Rab7 Silencing Prevents \(\mu\)-Opioid Receptor Lysosomal Targeting and Rescues Opioid Responsiveness to Strengthen Diabetic Neuropathic Pain Therapy

Shaaban A. Mousa,1 Mohammed Shaqura,1 Baled I. Khalefa,1 Christian Zöllner,2 Laura Schaad,1 Jonas Schneider,1 Toni S. Shippenberg,3† Jan F. Richter,4 Rainer Hellweg,5 Mehdi Shakibaei,6 and Michael Schäfer1

Diabetic neuropathy is a common long-term complication of diabetes mellitus. According to epidemiological studies, at least 20% of patients with diabetes have a manifestation of diabetic polyneuropathy, and approximately one-half of these individuals have pain (1) that is difficult to treat and is known to be less susceptible to opioid analgesics. Only high doses of opioids, e.g., 400–600 mg tramadol (2) or 40–60 mg oxycodone (3), are reported to achieve a comparable therapeutic effect (i.e., number needed to treat, 3–5), resulting in a high incidence of opioid-associated side effects such as sedation, cognitive dysfunction, constipation, nausea, and vomiting (4). Particularly in the elderly population, these side effects may have a great impact on quality of life (5).

In rodent models of streptozotocin (STZ)-induced diabetic neuropathy, the antinociceptive efficacy of opioids after their systemic (6), spinal (7,8), or supraspinal (8) administration is reduced relative to controls. Although various mechanisms for the loss of antinociceptive efficacy have been investigated mainly at the level of the spinal cord, the findings remain discordant (7,9,10). According to the American Diabetes Association, diabetic neuropathy defines the presence of symptoms or signs of peripheral nerve dysfunction in diabetes (1). On these peripheral sensory neurons, opioid receptors have been identified (11) that contribute to the antinociceptive effects of opioids (12). Consistently, the antinociceptive effects of systemic delta-opioid agonists were significantly attenuated in conditional knockout mice lacking delta-opioid receptors in peripheral sensory neurons (13). Moreover, \(\mu\)-opioid receptor (MOR) expression, G-protein-coupling, and efficacy are enhanced during chronic inflammatory pain, resulting in increased opioid antinociception (11) and less tolerance, i.e., reduced antinociceptive efficacy on repeated administration (14). Interestingly, in animals with neuropathic pain attributable to a chronic constriction injury, axonal MORs at the constriction site are a potential target for local opioid application, resulting in potent analgesia (15).

In this study, we sought to investigate whether MOR density and functional coupling are impaired in peripheral sensory neurons as a consequence of diabetes, thereby decreasing opioid therapeutic efficacy. We hypothesized that the density of sensory neuron MOR is reduced because of enhanced Rab7-dependent lysosomal targeting of MOR in vivo. To test this hypothesis, we used the model of STZ-induced diabetes and used different strategies to reverse Rab7-dependent lysosomal targeting of sensory neuron MOR to restore their responsiveness to opioids.

RESEARCH DESIGN AND METHODS

Reagents. We used the following reagents: \([3H]\)DAMGO (50 Ci/mmol); \([35S]\) GTP\(_\gamma\)S (1250 Ci/mmol); STZ; morphine; penicillin; fentanyl, naloxone; mouse monoclonal Rab7 antibody; high-performance liquid chromatography–purified Rab7 small interfering RNA (siRNA) sense (5'-UACUGGUUCAUAGCAGAUUGCUUUC-3') and antisense (5'-GAAAGACACUGCAUAGCAGACCUAGA-3'); negative control siRNA (scrambled sequence; Sigma-Aldrich, Taufkirchen, Germany) (16); Max Suppressor In Vivo RNA-LANCEr II, a formulation that enables highly efficient delivery of siRNA into animals (Bio Scientific Corporation); scintillation fluid (Perkin Elmer Wallac, Turku, Finland); artificial cerebrospinal fluid; nerve growth factor (NGF) (R&D Systems, Minneapolis, MN); D-lactic acid (BD Biosciences, San Jose, CA).
MN), rabbit polyclonal MOR antibody (Graimsch Laboratories, Schwabhausen, Germany); mouse monoclonal GAD 65 antibody (Millipore GmbH, Schwalbach/Ts, Germany); rabbit polyclonal insulin (total insulin) antibody (Cell Signaling Technology, Danvers, MA); guinea pig polyclonal calcitonin gene–related peptide antiserum (Santa Cruz Biotechnology). Filters were lysosome–associated membrane glycoprotein-1 (Santa Cruz Biotechnology) and chicken polyclonal PGP9.5 antibody (EnCor Biotechnology).

