Evidence for Epigenetic Regulation of Gene Expression and Function in Chronic Experimental Diabetic Neuropathy

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Abstract
Diabetic polyneuropathy (DPN) is a common but irreversible neurodegenerative complication of diabetes mellitus. Here we show that features of sensory neuron damage in mice with chronic DPN may have altered epigenetic micro RNA (miRNA) transcriptional control. We profiled sensory neuron messenger RNA and miRNA profiles in mice with type 1 diabetes mellitus and findings of DPN. Diabetic sensory dorsal root ganglia neurons showed a pattern of altered messenger RNA profiles associated with upregulated cytoplasmic sites of miRNA-mediated messenger RNA processing (GW/P bodies). Dorsal root ganglia miRNA microarray identified significant changes in expression among mice with diabetes, the most prominent of which were a 39% downregulation of mmu-let-7i and a 255% increase in mmu-miR-341; both were identified in sensory neurons. To counteract these alterations, we replenished let-7i miRNA by intranasal administration; in a separate experiment, we added an anti-miR that antagonized elevated mmu-341 after 5 months of diabetes. Both approaches independently improved electrophysiologic, structural, and behavioral abnormalities without altering hyperglycemia; control sequences did not have these effects. Dissociated adult sensory neurons exposed to an exogenous mmu-let-7i mimic displayed enhanced growth and branching, indicating a trophic action. These findings identify roles for epigenetic miRNA alterations and enhanced GW/P expression in diabetic dorsal root ganglia that contribute to the complex DPN phenotype.

Key Words: Diabetes mellitus, Diabetic polyneuropathy, MicroRNA, mmu-let-7i, Peripheral neurons.

INTRODUCTION
Diabetic polyneuropathy (DPN) is a common but irreversible complication that develops in up to 50% of patients with diabetes and results in sensory loss, pain, and risk of amputation (1). Although the pathogenesis of DPN is complex, a common theme is that polyneuropathy is a unique sensory-centric neurodegenerative disorder that leads to retraction of axons from their targets (2). Diverse components of DPN pathogenesis identified in models studied to date include alterations in conduction of motor and sensory axons, loss of distal sensation, and a series of specific neuronal messenger RNA (mRNA) changes. The latter includes declines in structural proteins such as neurofilament and βIII tubulin, increases in heat shock protein 27 and receptor for advanced glycosylation end product, and declines in growth proteins such as growth-associated protein 43 (3, 4). Although many of the changes have been attributed to targeting by the advanced glycosylation end product—albeit controversial—impacts of oxidative stress, nitricergic stress, and hyperglycemia-associated glycosylation of proteins, it is plausible that these changes converge upon a direct impact on gene expression. The wide manifestations might be accompanied by primary alterations in the micro RNA (miRNA) profile of sensory neurons of experimental and human diabetes (5). To address this possibility, we examined alterations in and impact of combined mRNA and miRNA profile changes in a long-term mouse model of DPN that manifests the diverse features of human disease.

MATERIALS AND METHODS
Induction of Diabetes
Outbred adult male (22–25 grams) CD1 mice (Charles River, St Constant, Canada) were used in the study. Procedures were reviewed and approved by the University of Calgary Animal Care Committee, in conjunction with guidelines from the Canadian Council of Animal Care. Diabetes was induced by intraperitoneal injections of streptozotocin (Sigma, St Louis, MO) 85, 70, and 55 mg/kg dissolved in citrate buffer (pH 4.5) for 3 consecutive days. Control animals received intraperitoneal injections of citrate buffer alone. Diabetes status was confirmed by a fasting glucose level of 16 mmol/L or higher on a blood glucometer (One Touch Ultra; Lifescan Canada, Burnaby, Canada).
Electrophysiology

Multifiber motor and sensory conduction recordings were carried out in the left sciatic tibial fibers of mice anesthetized with isoflurane at a near-nerve subcutaneous temperature of 37°C (maintained by a thermosensitive heat lamp as described previously) (6).

