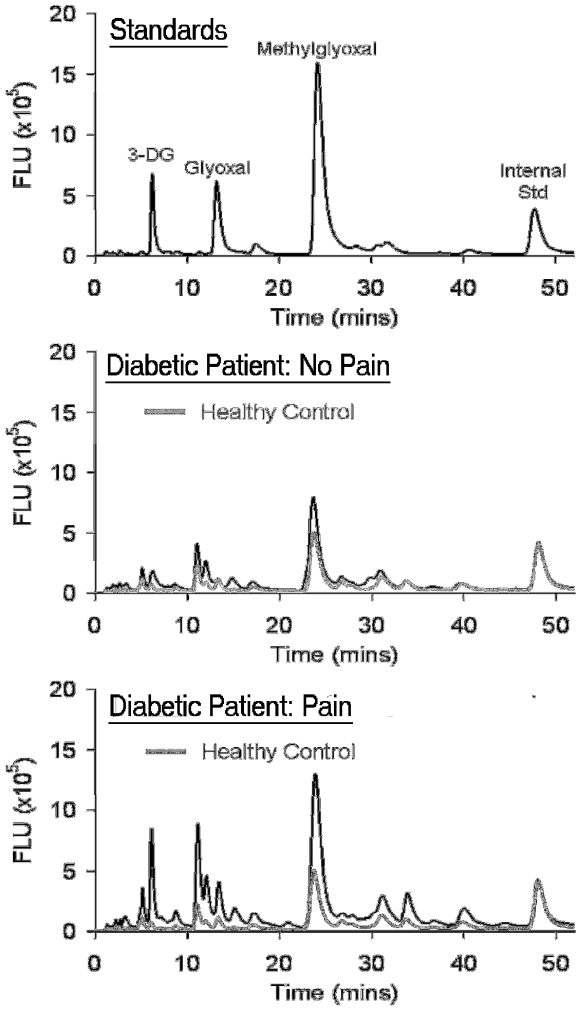
Supplemental Figure 1: ***Increased plasma methylglyoxal in type 2 diabetic patients with and without pain and the effect on COX2 transcription in vitro.***

a) Example of analytical chromatograms identifying glyoxal, methylglyoxal and 3-deoxyglucosone in plasma of type 2 diabetic patients with and without pain. The chromatograms of healthy subjects are inserted as a faint gray line.

b) Effect of methylglyoxal on COX-2 induction. Plasma from diabetic patient without pain was supplement with methylglyoxal and then used to stimulate healthy WT DRG neurons for 3h. COX-2 transcription was determined by real-Time-PCR. Data represent the mean ± s.d., *n* = 3 per group (ANOVA).

a.)



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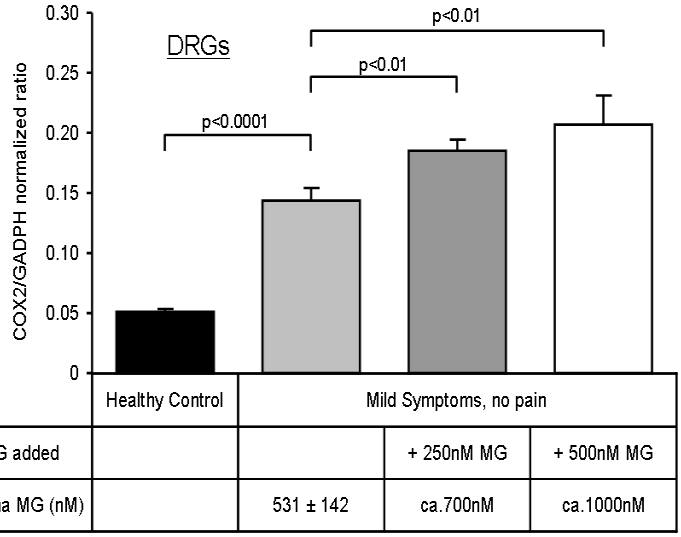


Supplemental Figure 2: *Effect of Exogenous methylglyoxal on thermal & mechanical hyperalgesia.*

a) Plasma concentration of methylglyoxal following injection of exogenous methylglyoxal (5µg) MG-plasma concentrations were determined by HPLC at time point 0h, 10min, 30min, 1h and 3h. Data represent mean ± s.d., *n* = 3 per time point (ANOVA).

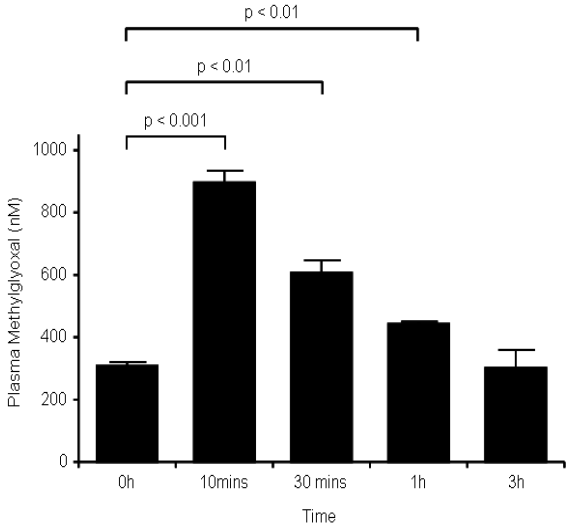
b) Effect of Methylglyoxal (5µg; 3h) on thermal hyperalgesia as determined by Hargreaves plantar assay. Data represent mean ± s.e.m, *n* = 10 per group (Unpaired, 2-tailed Student's t-test).

c) Effect of Methylglyoxal (5µg; 3h) on thermal hyperalgesia as determined by Tail-flick assay. Data represent mean ± s.e.m, *n* = 10 per group (Unpaired, 2-tailed Student's t-test).

d) Effect of increased temperature on methylglyoxal induced thermal hyperalgesia. Healthy WT mice were treated with exogenous Methylglyoxal (5µg; 3h). Thermal hyperalgesia was assessed by Hot plate assay at either 50 or 55ºC. Data represent mean ± s.e.m, *n* = 10 per group (Unpaired, 2-tailed Student's t-test).

e) Effect of Methylglyoxal (5µg; 3h) on mechanical hyperalgesia as determined by Von-Frey Filament Test. Data represent mean ± s.d., *n* = 10 per group (Unpaired, 2-tailed Student's t-test)

a.)



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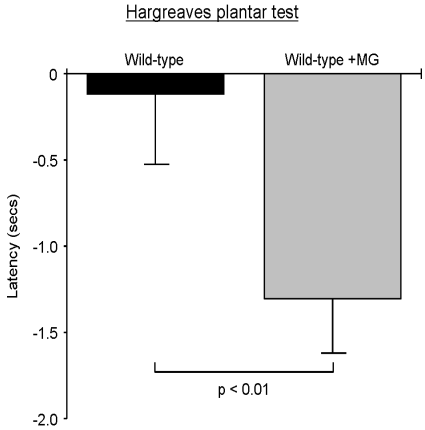

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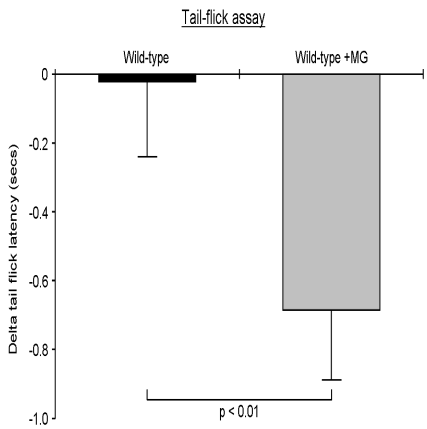
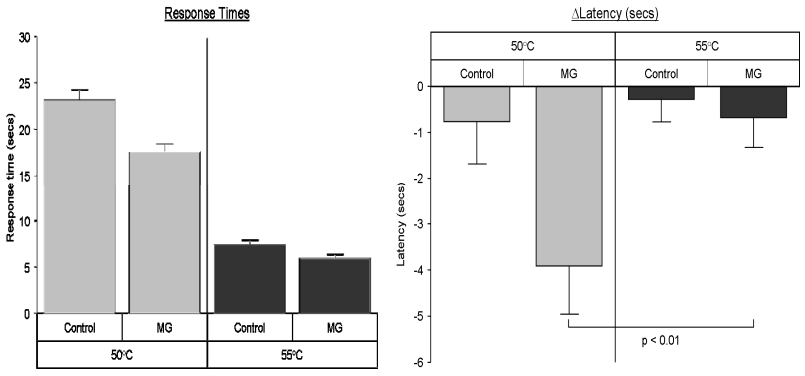
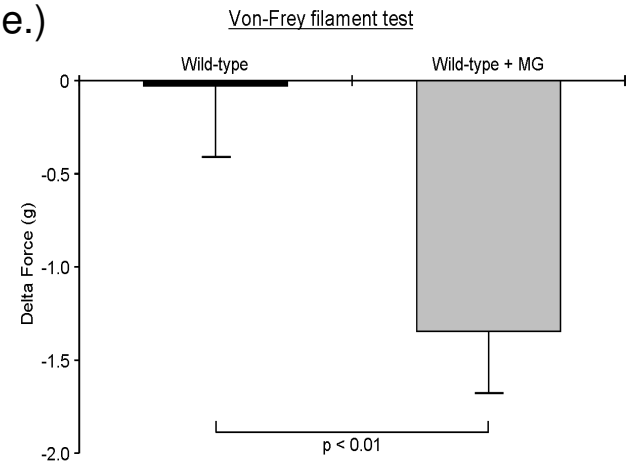


d.)

