

# Pharmacodynamics and Pharmacokinetics of Lidocaine in a Rodent Model of Diabetic Neuropathy

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## ABSTRACT

**Background:** Clinical and experimental data show that peripheral nerve blocks last longer in the presence of diabetic neuropathy. This may occur because diabetic nerve fibers are more sensitive to local anesthetics or because the local anesthetic concentration decreases more slowly in the diabetic nerve. The aim of this study was to investigate both hypotheses in a rodent model of neuropathy secondary to type 2 diabetes.

**Methods:** We performed a series of sciatic nerve block experiments in 25 Zucker Diabetic Fatty rats aged 20 weeks with a neuropathy component confirmed by neurophysiology and control rats. We determined *in vivo* the minimum local anesthetic dose of lidocaine for sciatic nerve block. To investigate the pharmacokinetic hypothesis, we determined concentrations of radiolabeled (<sup>14</sup>C) lidocaine up to 90 min after administration. Last, dorsal root ganglia were excised for patch clamp measurements of sodium channel activity.

**Results:** First, *in vivo* minimum local anesthetic dose of lidocaine for sciatic nerve motor block was significantly lower in diabetic (0.9%) as compared to control rats (1.4%). Second, at 60 min after nerve block, intraneural lidocaine was higher in the diabetic animals. Third, single cell measurements showed a lower inhibitory concentration of lidocaine for blocking sodium currents in neuropathic as compared to control neurons.

**Conclusions:** We demonstrate increased sensitivity of the diabetic neuropathic nerve toward local anesthetics, and prolonged residence time of local anesthetics in the diabetic neuropathic nerve. In this rodent model of neuropathy, both pharmacodynamic and pharmacokinetic mechanisms contribute to prolonged nerve block duration. (**ANESTHESIOLOGY 2018; 128:609-19**)

LOCAL anesthetics are widely used to block nerve conduction for surgical anesthesia or to manage acute and chronic pain. However, diabetic neuropathic nerves may react differently to blockade than healthy nerves.<sup>1</sup> We have previously shown in Zucker diabetic fatty rats with neuropathy that nerve blocks last substantially longer as compared to healthy control rats.<sup>2</sup> This is in concordance with recent experimental<sup>3</sup> and clinical<sup>4</sup> literature, but the mechanism of block prolongation remains unknown.

There are two hypotheses why nerve blocks could last longer in diabetic neuropathy; the first is pharmacodynamic and states that nerve fibers are more susceptible because their sodium channel expression<sup>5</sup> and function<sup>6</sup> has changed. The second hypothesis is pharmacokinetic and stipulates delayed wash-out of local anesthetic due to microangiopathy<sup>7</sup> and decreased nerve blood flow.<sup>8</sup> Both mechanisms have been assumed<sup>1</sup> but never directly investigated in the context of regional anesthesia.

Using the local anesthetic lidocaine in a rodent model of diabetic neuropathy, we examined the minimum local anesthetic dose of lidocaine for sciatic nerve block *in vivo*, used patch clamp measurements to quantify the effect of

### What We Already Know about This Topic

- Nerve blocks last substantially longer in animal models of diabetic neuropathy and in patients with diabetic neuropathy
- The prolonged block may be due to either delayed clearance of neuronal local anesthetic, increased sensitivity of diabetic neurons to the blocking effects of local anesthetics, or both

### What This Article Tells Us That Is New

- Rats with neuropathy secondary to type 2 diabetes had prolonged nerve block duration compared to control rats
- Radiolabeled lidocaine concentrations were higher in the nerves of diabetic rats 60 min after nerve block, when most diabetic nerves are still blocked
- The ED<sub>50</sub> of lidocaine for motor block in diabetic rats was 64% of that of control animals
- The *in vitro* inhibitory effect of a given lidocaine concentration was much more pronounced in primary sensory neurons harvested from diabetic rats

lidocaine on sodium currents *in vitro*, and determined intraneural concentrations of lidocaine *in vivo* over time. Our hypotheses were that as compared to healthy control nerves,

Submitted for publication December 15, 2015. Accepted for publication October 27, 2017. From the Department of Anesthesiology and Laboratory of Experimental Anesthesiology and Intensive Care (LEICA) (W.t.H., M.W.H., J.H., S.P., P.L.); Department of Nuclear Medicine (K.d.B., H.J.V.), Cardiology (A.O.V., H.L.T.), Anatomy, Embryology and Physiology (A.O.V.), and Neurology (C.V.), Academic Medical Center University of Amsterdam, Amsterdam, The Netherlands; Department of Anesthesiology and Intensive Care Medicine, Trauma Hospital, Graz, Austria (M.R.); Department of Anesthesiology, University of Heidelberg, Heidelberg, Germany (S.P.) and Department of Anesthesiology, Perioperative and Pain Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts (P.L.).

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(1) diabetic neuropathic neurons would be more sensitive to the blocking effects of lidocaine, and (2) the intraneural lidocaine concentration after nerve blockade would remain elevated over time.

## Materials and Methods

The present study protocol was approved on December 18, 2014, by the Institutional Animal Care and Use Committee of the Academic Medical Center, University of Amsterdam (Amsterdam, The Netherlands), protocol number LEI-CA75AA-1. Methods and results are reported according to ARRIVE guidelines.<sup>9</sup>

### Experimental Setup

Experiments were carried out in 25 male Zucker diabetic fatty and 25 control rats, obtained from Charles River Laboratories (France). Zucker diabetic fatty rats are an inbred model of type 2 diabetes mellitus (DM) that combines a dietetic component (Purina #5008 diet; Charles River) with a genetic predisposition. Diabetic animals exhibit a leptin receptor mutation (*fa/fa*, “diabetic”), whereas control animals carry a heterozygous mutation (*fa/+*, “Control”).<sup>10</sup>

Animals underwent two sets of experiments. In week 1, animals were tested for diabetic state and nerve conduction velocity, and subsequently we performed sciatic nerve block with varying concentrations of lidocaine to determine *in vivo* minimum local anesthetic dose for motor blockade in control *versus* diabetic animals. In week 2, we repeated and confirmed nerve conduction velocity measurements and performed sciatic nerve block with radiolabeled lidocaine to measure intraneural lidocaine concentrations over time. After these experiments were completed, nine animals from each group underwent excision of the lumbar dorsal root ganglia for patch clamp experiments to determine the effects of lidocaine on sodium currents in diabetic *versus* healthy nerves.