Functional Tests of Sensation

Mechanical and thermal sensitivity were measured as described previously (6).

mRNA and miRNA Analyses

Tissues were harvested under RNase-free conditions, and small RNA was extracted with a mirVana Isolation kit (Ambion, Carlsbad, CA). RNA quality was determined and RNA integrity number was measured with Agilent RNA Nano Chips on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Three hundred nanograms of RNA for each sample with RNA integrity number 8 or higher was labeled using the GeneChip Whole Transcript Sense Target Labeling Assay (Affymetrix, Santa Clara, CA) and hybridized to an Affymetrix GeneChip Mouse Gene 1.0ST Array at 45°C overnight. The final product was analyzed using 1.5% agarose gel. To determine whether our signals were delivered into tissues, we injected Arabidopsis thaliana miRNA miR171. Because these miRNAs are not expressed endogenously in mice, their presence in mouse tissues indicates that mimic sequences were delivered intranasally. The sequence of miRNA 171 (11) was synthesized in the DNA core laboratory (University of Calgary, Calgary, Canada). The reverse primer used was the universal reverse primer provided by the Qiagen miScript amplification kit, with analysis performed as described previously using the comparative C1 method (2.ΔΔCt).

Quantitative reverse transcription–polymerase chain reaction

Quantitative reverse transcription–polymerase chain reaction (qRT-PCR) was performed according to previous descriptions (10). Relative expression values were generated using the comparative C1 method (2.ΔΔCt) in which all genes of interest were standardized to RPLP0 expression. Primer sequences were as follows: RPLP0, MF5; TGGTGAAAACACGAGACC CA-3; insulin receptor, MF5; RGGATACCTCATGTCCT-3; Rb, MF5; TGGATAACCTGAGAGAGTCTG-3; and R5; RGGATACCTCATGTCCT-3. Relative expression values were generated using the comparative C1 method (2.ΔΔCt). Fold change, gene ontology, and hierarchical clustering data were not expressed endogenously in mice, their presence in mouse tissues indicates that mimic sequences were delivered intranasally. The sequence of miRNA 171 (11) was synthesized in the DNA core laboratory (University of Calgary, Calgary, Canada). The reverse primer used was the universal reverse primer provided by the Qiagen miScript amplification kit, with analysis performed as described previously using the comparative C1 method (2.ΔΔCt).

Adult Sensory Neuron Cultures

Three-day preinjured L4, L5, and L6 dorsal root ganglia (DRG) were dissociated and used for the sensory neuron culture protocol, as described previously, with minor modifications (12). For each slide, either of two reagents was added to the culture medium in accordance with the experimental design: Let-7i mimic, 5’-UGAG GUAGUAGUULGUGCUUGU-3’, miScript primer assay 171 Arabidopsis, 5’-TTCCTGTATTGAGCAGGCC-3’. Let-7 mimics complementary DNA was analyzed using a Qiagen miScript PCR complementary DNA synthesis kit and miScript primer assays (Qiagen, Toronto, Canada). Reverse transcription–polymerase chain reaction was performed using StepOnePlus PCR system (Applied Biosystems), and the final product was analyzed using 1.5% agarose gel. To determine whether our signals were delivered into tissues, we injected Arabidopsis thaliana miRNA miR171. Because these are not expressed endogenously in mice, their presence in mouse tissues indicates that mimic sequences were delivered intranasally. The sequence of miRNA 171 (11) was synthesized in the DNA core laboratory (University of Calgary, Calgary, Canada). The reverse primer used was the universal reverse primer provided by the Qiagen miScript amplification kit, with analysis performed as described previously using the comparative C1 method (2.ΔΔCt).
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**a** Glucose

![Bar chart showing blood glucose levels with diabetes status.](chart_a)

**b** Mechanical sensitivity

![Bar chart showing mechanical sensitivity time with diabetes status.](chart_b)

**c** Thermal sensitivity

![Bar chart showing thermal sensitivity time with diabetes status.](chart_c)

**d** Motor axons

![Bar chart showing motor axon conduction velocity with diabetes status.](chart_d)

**e** Sensory axons

![Bar chart showing sensory axon conduction velocity with diabetes status.](chart_e)

**f** Nondiabetic

![Image showing nondiabetic tissue.](image_f)

**g** Diabetic

![Image showing diabetic tissue.](image_g)

**h** Epidermal axon density

![Bar chart showing epidermal axon density with diabetes status.](chart_h)

**i** Total axons/mm²

![Bar chart showing total axons/mm² with diabetes status.](chart_i)

![Bar chart showing vertical axons/mm² with diabetes status.](chart_i)
**Intranasal Injections**

