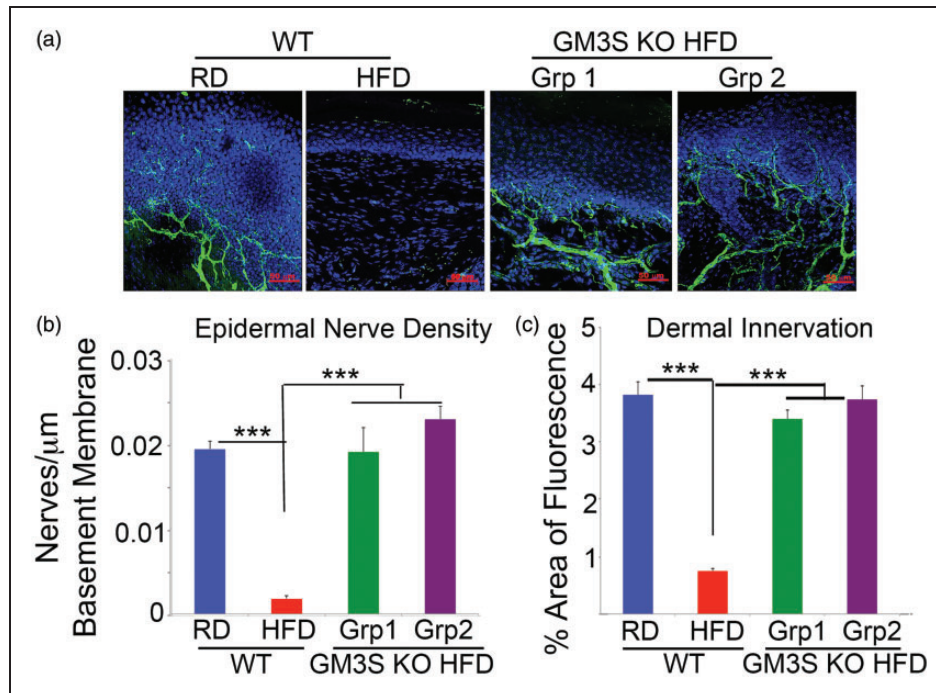
Figure 3. GM3 depletion rescues denervation in mouse footpad skin in diet-induced diabetes. (a) Representative confocal images of PGP 9.5 expression in 80 μm mouse footpad sections. Epidermal nerve density (b) is expressed as the number of nerves crossing the epidermis as a function of epidermal–dermal junction length. Dermal innervation (c) is expressed as the percentage of dermis with fluorescence. Data represent 16 feet (n = 8) from each group with three non-contiguous sections analyzed per foot. Grp 1 = diabetic GM3S KO HFD mice; Grp 2 = non-diabetic GM3 KO HFD mice. Values are expressed as mean ± SE. ***$p < 0.001$.

and was not different from non-diabetic control WT RD mice (Figure 2(b)), suggesting a primary neuroprotective effect of GM3 depletion.

GM3S is increased in diabetic nerves

In diabetes, GM3 is now known to be increased in canonical insulin target tissues, as well as epidermis. However, the expression of GM3S has never been assessed in peripheral nerves. Isolated WT HFD diabetic mouse DRGs and sciatic nerve had levels of expression of GM3S that were 1.7-fold and 24-fold, respectively, those in WT RD non-diabetic mice (Suppl. Figure 1). GM3S KO mice are unable to make GM3S or GM3.[22]

GM3S depletion rescues SFN in DIO diabetes

Denervation of epidermal footpad skin was observed in WT HFD diabetic mice, consistent with the presence of diabetic neuropathy. In contrast, even diabetic GM3S KO HFD mice had preservation of footpad skin innervation, as measured by the number of nerves crossing into the epidermis (0.02 nerves/μm basement membrane; Figures 3(a),(b)) and dermal nerve density (Figures 3(a),(c)).