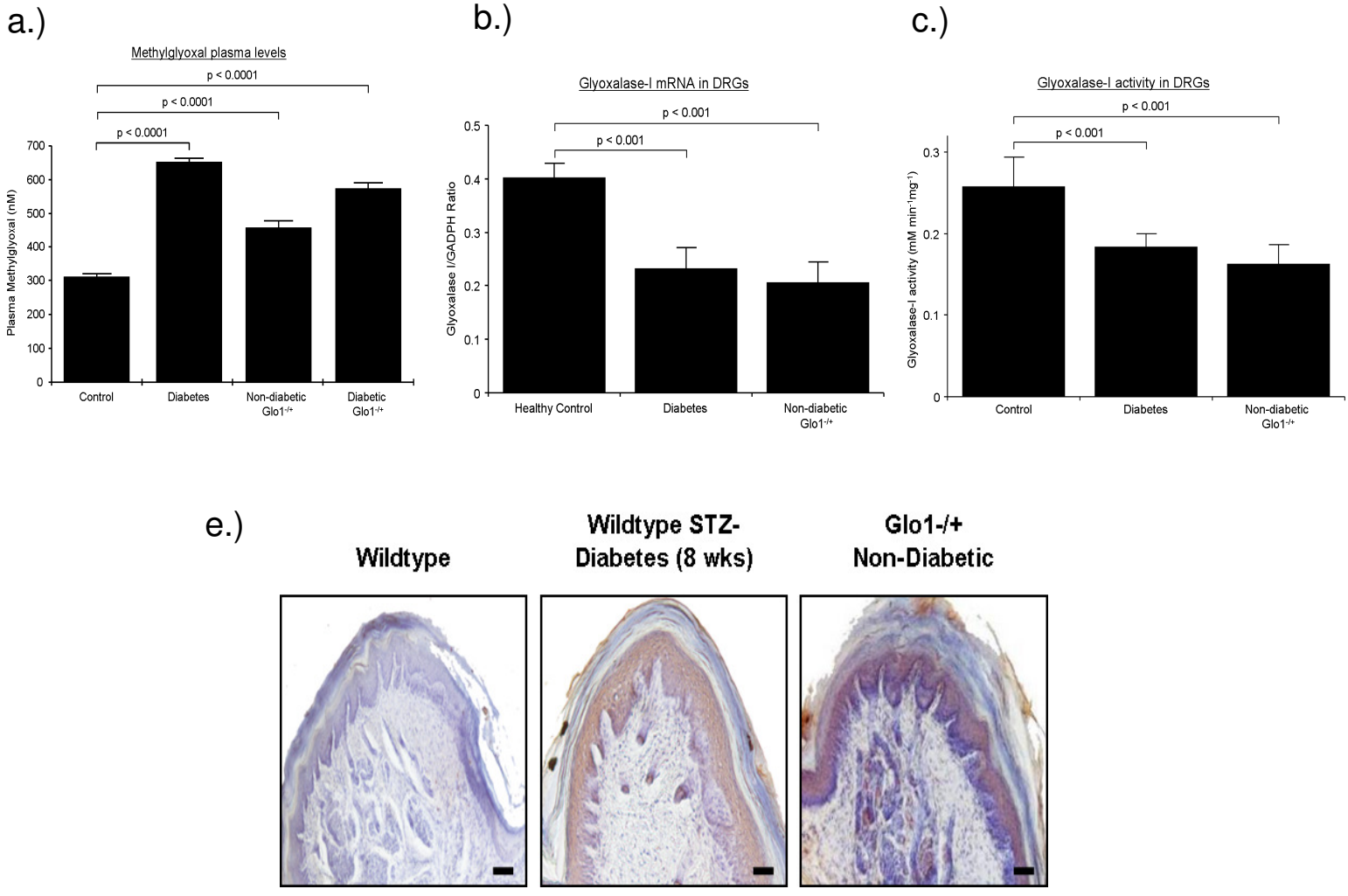


e.)



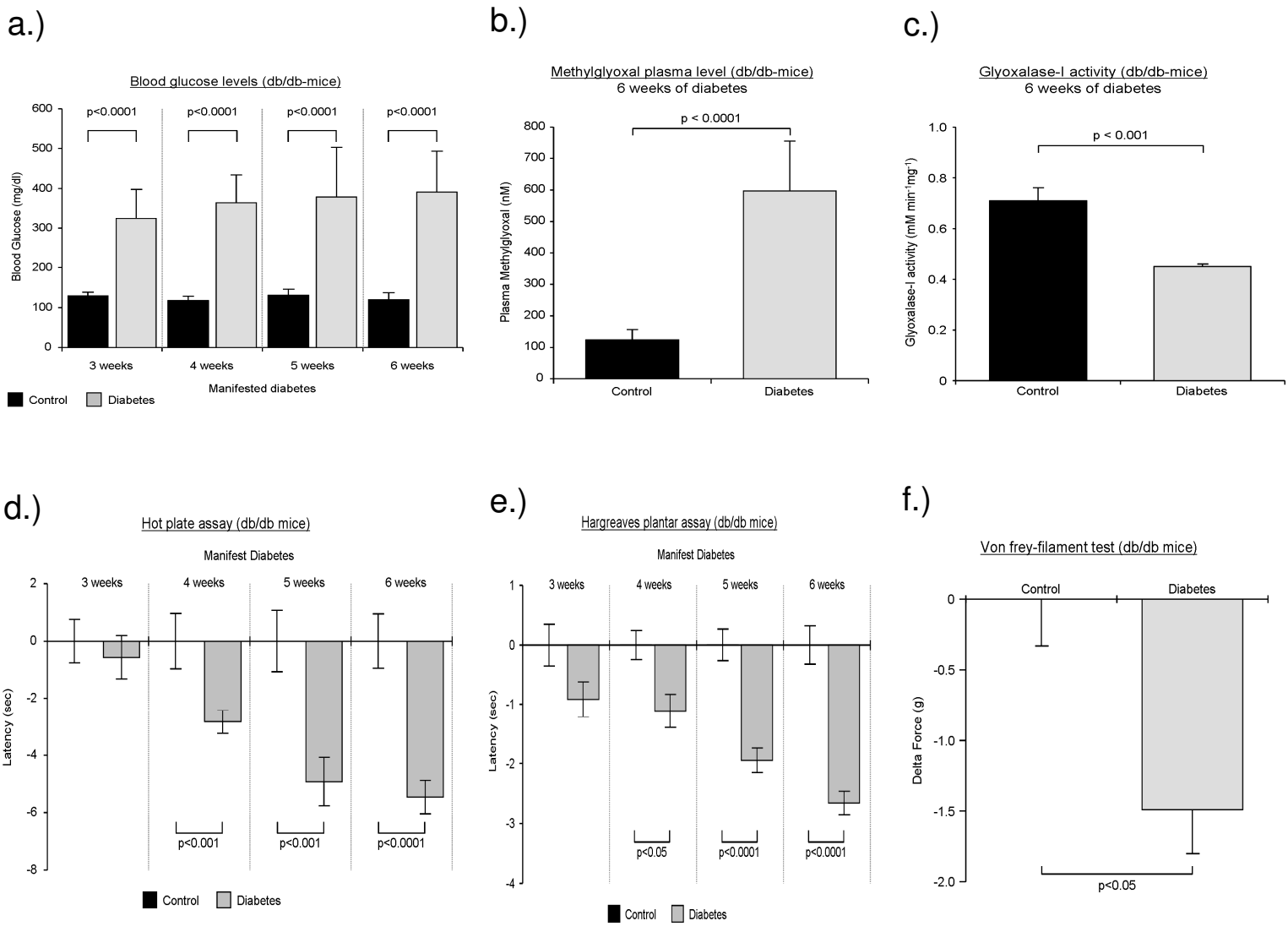
<u>Supplemental Figure 3</u>: Diabetes increases plasma methylglyoxal levels and reduced GLO1 transcription and activity in DRG neurons.

a) Plasma concentration of methylgloxal in the studied mouse models. Data represent the mean ± s.e.m., *n* = 20-25 (ANOVA).
b) GLO1 Transcription in DRGS neurons of the studied mouse models. Data represent the mean ± s.e.m., *n* = 10 (ANOVA).
c) GLO1 Activity in DRGS neurons of the studied mouse models. Data represent the mean ± s.e.m., *n* = 10 (ANOVA).
d) COX-2-expression in hind paws of healthy, STZ-induced diabetic WT and non-diabetic Glo1-/+-mice following heat-stimulation. Scale bar, 50µm.