**STZ-induced diabetes.** Experiments were conducted in age-matched male Wistar rats in accordance with the science-based guidelines for laboratory animal care of the National Research Council (2005) and were approved by the local animal ethics committee. Animals were received at 35–40 days of age and were intraperitoneally injected with STZ at a dosage of 40 mg/kg in 0.8 mL of citrate buffer (0.03 mol/L, pH 4.7). The age-matched control animals received an equal volume of citrate buffer alone. Diabetes was verified 3 days later by measuring blood hyperglycemia in the tail vein blood using a glucose strip Glucoflex (H&H DiabetesCare GmbH, Waiblingen, Germany).

**Antinociceptive testing.** Mechanical pain thresholds were assessed by a paw pressure algometer before (baseline) and after intraplantar injections of the opioid agonist fentanyl (0.5–1.25 μg/100 μL) as previously described (11,17). Paw pressure thresholds (PPT) were expressed as raw data in grams or as the ratio of [35S]GTP 

**Experimental groups.** Animals were subdivided into six groups (n = 6–8 rats per group): control, diabetic (STZ-treated), diabetic rats receiving intrathecal Rab7 siRNA or negative control siRNA, and diabetic rats with intrathecal NGF. Intrathecal catheters were implanted as previously reported (18). Rats received the following intrathecal treatments: for intrathecal β-NGF delivery, Alzet osmotic minipumps (200 μL; Alzet Corporation, Cupertino, CA) were filled with artifical cerebrospinal fluid and rat serum albumin (1 mg/mL) with or without 0.125 μg/1 μL NGF and connected to the intrathecal catheter to administer NGF or vehicle continuously at 1 μL/h over 7 days during the week 12 of STZ according to previous protocols (19). All tests were performed on day 8 after pump implantation. In vivo siRNA delivery was performed as follows: Rab7 siRNA and negative control siRNA were dissolved in Max Suppressor In Vivo RNA LANCE/II to a final concentration of 2 μg/10 μL β-NGF, which was administered as an intrathecal bolus. Rats were killed 1 week after the last injection of siRNA.

**Radioligand binding assay.** Membranes were obtained from the lumbar (L4–5) DRG as described previously (21,22). MOR-specific binding of [3H]DAMGO was performed as described previously (22). Briefly, cell membranes (200–300 μg) were incubated in assay buffer with increasing doses of DAMGO (0.02–2 nmol/L at 65 °C/cm; Amersham Pharmacia Biotech, Buckinghamshire, England) in the absence or presence of 10 μmol/L naloxone. Membranes were incubated for 30 min at 30°C in assay buffer. Filters were washed two times with 0.1% (w/v) polyethyleneimine solution for 30 min before use. Bound and free ligands were separated by rapid filtration under vacuum through Whatman GF/B glass fiber filters. Bound radioactivity was determined by liquid scintillation spectrophotometry at 70% counting efficiency for [3H] in overnight extraction of the filters in 3 mL scintillation fluid (EG&G Wallac, Turku, Finland). All experiments were performed in duplicate and performed three times. MOR [35S]GTP-γS binding assay in DRG membranes was performed as described previously (21). Various concentrations (0.05–2 nmol/L) of [35S]GTP-γS (1,250 Ci/mmol; New England Nuclear, Boston, MA) were incubated with 50 μg GTP-γS binding in assay buffer for 2 h at 30°C. At each concentration of [35S]GTP-γS, basal binding was assessed in the presence of GDP and absence of DAMGO, whereas specific binding was determined in the presence of 10 μmol/L naloxone. Basal (unstimulated) [35S]GTP-γS binding was subtracted from DAMGO-stimulated binding at each measurement to determine net DAMGO-stimulated [35S]GTP-γS binding. All experiments were performed in duplicate.

**NGF fluorometric two-site enzyme-linked immunosassay.** NGF concentrations in DRG of control animals, diabetic animals, and diabetic animals treated with siRNA were measured using a previously described ELISA for NGF (Fig. 1). Levels of NGF was quantitated after trypsinization of NGF with antibodies to determine reverse relationship of NGF with Rab7 that has been shown in vitro previously (23). After thawing and weighing the wet-weight DRG, tissue was homogenized on ice in 700 μL of homogenization buffer and then stored at −80°C until NGF was measured by a modified two-site enzyme-linked immunosassay for rat β-NGF, which was purchased from R&D Systems (Duoset ELISA Development Kit) (24). Endogenous NGF content was calculated as picograms of NGF per milligram of wet-weight tissue.