### Animals

Animals were obtained at 14 weeks of age and were given 6 weeks for acclimatization, with free access to water and diet. For all electrophysiologic measurements, sciatic nerve block, and measurements of weight and glucose, the animals were anesthetized using isoflurane (Baxter, The Netherlands) with an inspiratory concentration of 2 to 3 vol% because this regimen least affects electrophysiologic measurements in rodent models.<sup>11</sup> To minimize animal distress, the electrophysiologic measurements and nerve blocks were performed percutaneously. Buprenorphine (0.05 mg/kg body weight) was used as analgesic rescue.

Glucose was measured before experiments using a commercially available glucose meter (GlucoMen; Menarini Diagnostics, The Netherlands) able to detect glucose values of up to 33 mM. If glucose values exceeded the upper detection limit, we set these values at 33 mM.

Animal welfare (*e.g.* animal appearance and behavior) was assessed at least weekly by an animal care technician unaffiliated with the experimental team. Out of the 50 animals, 1 animal in the control group fulfilled predefined criteria for early termination of experiments (humane endpoints) when it developed a growing skin defect after percutaneous nerve block in week 1. The animal was euthanized. Further, one diabetic animal died during induction of anesthesia using isoflurane in week 2. Replacement of animals was done after consultation with the Animal Care and Use Committee. All other animals survived to the end of the experiment, no animal needed analgesic rescue, and welfare assessment showed no abnormalities concerning appearance or behavior at any time point. All animals showed full recovery from sciatic nerve block after the experiments of week 1. After the last experiments, while still under isoflurane anesthesia, animals were euthanized using CO<sub>2</sub> narcosis. For additional patch clamp experiments requested during the Journal review process, we received permission by the Animal Care and Use Committee to obtain an additional four Zucker diabetic fatty rats and four control rats.

### Sciatic Nerve Block

Nerve block was performed percutaneously combining the technique described by Thalhammer *et al.*<sup>12</sup> modified by nerve stimulation as described by Kroin *et al.*<sup>13</sup> In brief, a 25-gauge needle was introduced just caudal to the sciatic notch in a cephalad direction and connected with a clip to the electromyography system programed to deliver a pulse of 0.1-ms duration and 0.5-mA current, triggered manually. Ipsilateral hind-leg muscle contraction in the absence of local gluteal muscle stimulation was taken as sign of proximity of needle to nerve, and injection of 0.2 ml of lidocaine 2% was performed.<sup>14</sup> We defined a successful nerve block on the basis of two signs: successful nerve stimulation as described above before injection and subsequent behavioral testing showing absence of the toe-spreading reflex. The latter reflex, used to test sciatic nerve fibers, was evaluated as described before.<sup>14</sup> In brief, the animals were gently lifted, resulting in a physiologic vestibular reflex where toes are extended and spread.

### Electrophysiology

Animal temperature was maintained well above 34°C using a warming blanket (Harvard Apparatus, The Netherlands). We studied the sciatic nerve with monopolar needle electrodes as described previously,<sup>15</sup> using the PowerLab 4/25T nerve stimulator together with the Scope software package, version 3.8.7 (both from AD Instruments, United Kingdom). In brief, for motor conduction studies of the sciatic nerve, the recording cathode was placed in the muscles between the hallux and the second digit, and the recording anode was placed subcutaneously on the lateral surface of the fifth digit. Stimulating electrodes were inserted 3 mm apart at the medial ankle and just cranial to the sciatic notch. A

grounding electrode was attached between the stimulating and the recording electrodes. Supramaximal square-wave pulses of 0.1-ms duration were delivered. Supramaximal stimulation was achieved by increasing the intensity by 25 to 30% above the current that gave maximal muscle response. Motor nerve conduction velocity was calculated over the segment between the sciatic notch and the ankle.<sup>15</sup> Measurements were carried out by one investigator (P.L.), and neurophysiology graphs underwent blinded assessment and validation by an experienced neurophysiologist (C.V.).

### Pharmacodynamics

**In Vivo Minimum Local Anesthetic Dose.** For the determination of minimum local anesthetic dose, we performed sciatic nerve blocks using lidocaine (BBraun, Germany) in various dilution steps (in normal saline 0.9%; BBraun) in a total volume of 0.2 mL<sup>14</sup> and evaluated sciatic nerve motor block (yes/no) after 20 min. The starting concentration was 2%, and according to the Dixon up-and-down method, when a successful block was observed, this concentration was decreased by 0.2% for the subsequent test animal. Conversely, failed block led to a subsequent increase of the dose by 0.2%. The concentration of lidocaine at which a successful block would be achieved in 50% of the test animals ( $ED_{50}$ ) was determined by calculating the mean dose from consecutive rats in which successful nerve block was followed by failed nerve block three times.<sup>16</sup> *A priori*, we determined that a difference of 0.25% in minimum local anesthetic dose would be considered relevant.

**In Vitro Electrophysiology. Cell Preparation.** Single dorsal root ganglion (DRG) neurons were obtained by enzymatic dissociation after a protocol described previously.<sup>17</sup> In short, L4 and L5 dorsal root ganglia were excised and placed into cold (4°C) solution containing (in mM): 145 Na<sup>+</sup>, 4 K<sup>+</sup>, 2.5 Ca<sup>2+</sup>, 1 Mg<sup>2+</sup>, 5 L-malate<sup>-</sup>, 24 acetate<sup>-</sup>, 127 Cl<sup>-</sup> (Sterofundin; BBraun). The ganglia were dissected and stored in a modified Tyrode solution (20°C) containing (in mM): 140 NaCl, 5.4 KCl, 1.8 CaCl<sub>2</sub>, 1.0 MgCl<sub>2</sub>, 5.5 glucose, 5.0 HEPES, pH 7.4 (NaOH). Next, the ganglion pieces were placed in nominally Ca<sup>2+</sup>-free Tyrode solution (37°C), *i.e.*, modified Tyrode solution with 10 μM CaCl<sub>2</sub>, which was refreshed two times before the addition of liberase IV (0.25 to 0.29 U/ml; Roche, USA) and elastase (2.4 to 0.7 U/ml; Serva, Germany) for 10 min. During the incubation period, the tissue was triturated through a pipette (tip diameter, 2.0 mm). The dissociation was stopped by transferring the ganglion pieces into nominally Ca<sup>2+</sup>-free Tyrode solution (20°C). The tissue was triturated (pipette tip diameter, 0.8 mm) in Ca<sup>2+</sup>-free Tyrode solution for 4 min to obtain single cells. Finally, the nominally Ca<sup>2+</sup>-free Tyrode solution was replaced with normal Tyrode solution in three steps to increase the Ca<sup>2+</sup> concentration. In each step, approximately 75% of the solution on top of the cells was carefully replaced with Tyrode solution. The time interval between the solution changes was 10 to 15 min. For electrophysiologic measurements,

small aliquots of cell suspension were put in a recording chamber on the stage of an inverted microscope. DRG neurons were allowed to adhere for 5 min before superfusion was initiated.