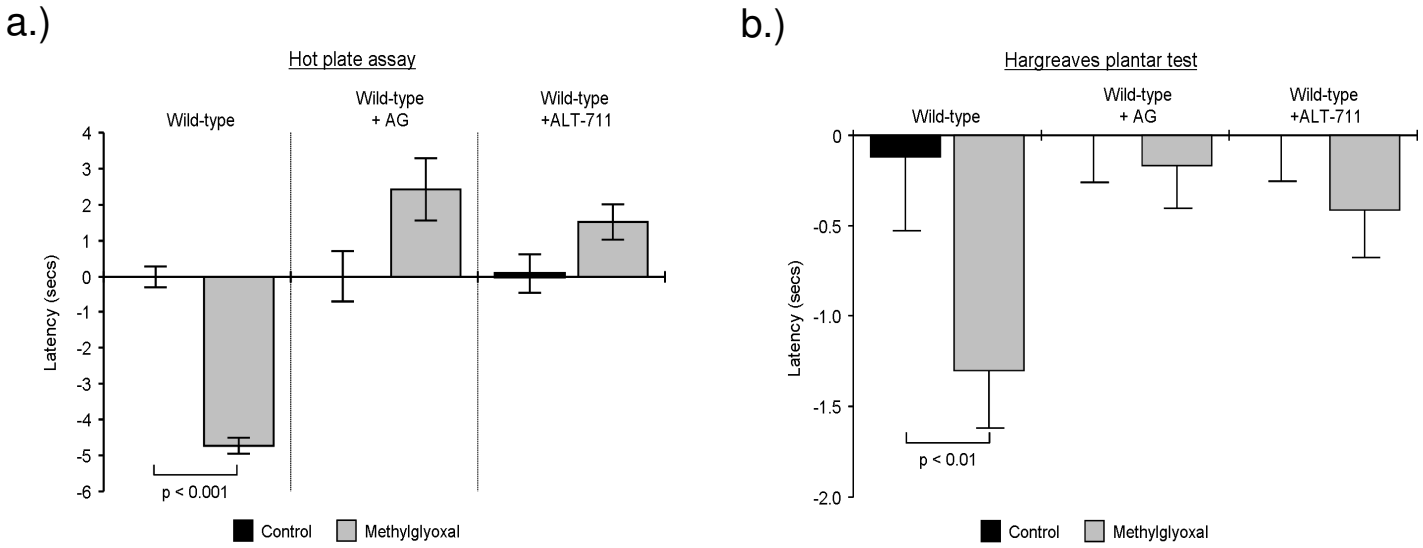
<u>Supplemental Figure 4</u>: Hyperglycemia in BKS-db/db mice increases plasma methylglyoxal levels, reduced GLO1 activity and increases thermal and mechanical hyperalgesia.

a) Blood glucose in BKS-db/db-mice of six week period. Date represents the mean ± s.d., *n* = 10, (Unpaired, 2-tailed Student's t-test).
b) Plasma concentration of methylglyoxal in control and diabetic (6 wk) BKS-db/db-mice. Data represent the mean ± s.d., *n* = 10 (Unpaired, 2-tailed Student's t-test).
c) GLO1 Activity in Sciatic Nerve tissue from control and diabetic (6 wks) BKS-db/db-mice. Data represent the mean ± s.d., *n* = 10 (Unpaired, 2-tailed Student's t-test).
d) Thermal hyperalgesia as determined by Hot plate assay in control and diabetic BKS-db/db-mice. Data represent the mean ± s.d., *n* = 10 (Unpaired, 2-tailed Student's t-test).
e) Thermal hyperalgesia as determined by Hargreaves plantar assay in control and diabetic BKS-db/db-mice. Data represent the mean ± s.d., *n* = 10 (Unpaired, 2-tailed Student's t-test).
f) Mechanical hyperalgesia as determined by Von-Frey Filament Test in control and diabetic BKS-db/db-mice. Data represent the mean ± s.d., *n* = 10 (Unpaired, 2-tailed Student's t-test).



***Supplemental Figure 5*: Effect of methylglyoxal scavenger and Advanced Glycation Endproducts Breaker on methylglyoxal-induced thermal hyperalgesia.**

Effect of the methylglyoxal- and AGE-inhibitors aminoguanidine (AG) and alagebrium (ALT-711) on methylglyoxal-induced hyperalgesia. Healthy WT mice were treated for 2 days with either aminoguanidine (*1g/l in drinking water*) or ALT-711 (*1mg/kg/day; oral gavage*) prior to treatment with methylglyoxal (5µg MG). Hyperalgesia was assessed 3h after methylglyoxal treatment by hot plate assay (**a**) and Hargreaves plantar assay (**b**). Data represent the mean ± s.d., *n* = 5 (Unpaired, 2-tailed Student's t-test).



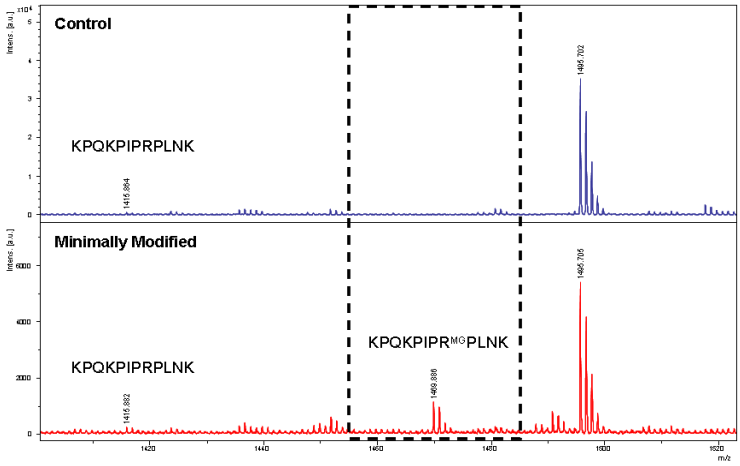
***Supplemental Figure 6*: Methylglyoxal present in plasma of diabetic patients binds to arginine in Nav1.8-inactivation gate.**

a) MALDI-TOF-MS analysis (*zoom view*) of tryptic peptides obtained by digestion of full length Nav1.8. The ion signals at m/z 1415.88 correspond to the non-modified tryptic peptide (aa 36-47), signal at m/z 1469.88 corresponds to the modified tryptic peptide (mass shift +54 Da). Signals at m/z 1495.7 correspond to the tryptic peptide LGGQDIFMTEEQK (aa 11-23).

b) Fragmentation analysis of the tryptic peptide modified with methylglyoxal at arginine. The peptide was obtained by digestion of the full-length inactivation gate peptide with trypsin.

c) Methylglyoxal present in patient's plasma binds to the Nav1.8 inactivation gate spanning peptide. Total mass chromatogram of the plasma from a non-diabetic control (*left*) or a diabetic patient (*right*) with severe pain, which were incubated with or without the Nav1.8 inactivation gate spanning peptide (*ca.*100µg) for 24h at 37°C. The ion signal at m/z 6032 corresponds to the non-modified peptide; the signal at m/z 6087 corresponds to methylglyoxal modified peptide. The peak area ratio of unmodified-to-modified peptide for the healthy control plasma is 0.95% and is increased, non-significantly, to 1.8% in the plasma of the diabetic patient.

a.)