Experiments were designed to test whether intranasal mmu-let-7i administration accesses cerebrospinal fluid and lumbar DRG. First, adult CD1 mice were given intranasal injections with labeled let-7 mimic (RNA-Quasar570; University Core DNA Services; mmu-let-7i-5p miScript miRNA mimic, 5′ UGAGGUAGAUUUGUCUGUU; Qiagen) on Days 0, 2, 4, and 6. On Day 6, after 5 hours of intranasal injection, another intranasal injection was given; after 20 minutes, the olfactory bulb was harvested and the samples were fast frozen and stored at −80°C. Fourteen-micrometer sections were prepared and visualized without washing or coverslipping the slides. The second group of mice was administered intranasal *A. thaliana* miR171 as described previously and, on Day 7, underwent harvesting for qRT-PCR amplification of miR171 in DRG and olfactory bulb samples. For functional studies of mmu-let-7i mimic replacement by intranasal injection, 5-month-old mice with diabetes and control CD1 mice both received let-7i intranasal treatment or control treatment (4 groups). Solutions for let-7i treatment (performed by investigator Chu Cheng) included 3 μL of mmu-let-7i mimic 20 nmol/L (Syn-mmu-let-7i-5p miScript miRNA mimic, 5′-UGAGGUAGAUUUGUCUGUUU-3′; cat no. MSY0000122; Qiagen), 6 μL of HiPerFect Transfection Reagent (cat no. 301707; Qiagen), and 11 μL of saline. Solutions for control injections included 3 μL of miScript target protector 20 nmol/L (NEG; control target protector, 5′-3′; cat no. MTP0000002; Qiagen), 6 μL of HiPerFect Transfection Reagent, and 11 μL of saline. Solutions for mmu-miR-341 anti-miR were prepared and administered identically but by a different investigator (Masaki Kobayashi) at a different time (MMU-MIR-341-3P; cat no. MIN0000588; Qiagen), with its own control group (negative siRNA control; AllStars Negative Control siRNA; cat no. 1027281; Qiagen). The duration of diabetes was slightly shorter in this latter cohort (3–4 months). Under brief anesthesia, an intranasal injection of mimic or control was administered to each animal on Days 0, 2, 4, and 6 (20 μL divided in half for each nostril). Two weeks after the last injection, electrophysiologic and behavioral tests were repeated. Finally, blood glucose was tested, and samples were harvested at 4 weeks.

**Immunohistochemistry**

Immunohistochemistry, including epidermal analysis, was carried out as described previously(12, 13). Individual GW/P body foci were counted with Cell-Profiler image analysis software using 3 slides from each of the 4 mice in each group, with the examiner blinded and assessing 4 distinct organized quadrants within each slide (14). Samples were fixed in 2% PLP (i.e. 2% paraformaldehyde, l-lysine, and sodium periodate) for 18 hours at 4°C and cryoprotected overnight in 20% glycerol/Sorenson phosphate buffer 0.1 mol/L at 4°C. Skin sections 25 μm thick were blocked in 10% goat serum for 1 hour at room temperature. Primary antibody PGP 9.5 (rabbit polyclonal, 1:1000; EnCor Biotechnology Inc, Gainesville, FL) was applied overnight at 4°C followed by goat anti–rabbit Cy3 (1:100; Jackson Immunoresearch, West Grove, PA) secondary antibody for 1 hour at room temperature. Images were captured using an Olympus laser scanning confocal microscope (original magnification: 100×; step size, 1 μm).

**In Situ Hybridization**

For mmu-let-7i miRNA or mmu-miR-341 detection, the protocol of Obernosterer et al (15) was used, with some modifications. For hybridization, 1 μL (10 pmol) of has-let-7i miRCURY LNA detection probe (Exiqon, Woburn, MA) was added to 150 μL of hybridization buffer (50% formamide, 5× saline sodium citrate, 1× Denhardt solution, yeast transfer RNA 0.25 mg/mL, salmon sperm DNA 0.25 mg/mL, polyA 0.1 mg/mL, and 10% dextran sulfate). The slides were incubated in anti–digoxigenin alkaline phosphatase antibody (Roche Applied Science) and in nitro blue tetrazolium/5-bromo-4-indolyl-phosphate 50 mg/mL, 2.4 blue tetrazolium 50 mg/mL, 3.5 periodate) for 18 hours at 4°C. Secondary antibody (goat anti–rabbit Cy3) was applied overnight at 4°C. Polyclonal antibody PGP 9.5 (rabbit, 1:100; Jackson Emergency Research, West Grove, PA) was applied overnight at 4°C. Samples were washed and incubated with anti–digoxigenin alkaline phosphatase antibody (Roche Applied Science) and in nitro blue tetrazolium/5-bromo-4-indolyl-phosphate for 20 hours and fixed and processed for immunocytochemistry with anti–neurofilament 200 antibody (1:800; Sigma). Outgrowth was analyzed as described previously (12).