GM3S depletion prevents hyperexcitability-associated calcium influx into diabetic DRG neurons

SFN is a well-recognized complication of T2D,[5,9–11] and we observed denervation of the epidermal footpad skin in our animal model of HFD-induced diabetes (Figure 3(a), WT HFD). Calcium toxicity has recently been shown to contribute to DRG neuritis and small fiber degeneration in a genetic form of SFN associated with a mutation in the Nav 1.7 sodium channel.[25] To examine a potential role for neuronal hyperexcitability in diabetic mice using $(Ca)_I$ into DRG neurons as a surrogate for overall excitability, we performed Ca imaging studies in response to different concentrations of known DRG neuron excitatory stimuli: high potassium (K^+) and capsaicin in acutely isolated HFD-induced diabetic and RD-fed non-diabetic control mouse DRGs.[21]

DRG neurons from WT HFD mice were much more sensitive to both high K^+ and capsaicin stimulation than WT RD DRG neurons. Even low concentrations of K^+ (10 mM) and capsaicin (1 μM) increased $(Ca)_I$ in WT HFD-induced diabetic DRGs (25.0 ± 2.7% are responsive, mean ± SE, Figures 4(b) and 4(e) and 39.0 ± 3.0% responsive, Figures 4(g) and 4(j), respectively) but not in non-diabetic WT RD mouse DRGs (5.0 ± 0.9%
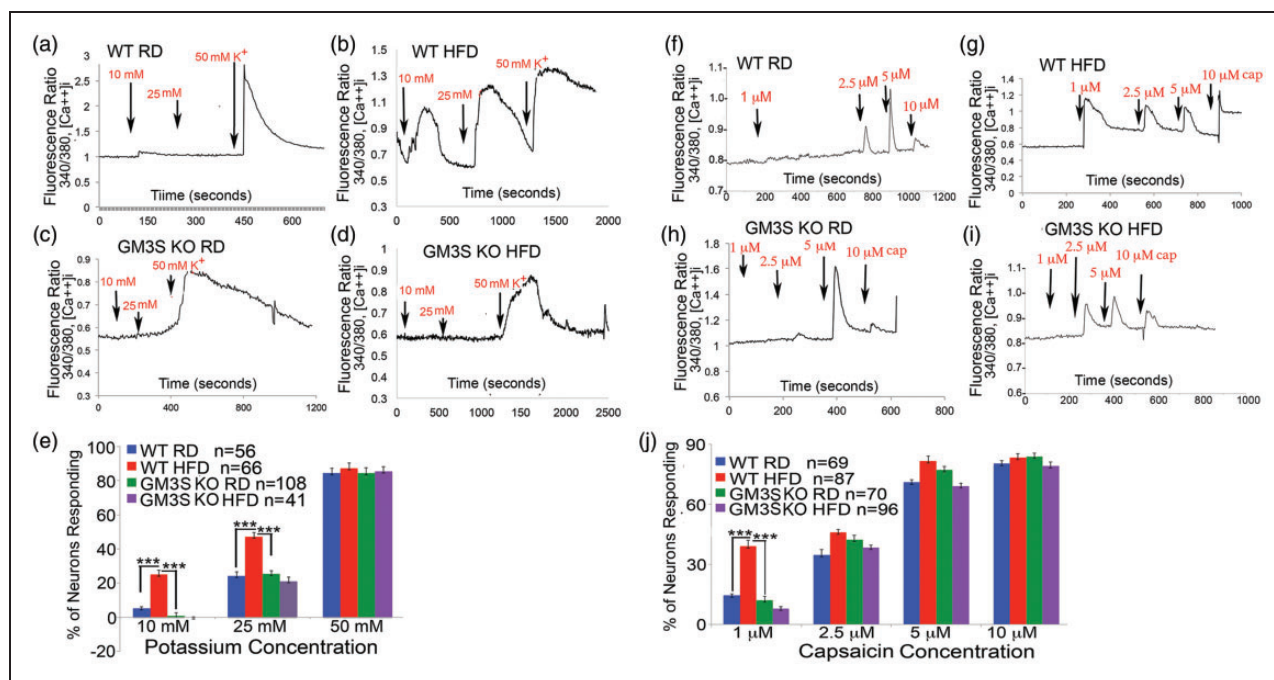
Figure 4. GM3S depletion prevents increased intracellular calcium influx in DRG neurons in diet-induced diabetes. (a–d) Representative tracings of intracellular calcium responses ($[Ca++]_i$) to different concentration of potassium (K^+) (10–50 mM) of acutely cultured DRG sensory neurons from control WT RD (a, n = 56), WT HFD (b, n = 66), GM3S KO RD (c, n = 108), and GM3S KO HFD (d, n = 41) mice. (e) Percentage of DRG neurons from each group responding to different concentration of potassium (K^+) (10, 25, and 50 mM). Values are expressed as mean ± SE. ***$p < 0.001$. (f–i) Representative tracings of intracellular calcium responses to different concentrations of capsaicin (cap) (1–10 μM) of DRG sensory neurons from the subgroups. (i) Percentage of DRG neurons from WT control mice fed with regular diet (WT RD), WT diabetic mice fed with high fat diet (WT HFD), GM3KO mice fed with RD (GM3KO RD), and GM3KO mice fed with high fat diet (GM3KO HFD) responding to different concentration of capsaicin (1, 2.5, 5, and 10 μM). Values are expressed as mean ± SE. ***$p < 0.001$.