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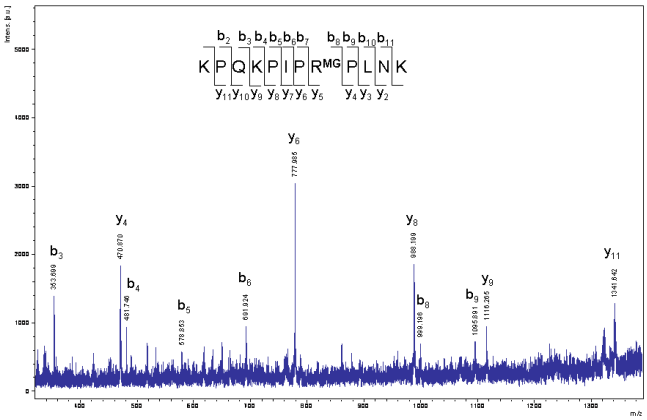
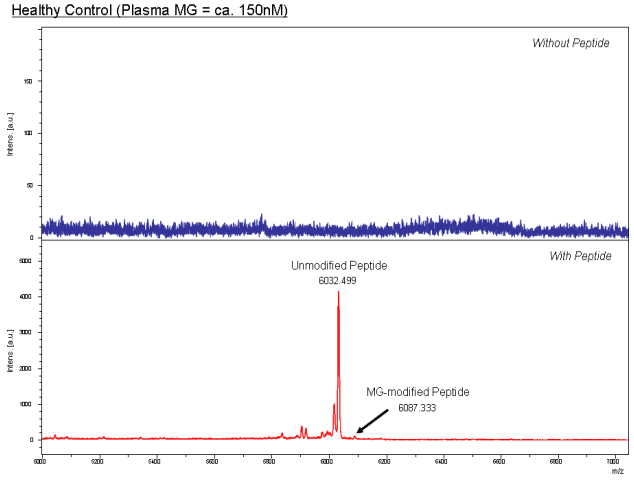
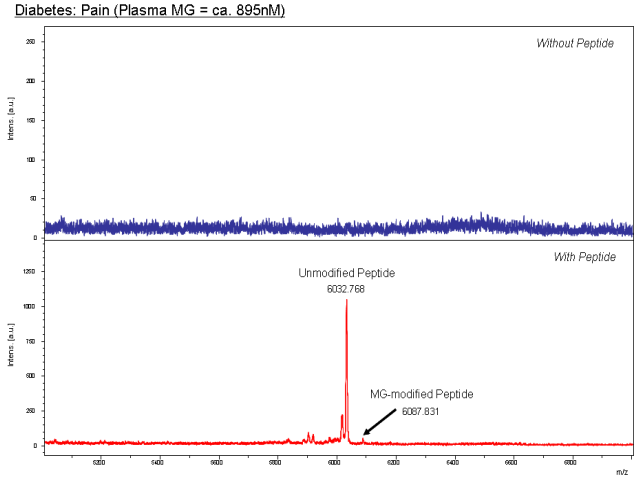


c.)





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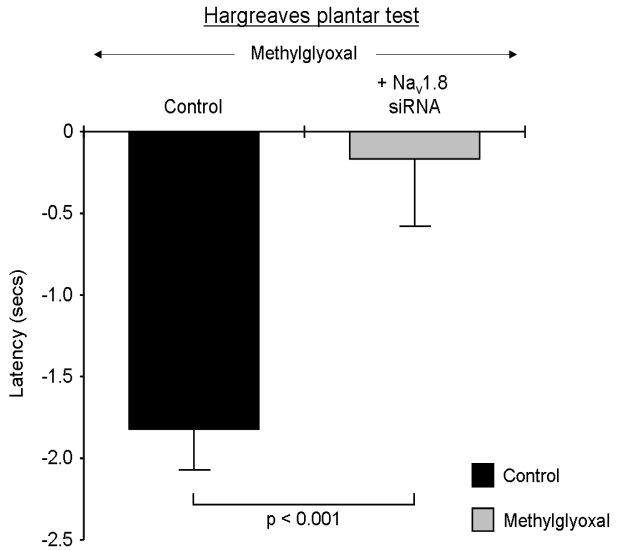
***Supplemental Figure 7*: Loss of Nav1.8 on methylglyoxal-induced thermal hyperalgesia.**

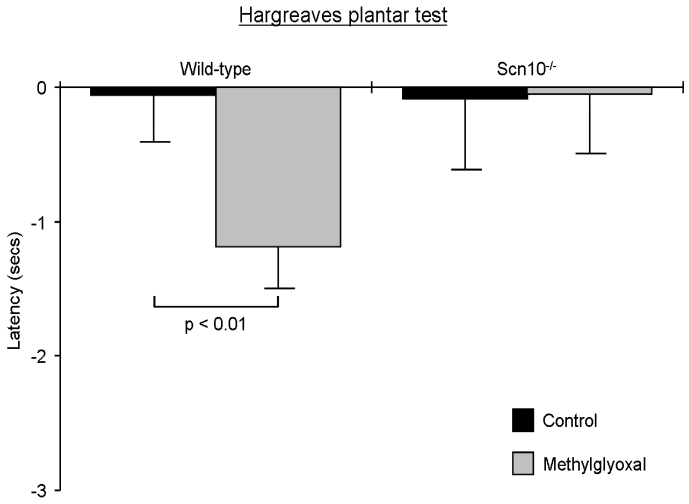
a) Nav1.8-specific siRNA was administered to healthy WT mice by i.v. injection in liposomal transfection reagent every 3rd day for a total of 10 days. On day 10, mice were injected with 5µg methylglyoxal. Hyperalgesia was assessed 3h after injection by the Hargreaves plantar test. Data represent the mean ± s.e.m., *n* = 10 (Unpaired, 2-tailed Student's t-test).

b) Effect of methylglyoxal on hyperalgesia, determined in healthy WT, Nav1.8 knockout (Scn10-/-) mice. Mice were treated with 5µg methylglyoxal, and hyperalgesia was assessed 3h later by the Hargreaves plantar assay. Data represent the mean ± s.d., *n* = 5-6., *n* = 10 (Mann-Whitney U-test).

a.)



b.)

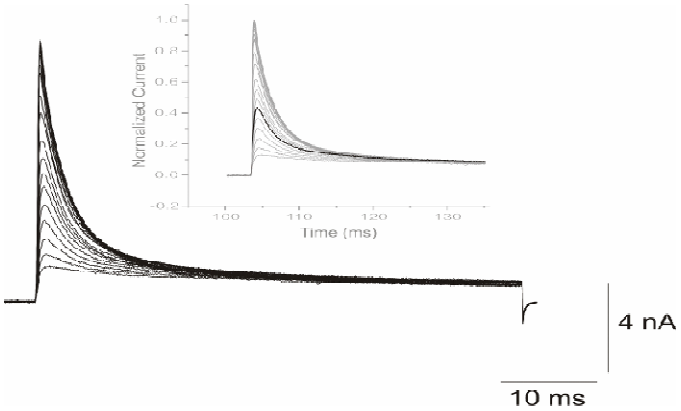


***Supplemental Figure 8*: Raw current records and current voltage relationship in voltage clamp studies.**

Raw current records and current voltage relationship in voltage clamp studies. MG induces a hyperpolarizing shift of steady-state fast inactivation of Nav1.8. Steady-state fast inactivation: 50 ms long pre-pulses from -140 mV to +20 mV in steps of 5 mV, followed by a 50 ms test-pulse to +50 mV (Vh = -140 mV). Untreated cells (**a**) were compared to cells treated with MG (100 µM) for 3h (**b**). Inserts show currents normalized to the first test-pulse. Note the difference in degree of inactivation high-lighted (*black line*) for pulse number 20.

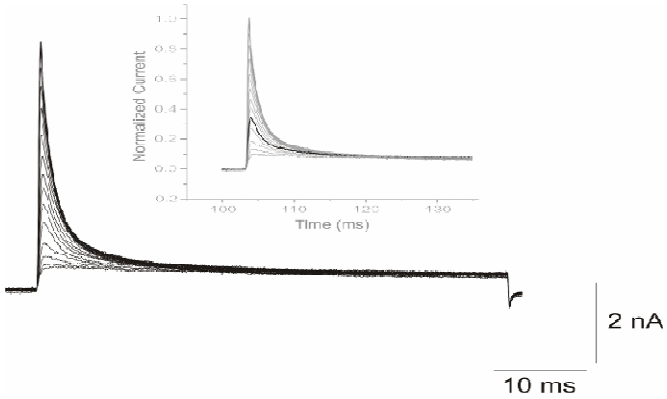
a.)

$Na_V1.8$ Control



b.)

$Na_V1.8$ 100 µM MG



SUPPLEMENTAL TABLES:

Supplemental table 1

Baseline characteristics of the studied type 2 diabetic patients with and without pain (*n* = 10 per group)

	No pain (n = 10)	**Pain** (n = 10)	**p-value**
Age (years)	62.4 ± 6.9	62.5 ± 5.6	n.s.
Gender (m/f)	9/1	3/7	0.004
Duration of Diabetes (years)	13 ± 7	11 ± 12	n.s.
HbA_{1c} (%)	6.6 ± 0.8	7.3 ± 1.0	n.s.
Creatinine (mg/dl)	0.95 ± 0.2	0.85 ± 0.27	n.s.
Urea (mg/dl)	38.7 ± 9.2	43.1 ± 15.0	n.s.
Neuropathic deficit score (NDS)	5.5 ± 2.9	5.4 ± 2.3	n.s.
Neuropathic symptom score (NSS)	7.2 ± 1.5	8.1 ± 1.37	n.s.
Pain, feet	**1.7 ± 1.16**	**7.7 ± 0.95**	**<0.0001**
Burning	**2.7 ± 3.09**	**5.3 ± 2.31**	**< 0.05**
Tingling	**3.0 ± 2.05**	**4.7 ± 2.21**	**0.091**
Plasma dicarbonyls (nM)			
Methylglyoxal	**531 ± 46**	**895 ± 55**	**< 0.0001**
Glyoxal	271 ± 37	434 ± 82	n.s.
3-Deoxyglucosone	396 ± 66	478 ± 56	n.s.