**Statistical Analysis**

Results are presented as mean ± SE and compared using 1-way analysis of variance with post hoc Tukey comparison or Student t-test (2-tailed unless specified; 1-tailed as indicated with an expected direction of change), as appropriate. Note that biologic replicates (separate mice from each group) were used for statistical analyses of miRNA and mRNA.

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**FIGURE 1.** Experimental diabetes of 5 months’ duration was associated with hyperglycemia (a), higher thresholds of mechanical (b) and thermal (c) sensitivity, and slowing of motor (d) and sensory (e) conduction velocities. Epidermal and dermal innervation is illustrated in a mouse without diabetes (f) and in a mouse with diabetes (g). *Superficial side of the skin section. Scale bar = 20 μm. Mice with diabetes had lower densities of all axon profiles (h) (oriented in all directions) and vertically directed axons (i). p < 0.0001, Student t-test. (h) p = 0.04, 1-tailed Student t-test. (i) p = 0.03, Student t-test. (a, b) n = 18 controls; n = 14 with diabetes. (d, e) n = 17 controls; n = 13 with diabetes. (h, i) n = 5 controls; n = 3 with diabetes. The duration of diabetes was 5 months (no insulin treatment was provided). (+) With diabetes present; (−) control (no diabetes).
RESULTS

Abnormalities in Mice With Diabetes

As detailed in previous work, the mouse model for chronic DPN—analyzed here for 5 months (without insulin treatment)—replicated critical features of the human disease, including persistent hyperglycemia (Fig. 1a); loss of mechanical (Fig. 1b) and thermal (Fig. 1c) sensitivity; electrophysiological abnormalities, including slowing of multifiber motor (Fig. 1d) and sensory (Fig. 1e) conduction (6); and loss of epidermal innervation (Figs. 1f–i) (1, 16). The final weight of mice with diabetes was reduced (41.3 ± 1.0 g; n = 16) compared with that of littermate controls (58.0 ± 1.5 g; n = 18) (p < 0.0001).

Evidence for Alterations in Gene Output in Diabetic Sensory Neurons

GW/P bodies are non–membrane-bound cytoplasmic organelles containing proteins of the RNA-induced silencing complex that function in posttranscriptional gene regulation through miRNA-mediated mRNA processing (17–20). Diabetic DRG sensory neurons had upregulation in the number of discrete cytoplasmic GW/P bodies, supporting the possibility of altered regulated mRNA processing (Figs. 2a–d). The expression of GW/P bodies reflects levels of altered cellular physiology, a feature of diabetic sensory neurons (19, 21).

We next performed gene expression microarray studies of DRG in mice with chronic diabetes (Figs. 2e, f; Figure, Supplemental Digital Content 1, http://links.lww.com/NEN/A771). Our analysis examined 28,869 genes and identified 261 mRNAs that differed between diabetic and nondiabetic DRG by at least 1.5-fold. This included 170 downregulated mRNAs and 91 upregulated mRNAs. Of these, 24 (5 downregulated and 19 upregulated) showed a change that attained a difference of p < 0.05 (Fig. 2f). These genes represented diverse functions. Although not further explored here, each altered mRNA may affect neuronal function, but most mRNAs have not been analyzed in diabetic polynephropathy or are of unknown function.

Altered miRNA Profiles in Sensory Ganglia of Mice With Diabetes

Micro RNAs, acting through mRNA regulation, may play a profound role in neuronal function (22–24). We examined miRNA expression and identified 19 miRNAs (12 downregulated and 7 upregulated) of higher abundance (signal intensity >500) that were significantly altered in mice with diabetes (among 1,042 examined) (Figs. 3a–c). In addition, 123 miRNAs (56 downregulated and 67 upregulated) of low abundance (signal intensity, 32–500) were significantly different between mice with diabetes and controls (Figure, Supplemental Digital Content 2, http://links.lww.com/NEN/A772).