**Quantitative Taqman RT-PCR.** Total RNA was extracted from L4–5 DRG using the RNeasy (Qiaozol Lysin Reagent) Kit (Qiagen, Hilden, Germany). The following specific primers were used: for rat Rab7 mRNA upstream, 5'-TACAAAGGCACAAATGAGACAGACT-3' and downstream, 5'-ATTTGAGCCGCTCCGGAAT-3' (accession no. AP298630) (25); for MOR mRNA upstream, 5'-TTCGCGCTGATGCTTACAG-3' and downstream, 5'-GTTGATCTCCGCGAGATTC-3' (accession no. AY172543) (26); for 18S rRNA upstream, 5'-CGGCTACACATTACCAAGGAA-3' and downstream 5'-GCTGAAATTCGGCGCT-3' (accession no. M11185.1) (27). Taqman quantitative RT-PCR was performed as described previously (20).

Western blot analysis. L4–5 DRG were solubilized according to Mousa et al. (26) to obtain total cell protein. For quantifying membrane-bound versus cytosolic (e.g., internalized) MOR, subcellular fractionation was performed as previously described (28). The protein distribution between membrane and cytosol fractions was assayed by Western blot analysis as previously described (26). The Western blot bands of MOR and Rab7 were quantified by Java Image processing and analysis software (ImageJ; open-source image software downloaded from the Internet) as described previously (18).

**Immunohistochemistry.** After perfusion of rats, pancreas, DRG, and skin were removed and further processed as described previously (11). The method of immunostaining DRG and skin and has been described in detail elsewhere (11,18). Briefly, we stained every fourth section of DRG that was serially cut at 10 μm for each animal (n = 5) with respective antibodies. DRG neurons with innervation of the DRG and skin were counted in every section. We determined the total number of neurons per transverse section and the number and percentage of neurons immunoreactive for MOR (n = 5 rats). Consistent with the total MOR number in DRG, the total number of MOR neurons per area (38.4 mm²) was counted in the skin (five tissue sections per animal) with the dermal–epidermal junction in the middle axis of the area. Immunostained PGP9.5-IR (immunoreactive) nerve fibers were counted at the dermal–epidermal junction of the skin as nerve fibers crossing or originating at the basement membrane excluding fiber branching. Five squares per each section were analyzed using a Zeiss microscope as described previously (29). For measurements of membrane-bound MOR immunoreactivity (outer ring) versus cytosolic enrichment in MOR-IR DRG neurons, representative images for DRG neurons from different treatment groups were taken. Measurements were conducted as previously described (30) using Java Image processing and analysis software. The number of nuclei per DRG neuron, a minimum of 100 neurons from 5 rats of each group were manually traced with line thickness set to 3 μm to measure membrane-associated immunoreactivity. The respective encircled areas served to measure intracellular immunoreactivity (cytosolic enrichment). The ratio of the fluorescence intensity of the outer borders (plasma membrane) to that of cytosolic enrichment (intracellular immunoreactivity) was used to assess the degree of membrane versus cytosolic enrichment. Data were expressed as means ± SEM.

**Statistics.** All statistics were performed using the Sigma Stat 2.03 (SPSS, Chicago, IL) software. Data were reported as means ± SD or SEM and were compared by one-way or two-way ANOVA if the normality test was passed (Kolmogrov–Smirnov test). Otherwise, the Kruskal-Wallis ANOVA on ranks was used. Post hoc multiple pair-wise comparisons were performed by the Dunn’s test. All data were expressed as mean ± SD or SEM. Two groups were compared by the two-tailed Student t test or Mann-Whitney U test. For the analysis of behavioral dose-dependent effects, a linear regression ANOVA was applied. P < 0.05 was considered significant.

**RESULTS**

**Increased blood glucose, reduced weight gain, and mechanical hyperalgesia in rats with STZ-induced diabetes.** Rats receiving intravenous 45 mg/kg STZ showed an apparent loss in insulin-producing GAD65-IR endocrine cells of the pancreas (Fig. 1A), concomitant with significantly increased blood glucose (P < 0.05; Fig. 1B), reduced weight gain (P < 0.05; Fig. 1B), and the development of mechanical hyperalgesia (decreased paw pressure thresholds) (P < 0.05; Fig. 2A; Fig. 24); however, there was no difference in the number of PGP9.5-IR nerve fibers per millimeter at the dermal–epidermal junction of the skin (Fig. 1D and E).