Supplemental table 2

Primers used for real-time PCR experiment:

mGlyoxalase I – Forward	5´- CCTCGTGGATTTGGTCACATT –3´
mGlyoxalase I – Reverse	5´- GGCACATTCTCCCGAAACTAA –3´
mGAPDH – Forward	5´ - AACGACCCCTTCATTGAC –3´
mGAPDH – Reverse	5´ - TCCACGACATACTCAGCAC -3´
mNa_v1.8 – Forward	5´- TCGAGATGTTGCTCAAGTGG –3´
mNa_v1.8 – Reverse	5´- TACCCTCATGCCTTCGAATC –3´
mNa_v1.9 – Forward	5´- AAGAGATGCAGGAGGCAAAA –3´
mNa_v1.9 – Reverse	5´- AGGCATCACATTCACCACAA –3´
mTRPV1 – Forward	5´- TTCCTGCAGAAGAGCAAGAAGC –3´
mTRPV1 – Reverse	5´- -CCCATTGTGCAGATTGAGCAT –3´
mCOX-2 – Forward	5´- GATGAGCAACTATTCCAAACCAG –3´
mCOX-2 – Reverse	5´- CCGCTCAGGTGTTGCACGTAG –3´

Supplementary Methods

Patient characteristics:

Patients with type-2 diabetes were selected from a previously described cohort[1]. Twenty patients were stratified according to self reported pain in the feet graded on 10 point numeric scales. Patients were considered to have severe pain when they reported $\geq$ 7 points on the numeric scale (n=10). Ten patients, who did not report pain as their main symptom, were selected as controls. Mean pain scores were 7.7±1.0 and 1.7±1.2 respectively ($p<0.0001$) while the groups did not differ significantly in the neuropathy deficit score (NDS)[2] as well as in a range of metabolic variables (*Suppl. table 1*). Although the groups were not stratified for these symptoms, patients with pain also reported significantly more burning sensations (5.3±2.3 vs. 2.7±3.0, p=0.047) and had a trend towards increased dysesthesia (4.7±2.2 vs. 3.0±2.0, p=0.9). Patients with clinical signs of peripheral arterial disease or other possible causes of neuropathy were excluded from the study[1]. The study protocol was approved by the University of Heidelberg Ethics Committee and all patients gave written informed consent.

Human material:

Human sciatic nerves were received from the Gewebebank für Entzündliche Erkrankungen (GEZEH) of the University Heidelberg (www.gezeh.de). Human skin biopsies were obtained from the skin biopsy bank of Prof. Dr. D. Ziegler at the DDZ in Düsseldorf, Germany.

Mouse models:

GLO$^{-/+}$ mice[3] and *Na*$_v$*1.8*$^{-/-}$ mice, obtained from Dr. John Wood, London, UK[4], are viable and display normal reproductive function. All strains were backcrossed on the C57BL/6-background for more than 10 times and C57BL/6-mice (*Charles River, Boston, USA*) served as controls for all transgenic mice. *BKS-db/db*-mice (*BKS.Cg-m+/+Lprdb/Bom Tac*)[5] and the respective control littermates were purchased from Taconic (*Lille Skensved, Denmark*). Mice

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were housed individually with a 12-hour/12-hour light/dark cycle and free access to food and water. All procedures in this study were approved by the Animal Care and Use Committees at the Regierungspräsidium Tübingen and Karlsruhe, Germany (*35-9185.81/G-90/04 and 35-9185.81/G-182/08*)

Induction of diabetes using streptozotocin (STZ):

Diabetes was induced by i. p. administration of STZ at 60mg/kg, freshly dissolved in sterile sodium citrate (0.05M, pH4.5) on 6 subsequent days[6]. Control animals received sodium citrate only. Diabetes was verified 16-25 days later by measurement of blood glucose levels in samples from the tail vein[6]. In the first 2 weeks following the onset of diabetes, blood glucose was measured daily. If glucose levels occasionally recovered, an additional STZ injection was given on day 25-27. More than 90% of mice became diabetic within the first 4 weeks and were used throughout the experiment. Those mice, which did not develop diabetes, served as additional controls. As soon as blood glucose increased above 300mg/dl, individual supplementation with 1-2U Insulin Glargine (40U/ml) was started. Once glucose levels had stabilized between 400 mg/dl and 450 mg/dl, blood glucose was determined at least once a week for the first 2 months, and thereafter every second week. Tight blood glucose control kept individual blood glucose levels stable throughout the experiments (healthy controls: 136.2 ± 3.06 mg/dl diabetic mice: 408.3 ± 15.3 mg/dl; $p < 0.0001$). At the end of the experiments functional assays were performed, before the animals were sacrificed with CO_2 and either the sciatic nerves were dissected, weighed and snap-frozen in liquid nitrogen or the dorsal root ganglia (DRG) were isolated as described below.

Plasma methylglyoxal (MG) determination:

The concentration of MG in mouse plasma samples was determined by HPLC after derivatization with 1,2-diamino-4,5-dimethoxybenezene as previously described[7]. Blood

samples were drawn into EDTA containing tubes from the retrobulbar vessels at time of sacrifice and centrifuged (2000g; 5 min). Plasma was removed and acidified with 10% TCA (0.1 ml). Samples were either analyzed immediately or stored at -80 C.

Assay of glyoxalase-I (GLO-I) activity:

GLO-I activity was determined by using the spectrometric method, which monitors the initial rate of change in absorbance at 240 nm caused by the formation of S-D-lactoylglutathione[8]. For the conversion of the hemithioacetal to S-D-lactoylglutathione, the change in molar extinction coefficient was ε_{240} = 2.86 $mM^{-1}cm^{-1}$. The standard assay mixture contained 2mM MG and 2mM GSH in sodium phosphate buffer (50mM, pH 6.6, 37 C). The reaction mixture was allowed to stand for 10 min before the addition of the cytosolic protein fraction (20µg) to ensure the equilibrium of hemithioacetal formation.

Isolation of DRG and DRG culture for gene expression studies:

L2-L6 DRG neurons isolation and cultured was performed as described[9]. Briefly, DRGs were excised and roots and membranes were removed and placed in sterile calcium and magnesium-free PBS. Ganglia were collected and resuspended in DMEM/F12 (1:1, v/v) containing 10% horse serum, 2% penicillin-streptomycin solution, 1% L-glutamine and 0.8% D-glucose (w/v). 0.1% collagenase was added and the tissue incubated at 37 C for 45 min. The tissue was incubated for a further 45 min in growth medium containing 0.05% trypsin at 37 C. Cells were dissociated by trituration through a fire-polished Pasteur pipette. After centrifugation at 250g for 5 min, the resultant pellet was washed twice in growth medium. Finally, cells were plated immediately, either onto a 6-well plate or glass cover-slips which had previously been coated with poly-L-ornithine (1µg/ml) and laminin (25µg/ml) supplemented with murine nerve growth factor (100ng/ml). Cells were maintained in an incubator at 37 C with 5% CO_2. After 24h incubation, the culture medium was supplemented with cytosine arabinoside (10µM)

and incubated for a 12h, after which time, culture medium was changed every two days until 70-80% confluence was reached.

RNA extraction and quantitative real-time PCR:

Total RNA was extracted using Trizol *(Invitrogen*, *Karlsruhe, Germany*) and from cultured DRGs using RNeasy mini kit (*Qiagen, Hilden, Germany*) both according to the manufacturer's instructions. A total of 2µg of total RNA from either sciatic nerves or DRGs was annealed with 1µg of oligo d(T) (*Promega, Heidelberg, Germany*) at 65 C for 10 min and then used for reverse transcriptase with 15units AMV-reverse transcriptase (*Promega, Heidelberg, Germany*) at 42 C for 1h. cDNA was used immediately or stored at -80 C. Real time-PCR was performed on a LightCycler (*Roche, Mannheim, Germany*). In brief, 2µl cDNA, were mixed with the appropriate primers (5pmol) and SYBR Green reagents (*Roche, Mannheim, Germany*) to a final volume of 20µl. The primers used are summarized in *table 3.* Cycling conditions were (95°C, 10min); (95°C, 10sec; 57°C, 5sec; 72°C, 5sec) x45; (65°C, 15min). The expected PCR products were ca. 180bp.