We compared parallel microarrays of DRG mRNA and miRNA in the same DPN mouse cohort using Ingenuity Pathway Analysis. Selecting only 4 of the altered miRNA profiles that had achieved high statistical significance (p < 0.01), we identified significant and predicted changes in mRNA profiles of at least 1.5-fold. These included a 1.5-fold upregulation of connective tissue growth factor, a growth factor related to insulin-like growth factor (IGF)-1 and its downstream target, IGF-1 receptor (linked to downregulation of mmu-miR-106A); a 1.6-fold upregulation of dual-specificity phosphatase 1, a nuclear phosphatase (linked to downregulation of mmu-let-7i); a 1.6-fold upregulation of F3, a transmembrane receptor (linked to downregulation of mmu-miR-106A); and a 1.7-fold upregulation of TXNIP, a thioredoxin-interacting protein that may mediate oxidative stress (linked to downregulation of mmu-miR-106A). Linked relationships between mmu-let-7i and changes in mRNAs are listed in Table, Supplemental Digital Content 3, http://links.lww.com/NEN/A773. Downregulation of mmu-let-7i was linked to a predicted rise in insulin receptor. Although insulin receptor rose by only 1.17-fold (not significant), the finding was consistent with significant increases in IRβ mRNA expression identified by qRT-PCR in the work of Guo et al (13) and Sugimoto et al (25). Downregulation of mmu-let-7i was linked to a predicted decline in CACNG4. On array, CACNG4 only declined by 1.2-fold (not significant), but this decline was significant upon qRT-PCR analysis of samples from diabetic DRG. CACNG4 is a subunit of the calcium L-type channel. Two previous reports have linked abnormalities in this channel to experimental diabetes (26, 27). Because we focused on 4 examples of altered miRNAs and established tight selection criteria for linked mRNAs, it is likely that substantially more interactions might be predicted than identified in this work. For example, mmu-let-7i is predicted to target more than 900 conserved targets (Ingenuity Pathway Analysis and TargetScan analysis; http://www.targetscan.org/; Whitehead Institute, Cambridge, MA), which include NGF, CACNG4, IGF-1, IGF-1R, IGF-2BP1–3, MAP3K1/3, MAP4K3/4, and many others. Sorted by
function, mmu-let-7i is predicted to target at least 46 apoptotic cell death pathway miRNAs, 42 cardiovascular and diabetes-related mRNAs, 84 growth pathway mRNAs, 80 inflammation-related pathway mRNAs, 21 metabolism and diabetes pathway mRNAs, and 59 neurotransmitter and nervous system mRNAs (Ingenuity Pathway Analysis).

To assess the potential relevance of miRNA changes to diabetic phenotype, we selected mmu-let-7i, a member of the let-7 family, one of the best known and first characterized miRNAs. mmu-let-7i was also downregulated by 39% in diabetes—a highly significant shift \( (p < 0.01) \). We also confirmed this change by qRT-PCR (Fig. 3d). In contrast, mmu-miR-341 was the most abundantly upregulated (i.e. by 255%; \( p = 0.05 \)).

We next confirmed that mmu-let-7i was expressed in sensory neurons by in situ hybridization. Dorsal root ganglia neurons were stained with let-7i and mmu-341 miCURY LNA detection probes, including all neurons in the section, whereas labeling of perineuronal satellite cells, blood vessels, or other cellular components was not identified.

**Let-7i Offers Overall Trophic Support for Adult Sensory Neurons**

We speculated, on in vitro analysis, that downregulated mmu-let-7i might impact the growth properties of sensory neurons targeted by diabetes (12, 28). Neurons exposed to a let-7i mimic had greater total neurite outgrowth, maximal process length, and branching compared with controls (Figs. 4e–l).

**Intranasal Nucleotides Access Lumbar DRG**

Because intranasal injection of insulin accesses and signals DRG neurons in diabetes (29–31), we hypothesized that miRNAs might similarly access the cerebrospinal fluid and DRG within root sleeves (32). Mice without diabetes that were exposed to intranasal let-7i miRNA (administered every second day for 6 days) had upregulated let-7i miRNA levels in their DRG, as identified by qRT-PCR analysis on Day 7 (Fig. 5a). Next, we examined sections of the olfactory bulb (a structure within the blood-brain barrier adjacent to the nasal cavities) and confirmed that discrete puncta of fluorochrome-labeled let-7i were identified around the outer surface of the bulb (Fig. 5b). We repeated intranasal delivery with an unrelated plant species miRNA—*A. thaliana* miR171—not present in mammals. After administration for 6 days, miR171 was detected in both the olfactory bulb and the lumbar DRG ganglia (Fig. 5c). We also examined whether exogenous let-7i influenced mRNA levels of selected neuronal proteins associated with plasticity. No changes in phosphatase and tensin homolog, retinoblastoma 1, glycogen synthase kinase 3B, or peroxisome proliferator-activated receptor \( \gamma \) mRNA levels were detected, but there was a borderline rise in Akt mRNA levels of DRG (Fig. 5d).