**Recording Procedures.** Action potentials (APs) and the sodium current ( $I_{Na}$ ) were recorded with the patch clamp technique using an Axopatch 200B clamp amplifier (Molecular Devices, CA) and custom-made acquisition software (Scope; kindly provided by J. Zegers). Membrane currents and potentials were low-pass filtered (cut off at 5 kHz) and digitized at 40 kHz. APs and  $I_{Na}$  were analyzed offline using custom-made software (MacDaq; kindly provided by A. van Ginneken). Cell membrane capacitance ( $C_m$ ) was estimated by dividing the decay time constant of the capacitive transient, in response to 5-mV hyperpolarizing voltage clamp steps from -40 mV, by the series resistance. Series resistance was compensated for by at least 80%.

**Current Clamp Experiments.** APs were recorded at 36 ± 0.2°C in modified Tyrode solution by the amphotericin-perforated patch clamp technique. Pipettes (borosilicate glass; resistance, 1.5 to 2 MΩ) were filled with solution containing (mM): Kalium-gluconate 125, KCl 20, NaCl 10, amphotericin-B 0.44, HEPES 10, pH 7.2 (KOH). APs were elicited by applying 500-ms depolarizing current pulses of various amplitudes through the patch pipette. We analyzed the resting membrane potential as the potential just before the depolarizing current pulses. In addition, from the first AP during the depolarizing pulse, we measured: (1) the AP overshoot, *i.e.*, the maximal potential of the AP above the 0 mV level; (2) the maximal upstroke velocity ( $dV/dt_{max}$ ) by the first derivative of the AP; and (3) the AP duration (APD) at 50% repolarization (APD<sub>50</sub>).

**Voltage Clamp Experiments.**  $I_{Na}$  was recorded at 20°C with the whole cell ruptured patch clamp technique using a voltage clamp protocol with a holding potential of -60 mV and 300-ms depolarizing pulses to 0 mV. This depolarizing test pulse was preceded by a 700-ms prepulse to -50 and -120 mV. We defined the  $I_{Na}$  recorded with the prepulse to -50 mV as tetrodotoxin-resistant, whereas the tetrodotoxin-sensitive  $I_{Na}$  was obtained by digital subtraction of the tetrodotoxin-resistant  $I_{Na}$  from the total  $I_{Na}$ , measured after the prepulse to -120 mV. The cycle length was 5 s. Extracellular solution contained (in mM): 20 NaCl, 120 CsCl, 1.8 CaCl<sub>2</sub>, 1.2 MgCl<sub>2</sub>, 0.1 CdCl<sub>2</sub>, 11.0 glucose, 5.0 HEPES, pH 7.4 (CsOH). Pipettes were filled with solution containing (in mM) 3.0 NaCl, 133 CsCl, 2.0 MgCl<sub>2</sub>, 2.0 Na<sub>2</sub>ATP, 2.0 TEACl, 10 EGTA, 5.0 HEPES, pH 7.2 (CsOH).  $I_{Na}$  was defined as the difference between peak current and the current at the end of the depolarizing voltage step. Dose-response curves of the effects of lidocaine on  $I_{Na}$  were fitted to the Hill equation:  $I_{drug}/I_{control} = 1/[1 + (dose/IC_{50})^n]$ , where  $I_{drug}/I_{control}$  is the normalized  $I_{Na}$  current, dose is the bath concentration of the drug,  $IC_{50}$  is the concentration required for 50% current block, and  $n$  is the Hill coefficient.

### Pharmacokinetics

**Intraneural Lidocaine.** Nerve block was performed on 24 control and 25 diabetic rats using radiolabeled  $^{14}\text{C}$ -lidocaine obtained from American Radiolabeled Chemicals (USA). Nerves were harvested at 5, 10, 30, 60, and 90 min after injection. Animals with excision at 5 and 10 min were kept under anesthesia until tissue harvesting, and animals with excision at 30, 60, and 90 min were awakened and subjected to behavioral testing until tissue harvesting. Sciatic nerves were excised from a point proximal to the sciatic notch to the popliteal fossa guided by methylene blue injectate. Nerves were homogenized in 0.5 ml of Solvable tissue solubilizer (Solvable Packard Chemical Operations, The Netherlands), before being incubated with 5 ml of InstaGel scintillation solution (PerkinElmer, The Netherlands). The radioactivity was determined after 10 min by liquid scintillation counting (2000 CA; Packard, The Netherlands) and corrected for background activity. The measured radioactivity is expressed in disintegrations/min. The animals were assigned to treatment groups (duration) using a predefined random number list (random.org list randomizer, accession date May 20, 2015).

### Statistical Analysis

For single cell measurements, normality and equal variance assumptions were tested with the Kolmogorov–Smirnov and the Levene median test, respectively. Groups were compared with unpaired  $t$  test or, in case of a failed normality and/or equal variance test, Mann–Whitney rank sum test. Paired  $t$  tests were used to compare drug effects between groups of cells.

Data for motor blockade was not normally distributed. Therefore means and bias corrected and accelerated 95% CIs were derived after bootstrapping, drawing 1,000 samples of the same size as the original samples.  $P < 0.05$  was considered statistically significant.

For measurements of intraneural lidocaine concentration, data not normally distributed were log transformed. The data are presented in scatterplots on a log scale by time in min, as median (log) with the interquartile range (25 to 75 percentile). Box plots of the control arm and DM arm were constructed. The log transformed data were analyzed using a two-way ANOVA for repeated measurements. A *post hoc* subgroup analysis at the different time points was performed using the Welch  $t$  test for unequal variances to test for significant differences between the DM group and the control group. Considering the multiple testing of hypothesis ( $P$  values), we added Bonferroni adjusted  $P$  values for the 30- and 60-min levels. Therefore, we multiplied the unadjusted  $P$  values by the number of hypotheses tested, 5. If the Bonferroni adjusted  $P$  values are still less than the original  $\alpha$  set for the study (0.05), we rejected the null hypothesis. A two-sided  $P$  value of less than 0.05 was considered to be statistically significant.