**Loss of opioid antinociceptive efficacy in diabetic rats.** Intraplantar injection of low, systemically ineffective doses of fentanyl to control rats significantly increased PPT on the ipsilateral but not contralateral paw (P < 0.05; Fig. 1A and D), which was even more pronounced in...
animals with CFA hindpaw inflammation ($P < 0.05$; Fig. 2A and C). In contrast, STZ-induced diabetes significantly reduced fentanyl-induced PPT elevations consistent with a loss in opioid antinociception ($P < 0.05$; Fig. 2A and C). The antinociception of intraplantar fentanyl was antagonized by increasing doses of the intraplantar opioid antagonist naloxone, confirming an opioid receptor–specific effect ($P < 0.05$; Fig. 2B). Dose-dependent antinociception of intraplantar fentanyl in diabetic animals was approximately two-fold or three-fold lower than in control or CFA-treated rats, respectively ($P < 0.05$; Fig. 2C).

**FIG. 1.** Pathophysiological changes in rats with STZ-induced (45 mg/kg intravenous) diabetes. **A:** Representative double-immunofluorescence images showing markedly reduced colocalization of insulin (red fluorescence) and GAD65 (green fluorescence) in pancreatic β-cells of STZ-induced diabetic compared with control rats. Bar = 40 μm. **B:** After the intravenous injection of STZ (red, $n = 18$), blood glucose levels significantly increased up to 12 weeks compared with vehicle-treated rats (black, $n = 18$; *$P < 0.05$, two-way repeated measures (RM)-ANOVA, Tukey test). **C:** During the same time period the gain in body weight was significantly less in STZ (red, $n = 18$) vs. vehicle-treated rats (black, $n = 18$; *$P < 0.05$, two-way RM-ANOVA, Tukey test). Bar = 50 μm. **D:** Immunofluorescence images of PGP9.5-IR nerve fibers in control rats and rats treated with intravenous 45 mg/kg STZ. **E:** The number of nerve fibers crossing or originating at the dermal–epidermal junction was not different between both groups. All data are shown as means ± SD. (A high-quality color representation of this figure is available in the online issue.)

**Loss of MOR in DRG sensory neurons, axons, and peripheral nerve terminals.** Confocal immunofluorescence showed colocalization of all MOR-IR DRG neurons with calcitonin gene–related peptide in diabetic and control rats (Fig. 3A). The percentage of MOR-IR per total cells in DRG was significantly reduced in diabetic compared with control animals ($P < 0.05$; Fig. 3B). Consistently, the integrated optical density of MOR-specific bands was significantly lower in diabetic rats compared with controls ($P < 0.05$; Fig. 3C and D). This loss was not attributable to reduced MOR gene transcription, because
MOR mRNA levels did not differ between diabetic and control animals (\( P < 0.05 \); one-way ANOVA). Consequently, the number of MOR-IR nerve fibers at the dermal–epidermal junction in diabetic animals was significantly decreased (\( P < 0.05 \); Fig. 4A and B).

**Enhanced MOR targeting to lysosomes in DRG sensory neurons.** Using fluorescence microscopy, MOR were localized predominantly to the plasma membrane of control DRG neurons with almost no evidence for a colocalization with Lamp-I or Rab7 (Fig. 5A). In contrast, in DRG of diabetic rats, MOR exhibited extensive colocalization with Rab7 in Lamp-I-IR perinuclear lysosome compartments. Quantitative analysis revealed a significantly decreased membrane-to-cytosol ratio of MOR within DRG neurons of diabetic animals compared with controls (\( P < 0.05 \); Fig. 5B). In support, DRG subfractionation studies revealed that the majority of MOR protein was confined to the cytosol and not to the membrane fraction in diabetic compared with control animals (\( P < 0.05 \); Fig. 5C). In addition, the maximal number of membrane MOR binding sites was significantly decreased in DRG of diabetic rats.

**Scatchard analysis revealed a** \( B_{\text{max}} \) value of 28 ± 6.4 fmol/mg protein for the DRG membranes of control and 14 ± 1.3 fmol/mg protein for diabetic animals (\( P < 0.05 \); Fig. 5E). Consequently, DAMGO-induced [\(^{35}\)S]GTP\(_{\gamma}\)S binding was significantly reduced in the DRG of diabetic relative to control rats (\( P < 0.05 \); Fig. 5F).