**miRNA Manipulation Reverses Features of Experimental Neuropathy**

To replenish abnormal diabetic miRNA levels, we supplemented mmu-let-7i using a mimic; to downregulate abnormal diabetic miRNA levels, we inhibited mmu-miR-341 with an anti-miR. Each was delivered independently, with its own control, by intranasal injection. Intranasal mmu-let-7i, but not mmu-miR-341 inhibitor, improved impaired diabetic mechanical sensitivity to the levels of mice without diabetes (Figs. 6a, 7a). Similarly, loss of thermal sensitivity was significantly improved after intranasal let-7i or mmu-miR-341 anti-miR administration (Figs. 6b, 7b). Motor and sensory conduction velocity were also improved by either let-7i or mmu-miR-341 anti-miR (Figs. 6c, d, 7c, d). Finally, there was a significant improvement in footpad epidermal innervation in 5-month-old mice with chronic diabetes or in littermate mice 3 weeks after the 6-day course of intranasal let-7i miRNA (not tested with mmu-miR-341 inhibitor) administration (13) (Figs. 6e–j). In mice without diabetes, let-7i also increased the density of epidermal axons.

**DISCUSSION**

In both human and animal models, sensory neurodegeneration in chronic diabetes is associated with early withdrawal of distal axons from their targets—a pattern that accounts for the “stocking-and-glove” loss of sensation in human DPN (2). These changes accompany widespread abnormalities involving electrophysiology and alterations in gene expression that indicate a degenerative phenotype (3, 4). How prevailing hypotheses on DPN development—including oxidative and nitric stress, polyol accumulation, microangiopathy, inappropriate AGE-RAGE signaling, or mitochondrial dysfunction—might account for diverse miRNA changes is unclear. Here, we suggest that altered miRNA expression patterns may accompany a diverse DPN phenotype. Direct connections between let-7i downregulation and insulin receptor upregulation offer one of several potential connections between miRNA supraregulatory control and trophic insulin signaling. Exogenous insulin renders direct trophic actions.

**FIGURE 3.** Heat map results comparing DRG miRNA microarrays of 5-month-old mice with diabetes and mice without diabetes at the \( p < 0.05 \) (a) and \( p < 0.01 \) significance levels (b). (c) Selected higher abundance miRNAs are shown indicating fold change and significance level at \( p \leq 0.10. \) * \( p \leq 0.05 \) and ** \( p < 0.01 \). Student t-test; \( n = 6 \) control mice; \( n = 6 \) mice with diabetes (for each mouse DRG, \( n = 1 \)). (d) Independent miRNA qRT-PCR analysis of relative mmu-let-7i levels confirms its reduction in diabetic DRG. * \( p = 0.001 \), Student t-test; \( n = 6 \) nondiabetic arrays; \( n = 5 \) diabetic arrays.
on sensory neurons and alters their patterns of mRNA expression, and near-nerve insulin reverses features of experimental diabetes (13, 33–36). Enhanced cytoplasmic processing of mRNAs by miRNA in GW/P bodies may account for the downregulation of specific mRNAs. For example, in long-term rat models of chronic diabetes, some key neuronal mRNA
FIGURE 4. In situ hybridization analysis of mouse DRG identifies mmu-let-7i and mmu-miR-341 expression prominent in neurons at lower power (a, d), with probe omitted control (con) (b) and mmu-let-7i at higher power (c). Scale bars = (a, d) 100 μm; (b, c) 50 μm. Confocal images of preinjured (axotomized) adult rat sensory neurons harvested from lumbar DRG and grown in the presence of control carrier solution (carrier, buffer without miRNA; e, f, both control examples) or a mimic molecule of mmu-let-7i (20 μmol/L in HiPerFect transfection buffer; g, h, both intervention samples) for 20 hours and stained with anti-neurofilament antibody. Scale bar = 100 μm. (i-f) Quantitation of neurite outgrowth. Exposure to let-7i (l-7i) was associated with a rise in total neurite outgrowth (l), maximal process length (j), and branching of neurons (k). There was also a nonsignificant trend toward more neurite processes in each neuron (l). (h) * p = 0.04, (i) p = 0.03, (j) p = 0.004, Student t-test; n = 5 independent experiments.