### Sample Size Calculation

For the pharmacodynamic experiments (Dixon up-and-down method), no sample size calculation was applicable. We estimated between 10 and 20 subjects necessary to reliably determine minimum local anesthetic dose. Therefore, sample size was determined on basis of intraneural lidocaine experiments, and no corrections for further experiments were made for findings obtained during pharmacodynamic experiments. The number of test animals was determined on basis of earlier experiments<sup>18</sup> in which epinephrine was used to modify the intraneural concentration of local anesthetic. Because the effects of diabetic neuropathy and added epinephrine on nerve block duration are comparable<sup>14</sup> and no preliminary results concerning diabetic lidocaine kinetics were available, we used the same group size of 10 nerves/measurement time point used by Sinnott *et al.*<sup>18</sup> This translated into 5 animals injected bilaterally (*i.e.* 10 nerves) per time point of analysis, resulting in 25 diabetic and 25 control animals being tested. This would allow detection of a difference in intraneural concentration of 30% between healthy and diabetic animals, with a power of 80% and with an  $\alpha$  of  $P < 0.05$ , assuming a common SD of 20% and one lost specimen per group. In addition, this number would be high enough to allow for the pharmacodynamic experiments. Power analysis was done with the aid of nQuery Advisor 7.0 (Statistical Solutions Ltd., Ireland).

## Results

### Diabetic Model

At the time of the experiments, the diabetic animals were heavier and had markedly elevated plasma glucose concentrations (table 1). These findings were consistent between weeks 1 and 2. In addition, motor nerve conduction velocity was significantly slower in the diabetic group as compared to control animals at both time points (table 1). There was no significant change in nerve conduction velocity in either group between experiments in weeks 1 and 2.

### Nerve Block Duration

Motor block duration in both groups were obtained. The motor block duration was significantly extended in the diabetic rats as compared to the control group ( $P = 0.036$ ; fig. 1).

### Pharmacodynamics

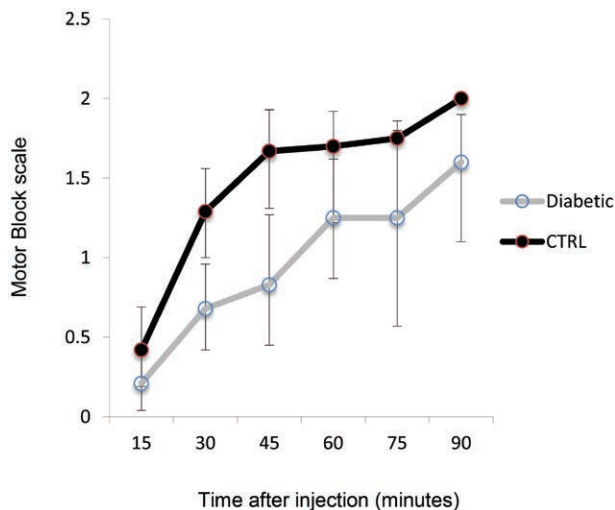
**In Vivo Minimum Local Anesthetic Dose.** Minimum local anesthetic dose  $\text{ED}_{50}$  was investigated in diabetic and control rats according to the Dixon up-and-down method. Diabetic animals showed a minimum local anesthetic dose  $\text{ED}_{50}$  for lidocaine-induced motor block of 0.9% compared to 1.4% in control animals ( $P < 0.018$ , CI 0.58 to 0.57) (fig. 2).

**In Vitro Electrophysiology.** Figure 3 shows basic AP characteristics and  $I_{\text{Na}}$  densities of freshly isolated control and DM DRGs with a size smaller than 25  $\mu\text{m}$ . Action potentials



**Table 1.** Animal Demographics (means  $\pm$  SD)

	Control	Diabetic	P Value
Week 1	n = 25	n = 25	
Weight (g)	344 $\pm$ 24	387 $\pm$ 50	< 0.001
Glucose (mmol/l)	10 $\pm$ 2	30 $\pm$ 4	< 0.001
Nerve conduction velocity (m/s)	44 $\pm$ 6	36 $\pm$ 4	< 0.001
Week 2	n = 24	n = 25	
Weight (g)	355 $\pm$ 24	372 $\pm$ 41	< 0.05
Glucose (mmol/l)	13 $\pm$ 4	30 $\pm$ 4	< 0.001
Nerve conduction velocity (m/s)	46 $\pm$ 10	38 $\pm$ 5	< 0.01



**Fig. 1.** Motor blockade scale over time (min). The values are shown as means with bias corrected and accelerated 95% CIs measured in diabetes mellitus (n = 25) and control (CTRL; n = 25). Motor block scores are noted as follows: 2, immediate and forceful reflex; 1, weak reflex; and 0, no reflex. All animals were tested before experiments and found to have no signs of sensory or motor deficit.

in response to 200-pA depolarizing pulses are shown in figure 3A.  $C_m$  was  $22 \pm 12.7$  pF (average  $\pm$  SD, n = 54), consistent with a previous finding in freshly isolated small diameter L4/L5 DRGs,<sup>19</sup> and did not differ significantly between Control and DM DRGs (fig. 3B). Average number of spikes and AP characteristics are shown in figure 3, C and D, respectively. DM DRG have more APs during depolarizing pulses compared to control DRGs. In addition, the maximum AP upstroke, dV/dt, was significantly higher, and the duration was significantly shorter in DM DRGs. Typical  $I_{Na}$  recordings and average  $I_{Na}$  densities are shown in figure 3, E and F. In DM DRGs, peak  $I_{Na}$  ( $I_{Na,t}$ ) was significantly greater than in control DRGs, due to a significant increase of both tetrodotoxin-resistant and tetrodotoxin-sensitive currents. The tetrodotoxin-resistant, *i.e.*, the  $I_{Na}$  recorded using the -50 mV prepulse, is rather small in both control and DM DRGs compared to the tetrodotoxin-sensitive  $I_{Na}$ , and the relative contribution of tetrodotoxin-sensitive is not altered due to DM ( $12 \pm 2.8\%$  (Control) and  $15.1 \pm 3.6\%$  (DM).

Thus, these experiments demonstrate that DM results in hyperexcitability, due to an increased density of  $Na^+$  currents, in freshly isolated DRGs, as described previously.<sup>19–21</sup>

To study whether DM changed the sensitivity of  $I_{Na}$  to blockade by lidocaine, a concentration–response curve was obtained by using concentrations between 1 and 1,000  $\mu$ M. Because the relative contribution of tetrodotoxin-sensitive is small and not changed by DM (fig. 3E), we focused on the inhibition of total  $I_{Na}$ , rather than on separate dose–response curves of tetrodotoxin-resistant and tetrodotoxin-sensitive currents. Figure 4A shows typical peak  $I_{Na}$  recordings in the absence and presence of different lidocaine concentrations; average effects are summarized in figure 4B. We found a significantly lower  $IC_{50}$  in DM compared to control DRGs, whereas the Hill coefficients were not significantly different. The higher  $I_{Na}$  sensitivity for lidocaine in DM DRGs suggests that the reduction of the action potential upstroke will be greater. To address this issue, we studied the effects of 10  $\mu$ M lidocaine on action potentials. Figure 5A shows typical action potentials during 200-pA depolarizing pulses in the absence of lidocaine (Control), in the presence of lidocaine, and upon washout of the drug. Lidocaine reversibly decreased the action potential upstroke in both control and DM DRGs, but the magnitude of reduction was significantly higher in DM DRGs (fig. 5B). In addition, the AP overshoot was significantly decreased in DM, but not in control, DRGs (fig. 5B). The slower AP upstroke velocity resulted in a significantly increased action potential duration ( $APD_{50}$ ) in DM DRGs (fig. 5B). Finally, we found a significantly lower number of spikes in diabetic neurons as lidocaine was added (fig. 5C). These single cell measurements demonstrate that DM DRGs have a higher sensitivity to lidocaine.