Preparation of total protein extracts:

Frozen tissue samples were grounded to a fine powder using liquid nitrogen. The homogenate was resuspended in ice-cold lysis buffer (10mM HEPES; 10mM KCl, 1.5mM $MgCl_2$, 1mM EDTA, 0.6% NP40, 0.5mM DTT, 0.2mM PMSF; pH7.9) and incubated on ice for 20 min. The tissue homogenates were then vortex and centrifuged (14000rpm; 10min; 4 C), and supernatants retained for analysis. The protein concentration for cell and tissue homogenates was determined by Bradford assay using BSA as a standard.

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Western blotting:

Total protein extract from sciatic nerves (20µg) were incubated in Laemmli loading buffer at 95 C for 5 min and separated under denaturing conditions on 10% Tris-glycine acrylamide gel and transferred to a nitrocelluose membrane and blocked with 5% nonfat dry milk at rt for 1h. Membranes were then incubated for a further hour with a goat polyclonal anti-GLO-I IgG antibody, 1:500 dilution, (*Santa Cruz, Heidelberg, Germany*) in 5% nonfat dry milk in PBS containing 0.05% Tween20 (PBS-T). After being washed with PBS-T, the membranes were incubated with horseradish peroxidase coupled donkey-anti-goat IgG secondary antibody, 1:1000, (*Santa Cruz, Heidelberg, Germany*) for 45min. Immunoreactive proteins were visualized on x-ray films using ECL-detection reagents according to the manufacturer's instructions and an exposure time of 10min as previously described[10].

Immunoprecipitation (IP) based western blot analysis of posttranslational modifications:

Healthy wistar rats (12 wks; male) received MG (5µg) or 0.0% NaCl i. v. After 3h, rats were sacrificed with CO_2 and the L4-L5 lumber DRGs were removed and processed immediately. Lumbar DRG (L2-L6) were also isolated from wild-type mice treated with MG, as well healthy and 8 week diabetic mice and non-diabetic GLO-/+ mice. For each group, DRGs were pooled from at least ten mice. DRG samples were homogenized in a glass dounce ice-cold lysis buffer (0.3M sucrose, 10mM Tris, 2mM EDTA; pH8.1) at 30µl/mg cell. Homogenates were kept on ice for 1h before centrifugation (2500rpm, 7min, 4 C) to remove nuclei and intact cells. The pellet was re-homogenized and spun as above. The supernatants from the two low-speed spins were combined and centrifuged at 45000 rpm for 2h at 4 C. The pellet, containing the total membrane fraction, was resuspended in 0.2M KCl and 10mM HEPES (pH7.4). Membranes were solubilized in an equal volume of 5% TritonX-100 and 10mM HEPES (pH7.4) and the suspension was kept on ice for 1h. Non-solubilized material was pelleted by centrifugation at 12000rpm for 10 min at 4 C, before the protein concentration of

the soluble material was determined by Bradford assay. Crude membrane fractions (500 µg) were pre-cleared by normal serum plus Protein A/G-agarose beads (*Santa Cruz, Heidelberg, Germany*) for 1h[10], before the supernatants were immunoprecipitated with 5µg of antibody for either $Na_v1.8$ (*Sigma, Deisenhofen, Germany*), $Na_v1.7$ or TRPV1 (both from *Santa Cruz, Heidelberg, Germany*) overnight at 4 C with gentle agitation. Protein A/G-agarose beads (*Santa Cruz, Heidelberg, Germany*) were added and incubated for a further 6h. Beads were collected by centrifugation (10,000g for 10 min at 4 C) and washed three times with buffer containing 20mM Tris (pH7.5), 150mM NaCl, 5mM EDTA, 0.2% SDS, 1% TritonX-100, 0.1mM PMSF, 1U/ml aprotinin. Protein were released from the agarose beads by incubation in denaturing buffer (125mM Tris-HCl, 4% SDS, 20% Glycerol, 10% ß-Mercaptoethanol; pH6.8; 50 µl) at 95°C for 10 min. The beads were collected by centrifugation, and the resulting supernatants retained and stored at -80° C for further analysis. For western blot analysis, immunoprecipitates were separated on 7.5% SDS-PAGE gels and transferred to nitrocelluose membranes. Visualization of the MG-modified proteins was achieved using the One-Step™ Complete IP-Western Kit (*GeneScript Corp, NJ, USA*) according to the manufacturer's instructions using an antibody for MG-derived AGEs (*Biologo, Kronshagen, Germany*; 10µg). Immunoreactive proteins were visualized on x-ray films using ECL-detection reagents (*GE Healthcare, Buckinghamshire, UK*) according to the manufacturer's instructions and an exposure time of 10min.

Determination of thermal hyperalgesia by the hot plate assays:

Hyperalgesia was studied using the hot-plate assay with an electronically controlled hot-plate analgesia meter (*Columbus Instruments, Columbus, Ohio, USA*) at 50 C[6,11]. Each mouse was removed from the hot plate when a jumping escape response occurred or hind paws were licked, or after a maximal cut-off time of 60 seconds. The latency until mice showed the first signs of discomfort (hind pare lifting, licking, or shaking, and jumping) were recorded by 2

investigators. Three measurements were taken per animal at each of the specified time points and averaged. The difference in latency times between control and treated animals was calculated as a measure of hyperalgesia.

Determination of thermal hyperalgesia by the Hargreaves Plantar test:

The foot withdrawal latency of the mice was also measured using the Hargreaves apparatus (*Ugo Basile, Comerio, Italy*) according to Hargreaves[12]. The cut-off time of the equipment was preset at 33 sec with an intensity of 60. Briefly, each mouse was placed into the testing enclosure and allowed time (30 min) to acclimatize before measurements were taken. Three measurements of foot withdrawal latency were made on each animal using alternating hind paws. Each trial was separated by an interval of 5min to decrease the possibly of skin injury and alteration of sensitivity of cutaneous nociceptors. As for the hot plate assay, the latency, until mice showed the first signs of discomfort, was recorded and the differences in latency times between control and treated animals determined.

Determination of the nociceptive threshold by the tail flick assay:

The nociceptive threshold was determined by recording the withdraw latency in tail flick tests [13]. In brief, mice were gently held with the tail positioned in the tail-flick apparatus (*Columbus Instruments, Columbus, Ohio, USA*) for radiant heat stimulation of the surface of the tail at 2-3cm approximately from the tail tip. The intensity of the radiant heat was adjusted to obtain basal or pre-treatment latency of 6-8sec in WT mice. The maximum cut off time was fixed at 10 sec.

***Determination of mechanical (tactile) hyperalgesia by the Frey-Filament test*:**

To assess the tactile allodynia, paw thresholds were measured using the Dynamic Plantar Anesthesiometer (*Ugo Basile, Comerio, Italy*)[14]. The electronic Von Frey device, using a single nonflexible filament, applies increasing force (0-20g) against the plantar surface of the hind paw over a 20 sec period. The nocifensive paw withdrawal response automatically turns off the stimulus, and the mechanical pressure that evoked the response is recorded. One day before the experiments, mice had to undergo a training session to get used to the procedure: The following day, mice were placed individually in plastic cages with a wire mesh bottom and allowed to acclimatize for at least 1h. After habituation, each mouse was tested three times alternately in each hind-paw, allowing at least 30sec between each measurement.

Repetitive *in vivo* determination of nerve conduction velocity in the tail of mice:

Nerve conduction velocity (NCV) was measured as basically described by Miyoshi and Goto[15]. For all measurements, the mice were anesthesized by i. p. injection of tribromoethanol (Avertin®) at a dosage of 250mg/kg. Immediately after loss of sensitive reactions the electrodes were placed at the tail which was warmed via a costum-made thermoblock to 37°C for 60 sec. Needle electrodes (10mm x 0.3mm (30G), *9013R0313, Alpine Biomed*) were inserted subcutaneously on the dorsal side of the tail, with recording electrodes placed on the hairline and the stimulating electrodes 20mm distal to the hairline. A ground electrode was placed between the proximal and the distal electrode. Compound action potentials in the dorsal *coccygeal* nerve were elicited by a Keypoint Interface (*Alpine Biomed*) with pulses varying between 4 and 13 mA amplitude and 0.2 ms in duration. The evoked potentials were amplified, recorded and analyzed on a Keypoint portable system *(Alpine Biomed*). Maximal NCV was calculated from the latency of the stimulus artifact to the starting point of the compound action potential and the distance between stimulating and recording electrode (20mm).