FIGURE 5. (a) Intranasal administration of let-7i for 7 days increased the endogenous levels of miRNA in DRG (mice without diabetes). * p = 0.046, Student t-test; n = 8 controls (let-7i supplementation). (b) Intranasal injection of fluorochrome-labeled let-7i mimic for 7 days and harvesting without fixation of olfactory bulbs to examine fluorochrome uptake (arrows) in cerebrospinal fluid and in the peribulb environment (left). Middle, autofluorescence; right, transmitted light. (c) Intranasal administration of A. thaliana miR171 for 7 days was associated with a new expression of the nucleotide in DRG neurons and olfactory bulb (c, control injection; i, miR171). (d) Relative miRNA levels of plasticity-related neuronal proteins after 7-day treatment with mmu-let-7i mimic or control. # p = 0.05, 1-tailed Mann-Whitney U test; n = 6-9 in both groups. Akt, protein kinase B; GSK3B, glycogen synthase kinase 3β; PPARg, peroxisome proliferator-activated receptor γ; PTEN, phosphatase and tensin homolog; Rb1, retinoblastoma 1.
signals (including neurofilament, tubulin, Trk receptors, and growth-associated protein 43) detected by in situ hybridization were downregulated (4).

It is remarkable that restoring mmu-let-7i or knocking down mmu-miR-341 (only two of the prominent miRNA alterations that we observed) rapidly reversed several key facets of the DPN phenotype. After administration for only 1 week and reanalysis after 3 weeks, there was improvement in the major features of the phenotype despite the fact that the changes had taken 5 months to develop. All changes were independent of hyperglycemia; glucose levels were not improved by the interventions. Moreover, ongoing hyperglycemia would be expected to accumulate alterations in systemic polyls and oxidative stress. Despite these continued diabetic “insults,” direct neuronal manipulation through exogenous let-7i miRNA treatment reversed indices of sensory neuron damage. Altered miRNA regulatory control may operate as a final downstream mechanism of diabetic neuronal damage. The dissociation of neuronal function from hyperglycemia argues against the hypothesis that these neuropathic changes are irreversible consequences of high glucose and glycosylation. On a related note, it is possible that a lasting epigenetic profile in human sensory neurons, in part generated by miRNA changes, may also account for “hyperglycemic memory”—the long-term persistence of altered phenotype from early intervention despite later relapses in control (37).

Because mmu-let-7i miRNA and mmu-miR-341 potentially target many individual mRNAs, identifying a single targeted intervention that accounts for DPN is a daunting task and has not been identified in any extensive work on the disorder to date. An important potential link between nuclear factor-κB (which is implicated in a number of diabetic complications, including neuropathy) and let-7i was suggested by let-7i downregulation in human biliary cells by lipopolysaccharide. A nuclear factor-κB p50-C/enhancer-binding protein-β silencer complex was demonstrated to bind to the let-7i promoter, which in turn promoted histone-H3 deacetylation (38). At this stage, the overall impact of let-7i on sensory neurons is trophic, altering its complex and extensive client targets in a way that favors growth. Zhang et al (39) suggested that miR-29b, downregulated in a model of streptozotocin-induced chronic diabetes in rats, protects DRG neurons, whereas Wang et al (40) identified similar findings in db/db mouse neurons with yet a further sequence, miR-146a. However, neither study examined animals with established features of DPN, characterized by epidermal loss of innervation or electrophysiologic abnormalities. The full context of these changes in relationship to a wider spectrum of miRNA changes was unknown. Furthermore, Wang et al (40) studied neurons exposed to short-term hyperglycemia, an intervention that cannot be considered a model of chronic diabetes, whereas Zhang et al (39) identified extensive apoptosis and neuron pathology—features that are not congruent with data that have not identified substantial apoptosis in models of long-term diabetes (4, 41). Although a new study has demonstrated linkage between specific miRNAs and overall alterations in pain function (42), their role is considered supraregulatory.