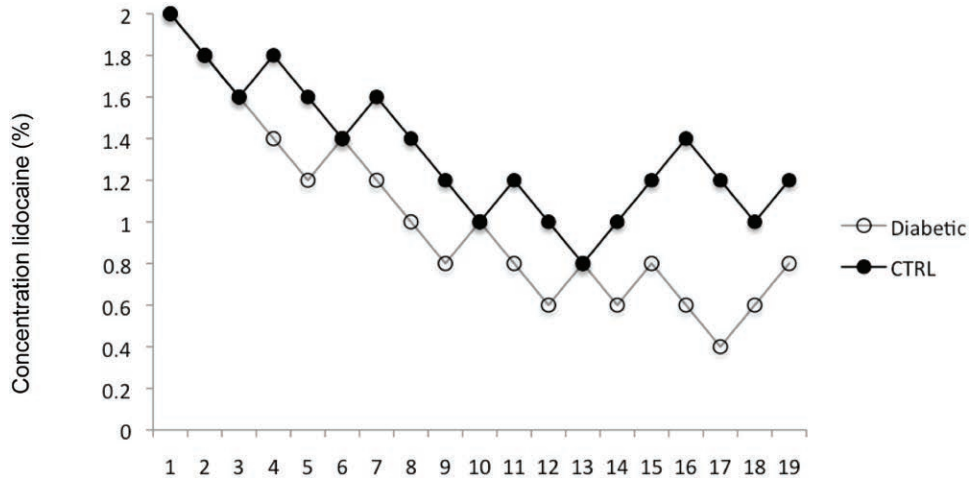
**Intraneural Lidocaine.** Although the absorption of radiolabeled lidocaine into the sciatic nerve at 5 min was similar between diabetic and control animals, we observed that at 60 min the neural content in nerves of control animals was (mean log) 5.4 *versus* 7.1 in diabetic animals ( $P = 0.005$ , CI -2.88 to 0.52). The results are given in figure 6.

## Discussion

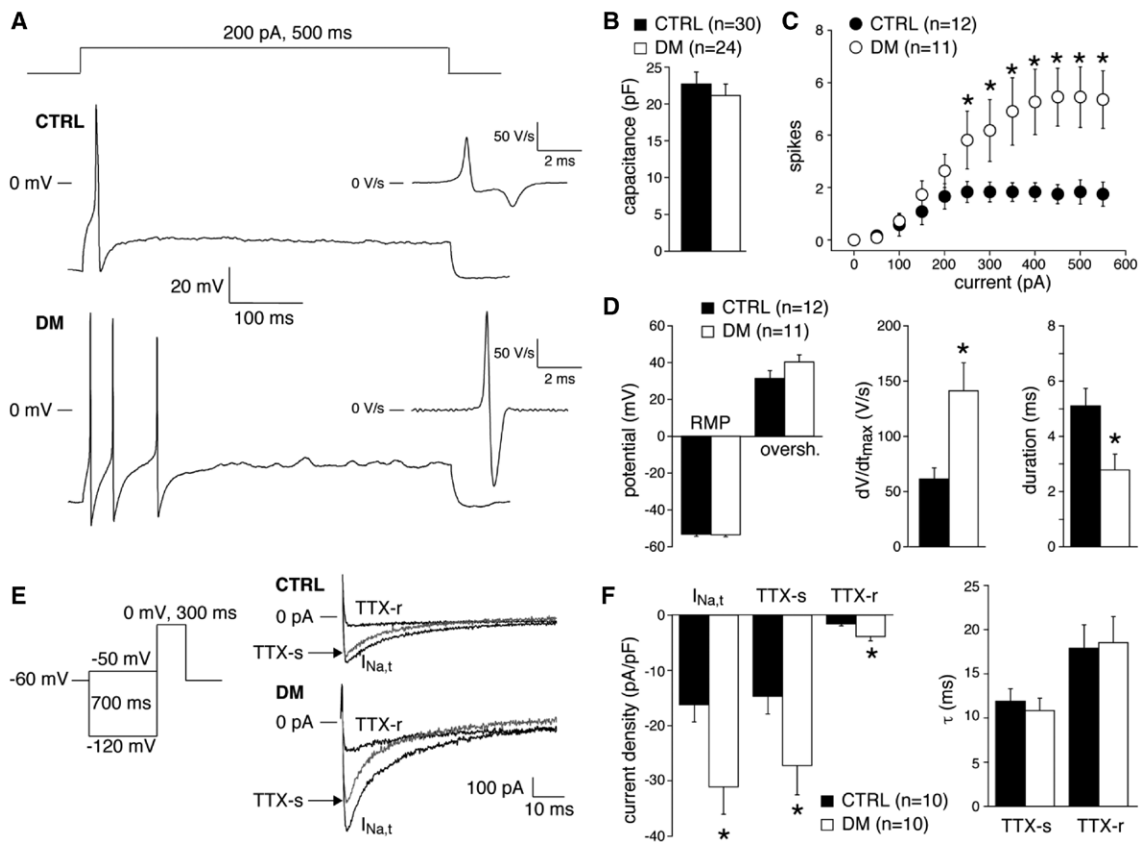
We demonstrate that both pharmacodynamic and pharmacokinetic mechanisms contribute to the overall phenomenon of prolonged nerve block duration.