Determination of cerebral blood flow (CBF) by autoradiography*:*

Neuronal activation in selected brain regions was determined by autoradiographic measurement of regional cerebral blood flow (CBF) as described previously[16,17]. Briefly, each mouse was placed into a restraint such that each hind paw was clearly accessible and a flexible intravenous catheter was inserted into the tail vein. Mice were permitted to rest quietly in the restraint for approximately 30min to recover from the stress of tail vein catheterization. Mice were then exposed to a direct radiant heat stimulus (55°C; as in the Hargreaves plantar assay) onto the exposed hind paw until paw withdrawal. All mice reacted within the 10 sec cut-off. Immediately after the pain response observed, 100 µl saline containing 2 mCi of [99m]technetium N,N'(1,2-ethlenediyl)bis-L-cysteine diethyl ester (Tc-99m-Neurolite®, IBA Molecular Imaging, prepared according to the manufacturers instructions) was injected into the tail vein as a bolus over 10 to 15sec. 2min after tracer injection, mice were euthanized by cervical dislocation. The brain was carefully removed from the skull, snap-frozen in liquid nitrogen-cooled 2-propanol and consecutive 20µm coronal sections were cut at -18°C starting at Bregma level zero[18]. Brain sections were mounted on glass slides and immediately desiccated at 70°C. Autoradiograms were generated by direct exposure of the mounted sections onto the emulsion side of Kodak BioMax™ MR-1 imaging film (*Kodak, Rochester, NY, USA*) for 2-14 h exposure. When the radioactivity level of the [99m]Tc labelled slides had returned to background, the slides were stained with cresyl violet. Structural identification was made by careful comparison of cresyl violet stained sections to coronal plates from the stereotaxic atlas of the mouse brain[16-19]. Densitometric analysis of autoradiograms was performed using Quantity One software (*Biorad, CA, USA*). Briefly, each brain section on

film was digitized to produce a high resolution (36.3 x 36.3 micron) gray scale image. Each image was corrected for the background and total brain slice density determined before a pseudo-colour-filter was applied to visualize areas of high and low density.

In vivo overexpression of glyoxalase I (GLO-I) by somatic gene transfer:

Full length murine GLO-I cDNA was cloned into the pCMV6-Neo expression vector under the control of a cytomegalovirus promoter. The pGL3-luciferase vector (*Promega, Heidelberg, Germany*) was used as a control. A concentration of 1.5 µg/g of vector or pCMV-Neo-GLO1 plasmid DNA was suspended in 150 µl liposomal transfection reagent (DOTAP; *Roche, Mannheim, Germany*) and incubated for 15 min at rt before administration by i. p. injection every 4th day in 8 weeks diabetic male WT mice (18-20 weeks old) for a total of 10 days[20]. Hyperalgesia was measured before injection. At the end of the experiment, all mice were sacrificed and plasma isolated for determination of MG concentration.

In vivo inhibition of glyoxalase I (GLO-I):

Healthy male WT mice (10-12 weeks old) were treated every 2nd day with 50µg/g (i.p) of the cell permeable GLO-I inhibitor, S-p-Bromobenzylglutathione Cyclopentyl diester[21] for a period of 15 days. Hyperalgesia was assessed by the hot plate assay at day 0 before application of the inhibitor. Thereafter, consecutive assessments of thermal hyperalgesia were made at selected time points, at which some mice also were sacrificed and plasma isolated for the determination of MG concentration.

In vivo siRNA treatment:

Pre-designed esiRNA for murine COX-2 (MU-03964-1) and scrambled esiRNA was purchased from Sigma (*Sigma, Deisenhofen, Germany*). siRNA for murine voltaged-gated sodium channels $Na_v1.7$ (Scn9A), $Na_v1.8$ (Scn10a) and TRPV1 and the respective control

siRNA was purchased from Ambion® (*Applied Biosystems, Darmstadt, Germany*). A concentration of 1.5µg/g of siRNA were suspended in 150 µl liposomal transfection reagent (DOTAP; *Roche, Mannheim, Germany*) and incubated for 15 min at rt before administration by intravenous injection every 3rd day for a period of 10 days in healthy WT mice (male; 10-12weeks). On the tenth day, mice were injected with either MG (5µg) or PBS as a control. After 3h, hyperalgesia was assessed and the mice were sacrificed. To confirm knock-down of treated mice, sciatic nerves and DRGs were removed and total RNA was extracted, before real-time PCR (qPCR) was performed.

Molecular modeling:

Receptor binding domain (RBD) analysis was performed to determine likely sites of MG-modification in Nav1.8 from human, rat and mouse. Briefly, the amino acid sequence for $Na_v1.8$ (*Accession No.Q9Y5Y9, Human; Accession No. Q62968, Rat; Accession No.Q6QIY3, Mouse*) were obtained from the UniProtKB/Swiss-Prot database (http://www.expasy.org). Residue hydrophobicity was calculated using Protscale (http://us.expasy.org/tools/protscale.html), and mean hydrophobic moment calculated using Embosswin (http://perso.wanadoo.fr/ablavier/embosswin/embosswin.html). Both hydrophobicity and the mean hydrophobic moment values were aligned with the sequence number to generate a plot of hydrophobicity *vs.* mean hydrophobic moment, sectioned as described[22]. Arginine and lysine residues found within the RBD were then highlighted as potential *hotspots* for modification by MG. Attempts to identify those arginine and lysine residues with a higher potential for modification based upon differences in residue pKa were not possible as the complete crystal structure for $Na_v1.8$ is not yet available. Instead, identification was made, with reference to the amino acid sequence, based upon the following criteria that arginine and lysine residues most reactive with MG are those with proximate arginine and lysine residues that decrease the pKa of both interacting amino groups and have

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glutamic or aspartic acid carboxylate side chain on the alternate side in the sequence to act as a catalytic base[23,24]. Using this selection criterion, the total number of glycation hotspots was established. Multiple sequence alignment was performed using *Bioedit* with in built *ClustW*.

In vivo scavenging of MG:

A synthetic peptide, *GERP* [*United State Letter Patent No. 61/182,203; May 29, 2009*], containing ten arginine residues per molecule and acting as an effective *in vivo* scavenger of MG, the synthetic control peptide GEAP (in which the arginine residues were substituted for alanine) and a synthetic peptide spanning sequence of the $Na_v1.8$ inactivation gate were designed in house and synthesized (*GenWay Biotech, Inc. San Diego, CA, USA*)**.** Healthy WT mice (male; 10-12 weeks) were injected i. p. with 1 mg of either *GERP* or *GEAP* 30 min prior to injection with either 0.9% NaCl or 5 µg MG. Hyperalgesia was assessed 3h after administration of MG. To determine the effect of GERP on diabetes induced hyperalgesia, STZ-induced 8 weeks male diabetic WT mice (18 weeks old) were injected i. p. once with either 1 mg of GERP or GEAP. Hyperalgesia was assessed before (day 0) and after treatment consecutively at day 1, 3 and 6 by hot plate assay. The DIII-DIV linker, containing the inactivation gate, of murine $Na_v1.8$ (*Q6QIY3*) was synthesized and incubated without (Control) with MG in either 1:5 (minimally modified) or 1:10 (maximally modified) molar ratio in 100mM sodium phosphate buffer (pH7.4) at 37°C for 24h[56]. All peptides were purified by RP-HPLC and analysized by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (*Ultraflex I, Bruker Daltonik, Bremen*) equipped with a nitrogen laser. For tryptic digestion 2µl of the peptide stock solution from either the control or minimally modified peptides (ca.200nmol/10µl 0.1% TFA) were incubated with 1µl □□sequencing grade trypsin (*Promega*; 17ng/□µl) in 40mM ammonium bicarbonate at 37°C overnight and the reaction was stopped by freezing. For the mass spectrometric analysis of tryptic digests samples were prepared by reverse phase extraction using ZipTip C18 according to the manufacturer

(*Millipore, CA, USA*). Elution and spotting was performed with a 50% acetonitrile/0.1% TFA solution saturated with HCCA matrix (HCCA: α-cyano-4-hydroxycinnamic acid). Mass spectra were acquired in the positive ion reflector mode on a matrix-assisted laser desorption ionization time-of-flight mass spectrometer (*Ultraflex I, Bruker Daltonik, Bremen*) equipped with a nitrogen laser. Mass spectra were obtained by averaging up to 500 individual laser shots. Acceleration voltage was set to 25 kV, reflector voltage was 26.3 kV. Fragmentation analysis was performed using the LIFT option of the instrument.