Here, we demonstrate delivery of small bioactive nucleotide molecules to the CNS by intranasal dosing. The findings support previous suggestions from our laboratory that central manipulation of DRG neurons is effective in altering the behavior of the entire neuronal tree, including its distal terminals. We believe that the functional impact of miRNAs, along with evidence that it accessed DRG neurons, indicates a potential new, nonviral approach toward altering CNS or peripheral nervous system gene function. Nonviral delivery obviates a number of concerns raised around the use of viral vectors, albeit higher transfection efficiency using viral delivery may yet supersede the approach used here. Nonviral approaches and noninvasive delivery may allow a more rapid translation of mRNA manipulation strategies to clinics.

**FIGURE 6.** Thresholds for mechanical sensitivity increased in mice with diabetes but decreased in mice exposed to the active mmu-let-7i miRNA mimic (a). Similarly, diabetes was associated with elevated thresholds for thermal sensation (b) and was improved by mmu-let-7i. (a) Mechanical, * p = 0.0008 diabetic baseline versus let-7i; mechanical control (con) nondiabetic (nondia) versus diabetic (dia), * p = 0.0002. (b) Thermal, * p = 0.028 diabetic baseline versus let-7i (1-tailed); con nondia versus dia, * p < 0.0001. Note pretreatment mechanical and thermal (* p < 0.0001) nondia versus dia (as in Fig. 1); Student t-test; n = 6 let-7i dia, 8 nondia; n = 6 con dia, 7 nondia. Both motor (c) and sensory (d) conduction velocities were reduced in mice with diabetes, with reversal after exposure to mmu-let-7i. (c) Motor, * p = 0.0003 diabetic control versus let-7i; motor con nondia versus dia, p < 0.0001; n = 9 let-7i dia, 10 con dia; n = 16 let-7i nondia, 14 con nondia. (d) Sensory, * p = 0.035 diabetic con versus let-7i (1-tailed); sensory con nondia versus dia, p = 0.004; n = 10 let-7i dia, con, 10 dia; n = 16 let-7i nondia, 14 con nondia. Note pretreatment motor and sensory (* p < 0.0001) nondia versus dia (as in Fig. 1); Student t-test. Epidermal innervation (e) or profiles directed 45 degrees or more toward the surface of the skin (vertical profiles) (f) mmu-let-7i was associated with a rise in epidermal profiles in mice without diabetes, improvement in total and vertical axon density in mice with diabetes, and a rise in vertical axon density in mice with diabetes that received active miRNA compared with control. (e) * p = 0.04, con nondia versus dia, 1-tailed Student t-test (as in Fig. 1); * p = 0.033, nondia con versus nondia let-7i, 1-tailed Student t-test. (f) * p = 0.03, con nondia versus con dia (as in Fig. 1); * p = 0.007, con nondia versus let-7i dia; p = 0.05, con dia versus let-7i dia; Student t-test; n = 5 let-7i dia, 3 con dia; n = 4 let-7i nondia, 5 con nondia. Samples of footpad sections with labeled PGP 9.5 immunopositive axons are illustrated in nondiabetic controls exposed to control (g) or mmu-let-7i (h) and in mice with diabetes that were exposed to control (i) or mmu-let-7i (j). Scale bar = 20 μm. The duration of diabetes was 5 months; no insulin treatment was provided.

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FIGURE 7. mmu00miR-341 i (inhibitor) did not affect elevated thresholds for mechanical sensitivity (a) in mice with diabetes (dia) but reversed elevated thresholds for thermal sensation (b). Mechanical, *p < 0.0001, nondiabetic (nondia) control (con) versus dia con. Thermal, *p < 0.0001 nondia con versus dia con. *p = 0.0006 dia con versus dia mmu-341i; Student t-test; n = 12 mmu-341i dia, 12 nondia; n = 12 con dia, 12 nondia. Both motor (c) and sensory (d) conduction velocities were reduced in mice with diabetes, with reversal after exposure to mmu-341i. (c) Motor, *p < 0.0001, nondia con versus dia con; * p = 0.0002, dia mmu-341i versus dia con. (d) Sensory, *p < 0.0001, con nondia con versus dia con; *p < 0.0001, mmu-341i versus dia con; n = 12 mmu-341i nondia, n = 12 nondia con; Student t-test. Duration of diabetes in mice was 3 to 4 months; no insulin was added.

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REFERENCES