### Sodium Channels in Diabetic Neuropathy

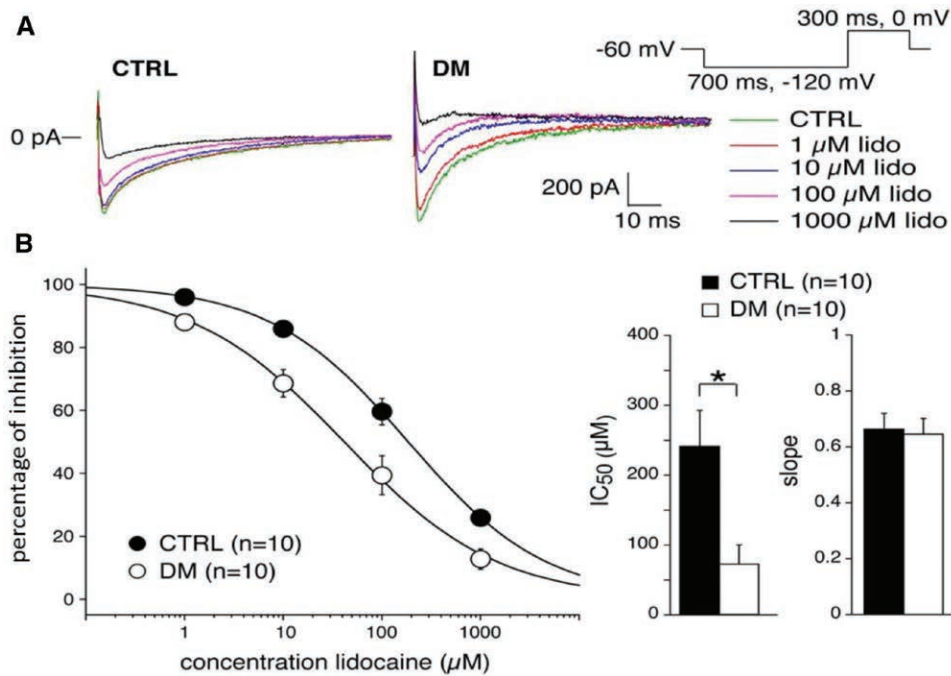
Next to being the main pharmacologic target of local anesthetics, sodium channels play a substantial role in the pathophysiology of diabetic neuropathy. On a neuronal level, the expression of specific sodium channels is differentially influenced during diabetic neuropathy. Literature has been undecided on the exact pattern of sodium channel alterations. In general, an increase in sodium currents is reported, and this increase correlates with progression of neuropathy in experimental models.<sup>6</sup> Depending on the type of neurons investigated, and even



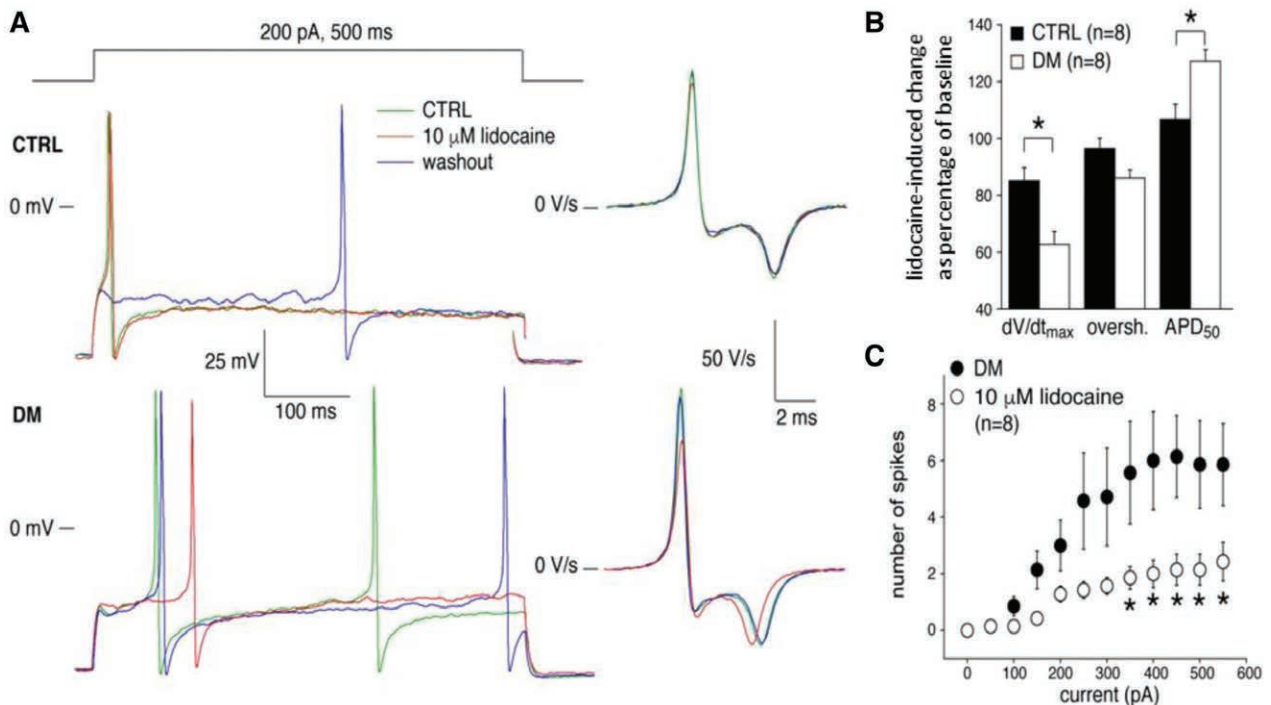
**Fig. 2.** *In vivo* minimum local anesthetic dose of lidocaine in sciatic nerve block. Minimum local anesthetic dose was investigated in diabetic and control (CTRL) rats according to the Dixon up-and-down method. Nineteen diabetic and nineteen control rats were necessary to determine the minimum local anesthetic dose. All animals were tested before experiments and found to have no signs of motor deficit, and they recovered fully after experiments.