**Reversal of MOR lysosomal targeting by silencing enhanced Rab7 expression.** Both quantitative RT-PCR and Western blot analysis in DRG neurons of diabetic rats showed a significant increase of Rab7, but not of Rab4, relative to controls (\( P < 0.05 \); Fig. 6A–D). A Rab7 gene silencing approach, in which diabetic rats received intrathecal Rab7 siRNA, caused a significant reduction of Rab7 mRNA and protein in DRG neurons but not of Rab4 mRNA, internal reference RNA, and protein (18S RNA and 28S RNAs).
β-actin protein) \((P < 0.05; \text{Fig. } 6A–D)\). This treatment prevented the entry of MOR-IR vesicles into the lysosomal degradation pathway and MORs were again abundantly relocated to the sensory neuron plasma membrane \((P < 0.05; \text{Fig. } 6C–F)\). The reduced membrane-to-cytosol ratio \((P < 0.05; \text{Fig. } 6E \text{ and } F)\) and optical density of MORs \((P < 0.05; \text{Fig. } 7A–C)\) in DRG of diabetic animals were significantly reversed by intrathecal Rab7 siRNA but not by

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**FIG. 3.** Loss of sensory neuron MOR and enhanced MOR targeting to lysosomes in DRG neurons of diabetic rats. A: Double-immunofluorescence images represent colocalization of MOR (red fluorescence) with the sensory neuron marker calcitonin gene–related peptide (CGRP, green fluorescence) in DRG sections of control and diabetic rats. Bar = 40 μm. B: The number of MOR-IR neurons was markedly reduced in diabetic rats compared with controls \((n = 5; *P < 0.05, \text{Student } t \text{ test})\). C: Western blot analysis of DRG from diabetic and control animals revealed a 55-kDa band specific for MOR and a 42-kDa band specific for β-actin. D: Densitometric image analysis (National Institutes of Health ImageJ software) of the respective protein bands showed that the density of the MOR protein band in DRG of diabetic rats was markedly reduced compared with control rats \((n = 6; *P < 0.05, \text{Student } t \text{ test})\). E: Quantification of MOR mRNA in DRG neurons of diabetic and control rats using Taqman quantitative RT-PCR shows the MOR mRNA was not significantly different from DRG neurons of diabetic vs. control rats \((n = 9; *P > 0.05, \text{Student } t \text{ test})\). All data are shown as means ± SEM. (A high-quality color representation of this figure is available in the online issue.)

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**FIG. 4.** A: Immunofluorescence images represent colocalization of MOR (red fluorescence) with sensory neuronal marker calcitonin gene–related peptide (CGRP, green fluorescence) on nerve endings within subcutaneous paw tissue of control and diabetic rats. Note, MOR-IR nerve fibers were reduced at the dermal–epidermal junction of the innervated skin of diabetic compared with control rats. Bar = 20 μm. B: Quantitative analysis showed that the number of MOR-IR nerve fibers was significantly reduced in diabetic compared with control rats \((n = 5; *P < 0.05, \text{Student } t \text{ test})\). All data are shown as means ± SEM. (A high-quality color representation of this figure is available in the online issue.)
negative control siRNA. Consequently, the reduced number of MOR-IR nerve fibers at the dermal–epidermal junction of the innervated skin recovered after intrathecal Rab7 siRNA application at days 1 and 3 of week 12 of STZ-induced diabetes (P < 0.05; Fig. 7D and E).

Reversing NGF deprivation normalizes Rab7 expression and restores sensory neuron MOR density and coupling in diabetic rats. NGF content was significantly reduced in the DRG of diabetic rats relative to controls (P < 0.05; Fig. 8A). Intrathecal infusion of NGF over the course of 7 days restored NGF concentration in diabetic rats to that of controls. In line with a previous report that NGF deprivation enhances Rab7 expression and activation (30), enhanced Rab7 protein expression in diabetic rats was significantly reversed by intrathecal NGF treatment (P < 0.05; Fig. 8B). Furthermore, when Rab7 expression in DRG of diabetic rats