responsive, Figures 4(a) and 4(e)) and $14 \pm 0.9\%$ responsive, Figures 4(f) and 4(j), respectively) (both $p < 0.001$). GM3S KO HFD DRGs, even from mice with 2 h GTT glucose levels $\geqslant 182$ mg/dL, had significantly lower increases in $(Ca)_I$ than WT HFD DRGs ($p < 0.001$) in response to 10 mM $K^+ (0 \pm 0.3\%)$ and 1 μM capsaicin ($8 \pm 0.9\%$) (Figures 4(d), 4(e), 4(i), and 4(j)). GM3S KO HFD DRG $(Ca)_I$ responses were no different from those in WT RD DRGs, confirming the ability of GM3 depletion to reduce hyperexcitability and associated $(Ca)_I$ into DRG neurons. The basal F_{340}/F_{380} ratios (measuring free cytosolic calcium concentration) for neurons in all four experimental groups were the same (Suppl. Table 1). Similarly, the number of neurons sensitive to the highest concentrations of K^+ (50 mM) and capsaicin (10 μM) was the same in all the groups (Figures 4(e) and 4(j)). Considering that TRPV1 expression, reflected by capsaicin sensitivity, is restricted to a major portion of nociceptors, this population of neurons is likely to be similar between DRG culture from WT RD and GM3S KO mice (Figure 4(j)).

Discussion

SFN is a well-recognized complication of T2D and has been shown to be responsible for both neuropathic pain and impaired wound healing.[26–28] In previous studies, we demonstrated that ganglioside GM3 depletion produced by GM3S knockout[15] or regional knockdown of GM3S by topically applied siRNA nanoconjugates[14] fully reverses impaired wound healing in DIO diabetic mice,[14,15] at least in part through increasing insulin/IGF-1 receptor signaling and accelerating epidermal cell migration. In the present studies, we have demonstrated that GM3S knockout, which results in GM3 depletion, also fully reverses neuropathic pain and small fibers degeneration in diabetes, possibly by preventing increased $(Ca)_I$ and neuronal hyperexcitability in DRG sensory neurons. While GM3S depletion ameliorates glucose tolerance in the mice, which could indirectly improve neuronal health, even diabetic GM3S KO mice showed preservation of cutaneous nerves, suggesting a primary effect of GM3 depletion on protecting nerves.

Neuropathic pain is associated with sustained pathological excitability of nociceptive primary afferent neurons, so that pain is produced in the absence of appropriate stimuli.[5–7] We have previously shown that DRG hyperexcitability is increased in diabetic DRGs[21] and animal models of neuropathic pain.[17,18] That GM3 depletion both reduces $(Ca)_I$ and prevents pathological nociceptor hyperexcitability further supports this association. In our experiments on cultured DRG neurons, increased $(Ca)_I$ in response to both high K^+ and capsaicin was evident in WT HFD mice, however increased thermal hypersensitivity was not observed.[11] Although culture conditions may alter DRG responses differently from DRG responses in vivo, our data suggest that thermal hypersensitivity is regulated by influences beyond TRPV1.