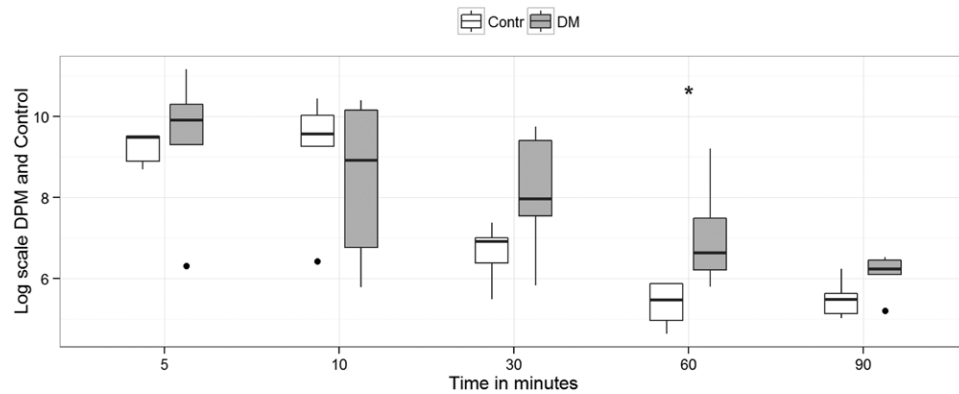
**Fig. 3.** Action potential characteristics of freshly isolated dorsal root ganglia under basal conditions. (A) Examples of action potentials recorded from a control (CTRL) and diabetic mellitus (DM) dorsal root ganglion (DRG) during a 200-pA, 500-ms-long depolarizing pulse. (Inset)  $dV/dt$  of the first action potential. (B) Average (SD) cell capacitance of CTRL ( $n = 30$ ) and DM ( $n = 24$ ) ganglia. (C) Average (SD) amount of spikes during depolarizing pulses of increasing amplitudes in CTRL ( $n = 12$ ) and DM ( $n = 11$ ) ganglia. (D) Average (SD) action potential characteristics of CTRL ( $n = 12$ ) and DM ( $n = 11$ ) DRGs during a 200-pA depolarizing pulse. Asterisks indicate adjusted significant differences between control and DM ganglia. (E) Typical  $Na^+$  current ( $I_{Na}$ ) in a CTRL and DM dorsal root ganglia recorded with the depicted protocol. Total sodium current  $I_{Na}$  ( $I_{Na,t}$ ) was recorded using the prepulse to  $-120$  mV, whereas tetrodotoxin-resistant (TTX-r) was defined as  $I_{Na}$  recorded using the prepulse to  $-50$  mV. Tetrodotoxin-sensitive (TTX-s) was obtained by subtracting TTX-r from  $I_{Na,t}$ . (F) Average current densities of CTRL ( $n = 10$ ) and DM ( $n = 10$ ) DRGs and time constants ( $\tau$ ) of current inactivation obtained by monoexponential fits. The data are given as mean  $\pm$  SD.



**Fig. 4.** Concentration dependence of  $I_{Na}$  block by lidocaine in control and diabetic dorsal root ganglia. (A) Typical  $I_{Na}$  recordings in control (CTRL) and diabetic mellitus (DM dorsal root ganglia in absence and presence of 1 to 1,000  $\mu\text{M}$  lidocaine. (Inset) Voltage clamp protocol. (B) Average effects of lidocaine in control ( $n = 10$ ) and DM ( $n = 10$ ) dorsal root ganglia. Please note that the currents are normalized to the current before application of the drug. Solid lines indicate Hill equation fits of average data. The data are given as mean  $\pm$  SD.



**Fig. 5.** Effects of 10  $\mu\text{M}$  lidocaine on action potential characteristics. (A) Examples of action potentials during a 200 pA depolarizing pulse in absence of lidocaine, presence of lidocaine, and upon washout of the drug. (Inset)  $dV/dt$  of the first action potential demonstrating a reversible decreased of the action potential upstroke in response to lidocaine. (B) Average (SD) effects of lidocaine on the action potential upstroke (left), action potential overshoot (middle) and action potential duration (right) in control (CTRL) and diabetic mellitus (DM) dorsal root ganglia. (C) Average (SD) number of spikes during depolarizing pulses of increasing amplitudes in DM ( $n = 8$ ) ganglia in absence and presence of lidocaine.



**Fig. 6.** Intraneural lidocaine  $^{14}\text{C}$  concentration over time. Intraneural lidocaine  $^{14}\text{C}$  concentration box plot given as median (log) (interquartile range, 25 to 75 percentile) values: diabetic mellitus (DM) versus control (Contr;  $n = 10$  measurements per time point). Asterisks indicate adjusted significant difference between CTRL and DM, and a dot signifies a data outlier, defined as a data point that is located outside 1.5 times the interquartile range above the upper quartile and below the lower quartile. DPM = disintegrations/min.

on the specific location within the neuronal membrane, a variety of changes has been demonstrated. For example, although expression studies suggest that globally,  $\text{Na}_v1.8$  may be decreased in diabetic dorsal root ganglia,<sup>5</sup> others have observed no decrease in  $\text{Na}_v1.8$ -mediated currents<sup>21</sup> or even an increase.<sup>20</sup> Notably, the latter increase was found in small-sized neurons, comparable to those investigated here. Adding to the complexity, Hong and Wiley<sup>21</sup> further described that diabetic neuropathy also leads to changes in the spatial distribution of sodium channel isoforms, with the nodal regions featuring decreased levels of  $\text{Na}_v1.6$  and  $\text{Na}_v1.8$ . An increase in the current density measured in cell soma coupled with a decrease in axonal (nodal)  $\text{Na}^+$  currents suggests that transport of channels down the axon may be impaired in diabetic animals. In addition, this finding may reconcile our findings of reduced nerve conduction velocity and increased excitability, because these two effects were measured at separate locations, the cell body versus the axon, which are differentially affected by neuropathic changes. Finally, expressed channels may be further modified in their functionality, e.g. inactivation, by secondary mechanisms such as methylglyoxal modification,<sup>22</sup> as well as serine-threonine or tyrosine phosphorylation.<sup>23</sup>

On the neuroanatomical level, diabetic neuropathy is characterized by the loss of nerve fibers, predominantly of unmyelinated sensory axons, in the setting of hyperexcitability. This leads to the unique combination of negative symptoms (loss of sensitivity to touch, pinprick, and temperature) and positive symptoms (hyperalgesia, allodynia, and ongoing pain)<sup>24</sup> combined with motor deficit.<sup>25</sup>

The clinical relevance of these changes for regional anesthesia is underlined by a recent report correlating a prolonged stimulus strength-duration time constant, which is strongly indicative of sodium channel (dys)function, with severity of neuropathy and decreased quality of life in diabetic neuropathic patients.<sup>26</sup>

### Pharmacodynamics

We report that the *in vivo*  $\text{ED}_{50}$  of lidocaine for motor blockade using a fixed volume was 1.4% in control and 0.9% in diabetic animals. The difference is significant and similar in magnitude to differences in local anesthetic requirement as a result of, for example, pregnancy.<sup>27</sup> This indicates that diabetic neuropathic nerves are more sensitive to local anesthetics. This finding was corroborated by *in vitro* investigations of sodium channel currents, which showed that the inhibitory effect of a given lidocaine concentration was much more pronounced in primarily sensory neurons harvested from diabetic as compared to control neurons. Diabetic DRGs are hyperexcitable as reflected by the increased number of action potentials during depolarizing pulses compared to control DRGs (fig. 3), but they are also susceptible to silencing by local anesthetics (fig. 5). In addition, the action potential upstroke and the current density of total  $I_{\text{Na}}$  was significantly higher in DM DRGs, due to upregulation of both tetrodotoxin-sensitive and tetrodotoxin-resistant  $I_{\text{Na}}$  (fig. 3). These findings correspond well to previous literature<sup>20</sup> and tie in with evidence from neurologic investigations demonstrating that nerve excitability is heightened in diabetic neuropathy.<sup>28</sup>