**FIG. 5.** Enhanced MOR lysosomal targeting occurs in parallel with a decrease in membrane-bound MOR and a loss in MOR G-protein-coupling in DRG of diabetic rats. A: Immunofluorescence images present colocalization of MOR (red fluorescence) with Rab7 or lysosomal marker lysosome–associated membrane glycoprotein-1 (LAMP-1; green fluorescence) in DRG. In control animals, MOR localized mainly to the cell surface of DRG neurons, whereas in diabetic rats MOR colocalized with Rab7 and Lamp1 (yellow) predominantly in the perinuclear lysosome compartment of the cytoplasm. Bar = 10 μm. B: Quantitative analysis of the relative MOR distribution in the plasma membrane (PM) vs. cytoplasm (CYT) showed that the PM/CYT ratio of MOR was significantly reduced in DRG of diabetic compared with control rats (P < 0.05, Student t test). C and D: Quantitative Western blot analysis of MOR in plasma membrane vs. cytosolic fractions of DRG neurons showed that the PM/CYT ratio of the integrated optical density of MOR was significantly reduced in diabetic compared with control rats, reflecting a reduction of MOR in the neuronal plasma membrane of diabetic rats (n = 4; *P < 0.05, Student t test). E: Saturation binding of [3H]DAMGO in membrane fractions of DRG from diabetic compared with control rats (n = 6). The maximal number of MOR binding sites in membrane fractions of diabetic rats was significantly reduced compared with controls (P < 0.05, two-way ANOVA). F: The stimulated [35S]GTPγS binding to MOR of DRG membranes showing a significant reduction in the maximum efficacy of diabetic compared with control rats (n = 6; *P < 0.05, two-way ANOVA, Tukey test). All data are shown as means ± SEM. (A high-quality color representation of this figure is available in the online issue.)
FIG. 6. Reversal of MOR lysosomal targeting by silencing enhanced Rab7 expression. A: Quantitative analysis of Taqman RT-PCR of Rab7 mRNA revealed a significant increase in Rab7 mRNA in diabetic rats that was abolished by intrathecal Rab7 siRNA, but not by a negative control siRNA treatment, on days 1 and 3 of week 12 of STZ-induced diabetes. *Significant differences compared with control nondiabetic as well as diabetic rats treated with intrathecal siRNA rab7 (n = 6; P < 0.05, one-way ANOVA, Dunn test). B: Quantitative analysis of Taqman RT-PCR of Rab4 mRNA revealed no change in Rab4 mRNA in diabetic rats with or without intrathecal Rab7 siRNA compared with vehicle (n = 6; P > 0.05, one-way ANOVA, Dunn test). C: Similar changes in the Rab7 protein band (24 kDa) were identified by Western blot analysis of DRG from diabetic rats treated with intrathecal Rab7 siRNA. D: Densitometric image analysis of Rab7 protein bands showed that the integrated density was significantly increased in DRG of diabetic rats; however, it was significantly reduced in diabetic rats treated with intrathecal Rab7 siRNA but not with negative control siRNA (n = 4; P < 0.05, ANOVA on ranks, Dunn test). E: Representative laser-scanning confocal micrographs showed a redistribution of perinuclear MOR (red fluorescence) in DRG neurons of diabetic animals to the plasma membrane of neuronal DRG after intrathecal Rab7 siRNA but not with negative control siRNA treatment. Bar = 10 μm. F: Quantitative analysis of the PM/CYT ratio of MOR showed that the integrated optical density of MOR is significantly increased in DRG of diabetic rats treated with intrathecal Rab7 siRNA but not with negative control siRNA (P < 0.05, one-way ANOVA, Dunn test). All data are shown as means ± SEM. (A high-quality color representation of this figure is available in the online issue.)
was restored to that of control animals, the loss of MOR receptor protein in DRG of diabetic rats was significantly reversed (Fig. 8C). In addition, after intrathecal NGF treatment, the impairment of MOR G-protein-coupling ([35S]GTPγS binding) was abolished ($P < 0.05$; Fig. 8D).

**Inhibition of MOR lysosomal targeting restores MOR-mediated analgesia in diabetic rats.** To determine whether these interventions enhance the antinociceptive effects of the MOR agonist fentanyl in diabetic rats, we assessed the effects of fentanyl on paw pressure thresholds.