Degeneration of myelinated and unmyelinated fibers in the DRG and dorsal horn has been noted previously from GM3 accumulation in double knockout mice missing the two enzymes that metabolize GM3 to more complex gangliosides, GM2/GD2 and GD3 synthases.[29,30] Double knockout mice have perineural cutaneous inflammation in association with neurological changes and develop acral ulcerations that heal poorly,[29] although the molecular mechanism of the neuropathy is not well understood.

How GM3 accumulation induces nerve degeneration is unknown. The present studies provide evidence that preventing GM3 accumulation in diabetic mice protects against increased $(Ca)_I$, which leads to DRG neurite degeneration and contributes to nerve degeneration in a genetic model of SFN.[25] Indeed, increased $(Ca)_I$ is widely thought to be a major contributor to pathways leading to axonal degeneration.[31–33] Our demonstration that GM3 depletion protects from both increased $(Ca)_I$ and small fiber degeneration in diabetic mice is consistent with the possibility. It is unclear, however, whether GM3 itself has a deleterious effect or largely affects neurons through its metabolism to more complex gangliosides, particularly ganglioside GM1. GM1 has been shown to have a direct excitatory effect on DRG neurons.[34] In addition, the association of tetrodotoxin-resistant channels (Nav 1.8) in DRG sensory neurons with lipid rafts, which are highly enriched with gangliosides, and in particular GM1, has been found to regulate sensory neuron excitability.[34] GM1 is also known to accumulate in association with endoplasmic reticulum stress and calcium-dependent mitochondrial apoptosis,[35] which have been described in diabetic neuropathy.[36] Nevertheless, the development of neuropathy in the double knockout mice, with increases in GM3 but depletion of GM1 through metabolic blockade, suggests that GM3 itself, rather than more complex gangliosides, is the effector.[14,15] Selective concurrent knockdown of GM2/GD2 synthase and GD3 synthase in cultured diabetic neurons, as we have previously described in keratinocytes,[14,15] will allow future investigation of the specific effect of GM3.

While our studies have discovered increased GM3S expression in peripheral nerves and a direct impact of ganglioside alteration on DRG neurons, the possibility that ganglioside modulation also affects neurons secondarily deserves future exploration. Disturbances in epidermal–neuronal signaling in inflamed or damaged skin lead to abnormal sensory transmission that underlies associated pain.[37,38] Keratinocytes are known to produce a variety of factors that can potentially modulate and directly initiate nociceptive responses. It has recently been shown that optogenetic activation of keratinocytes can evoke pain behaviors, suggesting that robust communication occurs between keratinocytes and sensory afferents in the skin that transmit nociceptive stimuli.[39] These findings support the idea that keratinocytes, as activators of cutaneous neurons, have a central role in the onset and maintenance of such abnormal transmission. We have already demonstrated that keratinocytes from GM3S KO mice have activated basal and ligand-stimulated IR and IGF1 R signaling. The result is accelerated migration and proliferation, particularly in the presence of excess glucose, which usually suppresses IR/IGF1R signaling and inhibits keratinocyte proliferation and migration.[15] In contrast, GM3 and exposure to inducers of GM3 synthesis, such as excessive glucose and chronic low-dose TNF, suppress mouse keratinocyte migration and proliferation.[15] It is possible that GM3 deletion modulates sensory afferents and pain behavior indirectly through activation of keratinocyte signaling pathways that affect nerves or the immune system, providing an accessible target for therapeutic intervention.

From a translational perspective, the results of the present experiments suggest a novel disease-modifying treatment for diabetic neuropathy and the recalcitrant wound healing impairment through systemic or possibly topically applied interventions to deplete GM3. Indeed, topically applied SNAs are able to penetrate skin and deliver GM3S-depleting siRNA,[14] raising the possibility that topical delivery of GM3S siRNA SNA to the footpad can prevent or reverse neuropathic pain.

Author contributions

DMM drafted the manuscript, which was then edited by ASP and RJM. All authors read and approved the manuscript.

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