Pathologic alterations seem particularly prominent in nodal regions.<sup>29</sup> All of this leads to a functional change in the axon that is reflected by a decrease in the strength-duration time constant, a measure of axonal membrane excitability, and an increase in rheobase, the minimal strength of a stimulus of indefinite duration to depolarize the axon.<sup>28</sup> The latter change may, at least in part, explain the increased threshold for peripheral nerve stimulation in diabetic neuropathy observed experimentally<sup>30</sup> and perioperatively.<sup>31,32</sup> The net effect concerning nerve blockade seems to be that the axonal membrane of diabetic neuropathic nerves is less excitable by electrical stimulation than normal membranes and that it is at the same time more sensitive to local anesthetics. Therefore, in diabetic neuropathy, a smaller dose of a local anesthetic may achieve the same result as a larger dose in a healthy



nerve. Reduction of doses in diabetic neuropathic patients has been suggested<sup>1</sup> and tested in a small clinical trial,<sup>33</sup> but until now, this has not been systematically investigated in clinical trials. Our experimental setup would support these contentions by showing that the *in vivo* minimum local anesthetic concentration was decreased by 35%, and the sensitivity to lidocaine during patch clamp experiments was increased in diabetic neuropathy.

The blockade of Na<sup>+</sup> channels by lidocaine can be complex,<sup>34</sup> and various DM-induced changes in Na<sup>+</sup> channel properties may contribute to our observed increased lidocaine sensitivity. Lidocaine can block the sodium channels in inactivated and resting state, thereby resulting in phasic (or use-dependent) and tonic blockade, respectively.<sup>34</sup> Typically, the IC<sub>50</sub> is lower for phasic lidocaine block compared to tonic blockade in both tetrodotoxin-resistant<sup>34</sup> and tetrodotoxin-sensitive<sup>35</sup> currents. Diabetes or high glucose levels result in slower recovery from inactivation, a negative shift in voltage dependence of inactivation,<sup>19,21,36,37</sup> all conditions that thus promote phasic lidocaine blockade and tonic block, when the membrane is at its resting potential, -60 mV in sensory neuron soma. The firing rate during depolarizing current pulses is significantly increased in DM, which will further promote use-dependent block by lidocaine. In addition and as mentioned before, DM results in an increase of various tetrodotoxin-sensitive and tetrodotoxin-resistant Na<sup>+</sup> channels. Tetrodotoxin-sensitive Na<sup>+</sup> channels are approximately five times more sensitive to lidocaine than tetrodotoxin-resistant for tonic block,<sup>34,38</sup> and the changes in the various isoforms may thus affect the lidocaine sensitivity. Moreover,  $\beta$ -subunits of Na<sup>+</sup> channels importantly modulate I<sub>Na</sub> densities and gating properties, and it is well known that they also affect lidocaine sensitivity, with a more pronounced block as  $\beta$ -subunits decrease.<sup>39-41</sup> DM increases the  $\beta$ 3 but does not change the  $\beta$ 1-subunit.<sup>42</sup> Due to the increase in  $\alpha$ -subunits, the ratio between  $\beta$ 1- and  $\alpha$ -subunits becomes lower, and we cannot exclude this as potential mechanism for the increased lidocaine sensitivity. Finally, DM results in multiple biochemical changes that may have an impact on lidocaine sensitivity. For example, DM increases protein kinase C/cyclic adenosine monophosphate in DRGs,<sup>19</sup> and cyclic adenosine monophosphate lowers the IC<sub>50</sub> for lidocaine in tetrodotoxin-resistant channels.<sup>43</sup> Thus, multiple mechanisms may be responsible for the observed increased lidocaine sensitivity. Further research should determine detailed dose-response curves in diabetic *versus* control animals, specifically investigating whether these alterations are found across different neuronal subgroups, are particular to specific sodium currents, and are different with various local anesthetics.

### Pharmacokinetics: Intra-neural Lidocaine

Another potential cause for the prolonged block duration may be impaired nerve blood flow in diabetic neuropathy.<sup>44</sup> This has been demonstrated for type 1 DM,<sup>45</sup> and

similar findings have been obtained in models of type 2 DM.<sup>7</sup> Further, blood flow is also reduced in autonomic and dorsal root ganglia.<sup>44</sup> The main pathogenic mechanisms is diabetes-induced vasa nervorum endotheliopathy,<sup>44</sup> and the net effect is a substantial decrease in axonal flow unit. Cameron *et al.*,<sup>45</sup> for example, found a reduction in blood flow of approximately 40% when measured using microelectrode polarography and hydrogen clearance in STZ rats. We demonstrated that the content of radiolabeled lidocaine was higher in the diabetic nerve than in control nerves at 60 min, which may at least contribute to the prolonged block duration in diabetic nerves. The time point at 60 min is the most important one in the measurement series, because it is the time point when functional deficits from motor block have typically recovered in healthy nerves, whereas most diabetic nerves are still blocked.<sup>14</sup>

### Limitations

Our model is not directly comparable to some previous investigations by other authors because we used type 2 DM animals, whereas most others used type 1 DM animals.<sup>13,46-48</sup> However, we believe that our methodology better reflects the growing patient collective of type 2 diabetics with neuropathy presenting for surgery.<sup>49</sup> We performed our experiments using the local anesthetic lidocaine, because this has been the most widely used drug when diabetic neuropathy was investigated. Last, we did not take active steps to blind experimenters to the experimental group allocation during *in vivo* experiments, because the diabetic animals were much more obese than the control animals, and therefore group allocation was immediately visible. To counteract this potential bias, we randomized animals to group allocation whenever possible.

### Conclusions

In a rodent model of regional anesthesia in animals with neuropathy secondary to type 2 Diabetes, we have observed increased nerve block duration, as previously described. Further, dose-finding and electrophysiologic experiments suggest that diabetic neuropathic neurons and nerves are blocked at lower concentrations of lidocaine than healthy nerves. Last, we report that at 60 min after nerve block, intraneural lidocaine is less in healthy than in diabetic nerves. Within the limitations of our preclinical model, our results support both pharmacodynamic and pharmacokinetic mechanisms to explain increased block duration with diabetic neuropathy.

### Acknowledgments

The authors thank Gary Strichartz, Ph.D., Harvard Medical School, Boston, Massachusetts, for his expert advice. We acknowledge the expert help of Susanne van Dieren, Ph.D., and Jan Binnekade, Ph.D., with statistical advice.

### Research Support

Support was provided solely from institutional and/or departmental sources.

## Competing Interests

The authors declare no competing interests.

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