**FIG. 7.** Silencing enhanced Rab7 expression in diabetic rats restores MOR in sensory neurons. **A**: Immunofluorescence images represent MOR-IR DRG neurons from control, diabetic, and diabetic rats treated with Rab7 siRNA or with negative control siRNA. Bar = 40 μm. **B**: The reduced number of MOR-IR DRG neurons in diabetic rats was significantly reversed after intrathecal Rab7 siRNA, but not negative control siRNA, on days 1 and 3 of week 12 of STZ-induced diabetes ($n = 5$; $^*P < 0.05$, ANOVA on Ranks, Tukey test). **C**: Similarly in Western blot, the reduced intensity of MOR protein bands in DRG neurons was reversed by intrathecal Rab7 siRNA. Densitometric image analysis showed that the reduced integrated density of MOR protein bands in DRG of diabetic rats ($n = 4$) was reversed by intrathecal Rab7 siRNA ($^*P < 0.05$, ANOVA on Ranks, Tukey test). **D**: Immunofluorescence images showing MOR-IR nerve fibers at the dermal-epidermal junction of the innervated skin of control rats, diabetic rats, and diabetic rats treated with intrathecal Rab7 siRNA or negative control siRNA. Bar = 40 μm. **E**: The significantly reduced number of MOR-IR nerve fibers was reversed by intrathecal Rab7 siRNA, but not by negative control siRNA ($n = 5$; $^*P < 0.05$, ANOVA on Ranks, Tukey test). All data are shown as means ± SEM. (A high-quality color representation of this figure is available in the online issue.)
in animals that received intrathecal Rab7 siRNA on days 1 and 3 or intrathecal NGF over the course of 7 days of week 12 of STZ-induced diabetes (Fig. 8E and F). Consistent with the functional opioid recovery, the impaired fentanyl-induced antinociception in diabetic animals was restored after both intrathecal Rab7 siRNA and NGF ($P < 0.05$; Fig. 8E and F), similar to the blockade of MOR lysosomal targeting and degradation in peripheral sensory neurons of diabetic rats.
DISCUSSION

This study demonstrates in the model of STZ-induced painful diabetic neuropathy that the impaired antinociceptive effects of opioids are associated with a loss in MOR number and G-protein-coupling of peripheral sensory neurons. Under normal conditions, MORs are localized primarily on the cell membrane of peripheral sensory neurons. In contrast, in diabetic neuropathy, a loss of MOR is associated with enhanced Rab7 expression and a colocalization of MOR with Rab7 in Lampl-positive perinuclear lysosome compartments. Different strategies such as Rab7 siRNA knockdown and intrathecal NGF treatment resulted in the normalization of Rab7 expression and the subsequent reversal of Rab7-dependent lysosomal targeting, which finally restored MOR density on the cell membrane of sensory neurons and rescued opioid responsiveness toward better pain control.

It is well-established in experimental (11) and clinical (12) studies that inflammatory pain leads to the upregulation of MOR on peripheral sensory neurons and consequently potentiates the peripheral antinociception elicited by local opioids (12,18,21). In contrast, in diabetic animals, the antinociceptive effects of intraplantar fentanyl were significantly larger than the differences in baseline pain thresholds. Because peripheral opioid antinociception is dependent on peripheral sensory neuron MOR number, coupling, and efficacy (11,22), we hypothesized that changes in sensory neuron MOR of STZ-induced diabetic rats are responsible for this loss in opioid responsiveness.

The number of small MOR-IR DRG neurons, which coexpressed the sensory neuron marker calcitonin gene-related peptide, as well as the total amount of MOR protein were significantly reduced, a fact that was not described in previous studies (6–8,31). In addition, the peripheral nerve terminals of these neurons showed a reduction in MOR immunoreactivity at the dermal–epidermal junction without changes in the number of PGP9.5 nerve endings. Consistently, previous studies have shown that even long-term STZ-induced diabetes is associated with a relative preservation of sensory neuron populations (32).

To follow-up the fate of the MOR, we performed immunofluorescence confocal microscopy in DRG sections of diabetic and control rats in which MOR immunoreactivity was highly concentrated in the plasma membranes of neurons from control DRG, whereas they were highly localized in Lampl-positive perinuclear lysosome compartments of diabetic animals (33,40). Intracellular receptor trafficking is important for the function of G-protein-coupled receptors (34). They can be internalized by agonist-induced endocytosis (34), heterologous protein kinase–mediated endocytosis (35), or by constitutive endocytosis (36), resulting in their delivery to the intracytoplasmatic compartment of early endosomes. There, they can be recycled back to the plasma membrane in a fully sensitized state (15) or delivered to lysosomes for degradation (34), dependent on small monomeric Rab GTPases such as Rab7 GTP-binding protein (37). Here, the number of MOR binding sites in the plasma membrane as well as the maximal efficacy in MOR G-protein-coupling was significantly reduced in DRG of diabetic rats, which contributes to a loss in agonist efficacy (38).