Measurement of CGRP-release:

In vivo, adult C57BL/6 and $Na_v1.8^{-/-}$-mice received an i. p. injection of MG (80µmoles in 80µl) or saline for control three hours prior to the final experiment. After sacrifice by suffocation in pure CO_2, the skin from both hind paws was subcutaneously excised below the knee level as described previously[25]. The skin flaps obtained were tied around acrylic glass rods with the corium side exposed. The preparations were washed for 30 min at 32°C in carbogen-gassed (95% O_2, 5% CO_2) synthetic interstitial fluid (SIF) containing 108mM NaCl, 3.48mM KCl, 3.5mM $MgSO_4$, 26mM $NaHCO_3$, 1.7mM NaH_2PO_4, 1.5mM $CaCl_2$, 9.6mM sodium gluconate, 5.5mM glucose, and 7.6mM sucrose. After the adaptation period, the skin flaps were first incubated for 5 min in test tubes containing control SIF to determine basal CGRP release. Thermal stimulation was performed by immersing the preparations in further test tubes containing pre-heated SIF at 45°C; this temperature was kept constant in a temperature-controlled shaking bath for 5 min of stimulation. For chemical stimulation, the preparations were incubated for 5 min in tubes containing the test solution of 60mM KCl dissolved in SIF and corrected for osmolarity. The CGRP content of the incubation fluid was measured using commercial EIA kits (*Bertin/SPIbio, Montigny le Bretonneux, France*) immediately after the experiment as previously described in detail. The EIA plates were determined photometrically using a microplate reader (*Dynatech, Channel Islands, UK*). The

antibodies used are directed against human α/β-CGRP but are 100% cross-reactive against mouse and rat CGRP. The detection level is about 2 pg/ml. Mean (± SEM) stimulus responses of the induced release are presented as columns reflecting the 5 min sampling periods. Both hind paw skin flaps of the same animal were tested and the mean of both CGRP values was taken for further statistical analysis. The t-test for dependent samples was employed to compare data within one experimental group and ANOVA followed by LSD post hoc analysis was used for comparison of different experimental groups and phenotypes.

Electrophysiology:

Isolation of DRG, DRG culture and transfection:

Adult WT C57Bl/6 and $Na_v1.8^{-/-}$- mice were killed by CO_2 suffocation. DRGs from all spinal levels were removed and incubated in a mixture of 1 mg/ml Collagenase (type IX, *Sigma, Deisenhofen, Germany*) and 0.2 mg/ml protease (*Sigma, Deisenhofen, Germany*) in Dulbecco's Modified Eagle's Medium (DMEM) for 45 min at 37°C. After trituration the dissociated cells were plated onto borosilicate glass cover-slips which had been treated with poly-D-lysine (0.1 mg/ml for 30 min), and cultured (37°C, 5% CO_2 in air) in serum free TNB 100 basal medium (*Biochrom AG, Berlin, Germany*), supplemented with penicillin/streptomycin 100 U/ml and 100 ng/ml nerve growth factor-7S (NGF, *Alomone Laboratories, Jerusalem, Israe*l). The neurons were used for recordings the day after the dissection. Recordings were carried out alternatively from neurons of control or conditioned culture dishes. MG was present at 100 M for ~3 h or at 1μM for 12-14h, before and during the actual recordings. In one series of experiments, to prevent posttranslational modifications by MG, aminoguanidine (AG) was added (100μM for 3-5h) to the culture medium followed (or not, for control) by MG 1μM for the subsequent 12-14 h. Rat $Na_v1.8$-cDNA was transiently transfected in the dorsal root ganglion neuroblastoma hybridoma cell line ND7/23 cells (*European Collection of Cell Cultures*) as described previously[26]. ND7/23 cells were

maintained in Dulbecco´s modified Eagle medium (*Gibco, Karlsruhe, Germany*), supplemented with penicillin/streptomycin 10000 U/ml (*Gibco, Karlsruhe, Germany*), HEPES 1M (*Gibco, Karlsruhe, Germany*), heat-inactivated FBS 10% (*HyClone Europe Ltd., Cramlington, United Kingdom*) and taurine 0.3 M (*Sigma, Deisenhofen, Germany*) at 37°C and 5% CO_2. All cells were transfected by the calcium phosphate precipitation method and included a reporter plasmid (CD8-pih3m, 1µg). Transfected cells were used for experiments within 3-4 days.

Electrophysiological recordings:

Whole cell patch clamp recordings were performed at rt using an Axopatch 200B amplifier and pClamp 8.2 software (*Axon Instruments, Union City, CA, USA*). Current-clamp recordings were made from small and medium-sized DRG neurons with a resting membrane potential more negative than -40 mV. Patch clamp pipettes with a resistance between 1.8 and 4 M were pulled from borosilicate capillary glass (*GB150F-8P, Science Products GmbH, Hofheim, Germany*). Membrane voltage was sampled at 10 kHz and filtered at 2 kHz. The extracellular solution contained (in mM): NaCl, 140; KCl, 4; $CaCl_2$, 2; $MgCl_2$, 1; HEPES, 10; NaOH, 4.55; glucose, 5 (pH 7.4 at 25 °C). The pipette solution contained (in mM): KCl, 135; $MgCl_2$, 1.6; EGTA, 2; HEPES, 10; Mg-ATP, 2.5 (pH 7.3 at 25 °C, adjusted with NaOH). Only one cell was used for recording from each coverslip. All recordings were stored on PC for off-line analysis using the pClamp 8.2 and Microcal Origin 7 software (*OriginLab Corp, Northampton, MA, USA*). Data are presented as mean ± SEM. Two-tailed Student's unpaired t test was performed using the Microcal Origin 7 software. A value of $p < 0.05$ was considered statistically significant. The following measurements were made on the action potential waveform: RMP (resting membrane potential), V_{thr} (voltage threshold, or take-off voltage, defined as the point at which the first derivative of the action potential starts to increase from an approximately constant level), RT (rise time, the time from V_{thr} to the overshoot (peak) of

the action potential), AP duration (the time from V_{thr} to the return to RMP), AP size (the voltage change from RMP to action potential overshoot), AHP (after hyperpolarization) amplitude and AHP duration (the time from the return to RMP to 80% recovery from the AHP). The current threshold for generating an action potential for a 5 ms rectangular pulse was also measured. After recording the response to current steps in current clamp mode, the recording configuration was switched to the voltage clamp mode, and capsaicin (2 M) was applied for 20 s at a holding potential of -60 mV. *Voltage-clamp* recordings were made from small and medium-sized DRG neurons. TTXr currents were studied in WT DRG neurons in presence of 250 nM TTX. TTXs currents were studied in $Na_v1.8^{-/-}$ DRG neurons. For recordings on DRG neurons, the extracellular solution contained (mM): NaCl 40, Choline-Cl 100, KCl 3, $MgCl_2$ 1, $CaCl_2$ 1, HEPES 10 (pH adjusted to 7.4). The pipette solution contained (mM) CsF 140, EGTA 1, NaCl 10, and HEPES 10, pH 7.4. For experiments on $Na_v1.8$-ND7/23 cells, standard extracellular solution was (in mM) 65 NaCl, 85 Choline-Cl, 2 $CaCl_2$ and 10 HEPES, pH 7.4. The pipette solution contained (in mM) 100 NaF, 30 NaCl, 10 EGTA and 10 HEPES, pH 7.4. Transfection-positive cells were identified by immunbeads (anti-CD-8 Dynabeads, *Dynal A.S., Oslo, Norway*). Only cells with an initial seal >1 GΩ and a leak current <500 pA throughout the recording were used for the analysis. Voltage errors were minimized using 70-80% series resistance compensation, and the capacitance artifact was canceled using the computer-controlled circuitry of the patch-clamp amplifier. Linear leak subtraction, based on resistance estimates from four hyperpolarizing pulses applied before the depolarizing test potential, was used for all voltage-clamp recordings. Membrane currents were filtered at 5 kHz and sampled at 20 kHz. Data were analyzed with the Clampex 8.2 software and Origin 6.0 (*Microcal Software, Northampton, MA, USA*). To calculate the midpoint of activation, the peak conductance g_m was estimated from the equation $g_m=I_{Na}/(E_m-E_{rev})$, where I_{Na} is the peak current, E_m is the corresponding voltage, and E_{rev} is the estimated reversal potential. The data were least-squares fitted with the Boltzmann equation

$g_m/g_{max}=1/[1+\exp((E_{0.5}-E)/k_E)]$, where g_{max} is the maximum conductance, $E_{0.5}$ is the voltage at which $g/g_{max}=0.5$, and k_E is the slope factor. To obtain the midpoint of steady-state inactivation, peak currents evoked by a test pulse were measured, normalized, and plotted against the conditioning prepulse potential. The data were least-squares fitted by the Boltzmann equation $y=1/[1+\exp((E_{pp}-h_{0.5})/k_h)]$, where $h_{0.5}$ is the voltage at which y=0.5 and k_h is the slope factor. Values of all experiments are presented as mean ± S.E.M. in the figures.

Statistics:

All data is expressed as mean values ± standard deviation (SD; n ≤ 5) or mean values ± standard error of the mean (SEM; n ≥ 10). Unpaired, 2-tailed Student's t-test was used to determine significance in all comparisons. The t-test for dependent samples was employed to compare data within one experimental group. ANOVA followed by LSD post hoc analysis was used for comparison of different experimental groups were indicated. Statistical analysis was performed using SPSS software version 15.0 (*SPSS Inc., Chicago, USA*). Any $p > 0.05$ was considered to be statistically non-significant.

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