Apparently, peripheral sensory neuron MORs in diabetic animals seem to enter the lysosomal pathway, undergo subsequent degradation, and result in the loss of opioid responsiveness. Rab7 has been considered in mammalian cells as a key regulatory protein for the intracellular vesicle traffic toward perinuclear lysosome compartments (39,40). Consistently, Rab7 mRNA and protein levels were highly upregulated in diabetic animals. Therefore, we applied intrathecal delivery of Rab7 siRNA to silence the endogenous expression of Rab7 in peripheral sensory neurons (20,41). As a result, intrathecal Rab7 siRNA, but not scrambled RNA, led to a dramatic reduction of both Rab7 mRNA and protein. Importantly, other members of the Rab GTPases family, such as Rab4, were not affected. Rab7 siRNA treatment also abolished the localization of MOR to Rab7-IR perinuclear lysosomal compartments and reversed the number of MOR-IR sensory neurons. Similarly, Balut et al. (41) showed that in vitro siRNA-mediated knockdown of Rab7 resulted in a significant inhibition of the plasma membrane–associated KCa3.1 channel degradation rate. To the best of our knowledge, these results represent the first in vivo report on the peripheral sensory neuron MOR downregulation through the Rab7-mediated lysosomal degradation pathway during diabetic neuropathy. Until now, agonist-induced downregulation of G-protein-coupled receptors via the lysosomal pathway has been reported mainly in vitro (34,42).

The maintenance of nociceptive sensory neurons depends on NGF, a neurotrophic factor with the ability to resist apoptosis and to foster regenerative capacity (43). NGF is expressed in peripheral tissues such as the skin, binds to the high-affinity receptor trkA of petidergic peripheral sensory nerve terminals, is retrograde-transported to the DRG, and regulates the expression of ion channels and receptors of these neurons (43). Recently, we have demonstrated that >80% of sensory neuron MOR colocalize with trkA in the NGF-dependent subpopulation of small DRG neurons (11). In diabetic rats, peripheral sensory neurons suffer from a persistent deficit in NGF concentration (24,44,45). Moreover, growth factor deprivation leads to the upregulation of Rab7 expression (23). In this study, we observed a significant reduction in NGF concentrations of sensory DRG neurons from diabetic rats concomitant with a high increase in Rab7 mRNA and protein levels, suggesting a causal relationship. Intrathecal NGF treatment of diabetic animals clearly reduced the enhanced Rab7 mRNA and protein. Subsequently, the total number of MOR-IR sensory neurons, the relative number of membrane spanning MOR in DRG, and the MOR on peripheral sensory nerve endings returned back to baseline values, supporting the interdependence between NGF and Rab7 (23). Finally, behavioral experiments showed that the antinociceptive effects of opioids were clearly improved in diabetic animals by the normalization of Rab7 expression as well as by intrathecal NGF treatment.

These results provide novel insights into the loss of the MOR number, functional coupling, and antinociceptive efficacy of peripheral sensory neurons suffering from diabetic neuropathy. Moreover, they show for the first time in vivo that enhanced Rab7-dependent lysosomal targeting and degradation of MOR during diabetic neuropathy contribute to the downregulation of peripheral sensory neuron MOR. This may explain why diabetic patients sometimes do not respond to increasing doses of opioids at all, except with an increase in the occurrence of serious side effects. Importantly, the blockade of the enhanced Rab7 expression in diabetes, e.g., by substitution of NGF, prevented MOR targeting to lysosomes, restored MOR density in the cell membrane of sensory neurons, and rescued opioid...
responsiveness toward better pain relief. This is in contrast to other pathological pain states such as inflammatory pain in which the sensory neuron MORs and antinociception are enhanced and provides intriguing evidence that regulation of opioid responsiveness varies as a function of pain pathogenesis. After all, these findings may caution the unrevised use of the anti-NGF monoclonal antibody (46), which has been developed recently for the treatment of chronic pain.

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S.A.M. conducted immunostaining, designed experiments, analyzed the data and drafted the paper. M.Shaq performed binding experiments, analyzed the data, and revised the manuscript. B.I.K. and L.S. carried out behavioral experiments and revised the manuscript. C.Z. contributed to the binding experiments. J.S. participated in the immunostaining and revised the manuscript. T.S.S. assisted with the study design, analysis and writing of the manuscript. J.F.R. assisted in the confocal immunofluorescence analysis and revised the manuscript. R.H. conducted the NGF fluorometric two-site enzyme-linked immunoassay and revised the manuscript. M.Shak conducted Western blot experiments and revised the manuscript. M.Sc. conceived and designed experiments, supervised the study, analyzed the data and wrote the manuscript. M.Sc. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of data and the accuracy of the data analysis.

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