Impaired Hedgehog signalling-induced endothelial dysfunction is sufficient to induce neuropathy: implication in diabetes

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Aims Microangiopathy, i.e. endothelial dysfunction, has long been suggested to contribute to the development of diabetic neuropathy, although this has never been fully verified. In the present paper, we have identified the role of Hedgehog (Hh) signalling in endoneurial microvessel integrity and evaluated the impact of impaired Hh signalling in endothelial cells (ECs) on nerve function.

Methods and results By using Desert Hedgehog (Dhh)-deficient mice, we have revealed, that in the absence of Dhh, endoneurial capillaries are abnormally dense and permeable. Furthermore, Smoothened (Smo) conditional KO mice clarified that this increased vessel permeability is specifically due to impaired Hh signalling in ECs and is associated with a down-regulation of Claudin5 (Cldn5). Moreover, impairment of Hh signalling in ECs was sufficient to induce hypoalgesia and neuropathic pain. Finally in Leprdb/db type 2 diabetic mice, the loss of Dhh expression observed in the nerve was shown to be associated with increased endoneurial capillary permeability and decreased Cldn5 expression. Conversely, systemic administration of the Smo agonist SAG increased Cldn5 expression, decreased endoneurial capillary permeability, and restored thermal algesia to diabetic mice, demonstrating that loss of Dhh expression is crucial in the development of diabetic neuropathy.

Conclusion The present work demonstrates the critical role of Dhh in maintaining blood nerve barrier integrity and demonstrates for the first time that endothelial dysfunction is sufficient to induce neuropathy.

Keywords Desert hedgehog • Blood nerve barrier • Permeability • Diabetic neuropathy

1. Introduction

Diabetic neuropathy, which results in a serious decline in patient quality of life and can lead to a number of severe complications including limb amputation and the Charcot foot, is one of the main complications of diabetes with 75% of diabetics suffering from it. Unfortunately, no treatment for neuropathy exists save keeping blood sugar within acceptable levels or treatment of symptoms. Diabetic neuropathy is classified among diabetes-associated microangiopathies; however, the contribution of neuropathy-associated microangiopathy to the development of the disease has not been fully demonstrated. Two hypotheses are proposed to explain the aetiology of diabetic neuropathy: the metabolic theory, in which neural cell function is initially impaired, and the vascular theory, in which diabetic microangiopathy is suggested to promote neuropathy.1

Diabetes-associated nerve microangiopathies are characterized by a vessel wall thickening2–6 together with an increased vasoconstriction7 leading to a deleterious decreased tissue perfusion. Accordingly, vasodilators (angiotensin-converting enzyme inhibitors, angiotensin II receptor antagonists)5 or pro-angiogenic factors [VEGFA, Angiopoitein-1 (Angpt1)]8,9 have been proposed to treat peripheral neuropathy. Nevertheless, few studies have reported that more advanced stages of neuropathy-associated microangiopathy are associated with increased capillary density within the nerves,5,10,11 increased endoneurial vessel
permeability, suggesting that the proposed "pro-angiogenic" strategies to treat microangiopathy in nerves might not be suitable at every stage of diabetic neuropathy.

Desert Hedgehog (Dhh) belongs to the Hh family of morphogens together with Shh and Indian hedgehog (Ihh). This family of proteins was identified nearly three decades ago in drosophila as a crucial regulator of cell fate determination during embryogenesis. Dhh is mainly expressed in the peripheral nerves and male gonads. In peripheral nerves, Dhh is expressed by myelinating Schwann cells and has been shown to orchestrate the organization of nerve sheaths (i.e. Epineurium, Perineurium, and Endoneurium) by signalling to perineurial cells. In Dhh knockout mice, epineurial collagen is reduced; the perineurium is thin and disorganized and fails to express connexin 43, the basal lamina is patchy. Moreover, administration of Shh was shown to improve nerve function in Diabetic rats.

The role of Dhh is the least documented of the Hh morphogens. During development, Dhh has been shown to be expressed in the endothelium of large vessels. In adults, the Hh pathway has been shown to be reactivated after ischaemic injury, including hind limb ischaemia (HLI) and myocardial infarction. Administration of Shh, as either recombinant protein or via gene therapy, has been shown to promote neovascularization of ischaemic tissues by encouraging angiogenesis and endothelial progenitor cell recruitment. We found that endogenous Dhh is an essential actor of Hh-dependent ischaemia-induced angiogenesis by stimulating peripheral nerve survival and subsequent nerve-driven angiogenesis.

We hypothesize that the decrease in Dhh expression observed in diabetic nerve contributes to the development of neuropathy, at least in part, through its action on vasa nervorum. The objective of the present study is to characterize the specific role of Dhh in vasa nervorum homeostasis and to determine whether the loss of Dhh may contribute to the physiopathology of microangiopathy and development of neuropathy.

2. Methods

2.1 Mice

C57BL/6 and C57BLKS-m Lepr+/+ mice were obtained from Charles River Laboratories and bred in our animal facility. C57BLKS-m Lepr+/+ mice were bred together to obtain Lepr+/+ mice. Dhh+/−/− mice and Smoothenoned Floxed (Smo+/−) mice were obtained from the Jackson laboratory. Dhh+/−/− mice were bred together to obtain Dhh+/−/− mice and WT control mice. Pad6b-CreERT2 mice were kindly donated by M. Fruttiger and bred with Smo+/− mice. Rosa26R mice were obtained from the Jackson laboratory. Mice were handled in accordance with guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and approved by the local Institutional Animal Care and Use Committee. Cre recombinase was activated by intra-peritoneal (IP) injection of 1 mg tamoxifen (Sigma-Aldrich) for 5 consecutive days, and the phenotype was assessed 4 weeks later. Mice were sacrificed by cervical dislocation.

2.2 Clodronate treatment

Prolonged peripheral macrophage depletion was obtained by IP injection of 100 μL Clodronate liposome suspensions (ClodronateLiposomes.com, Haarlem, The Netherlands) every 5 days for 3 weeks. Control mice received 100 μL PBS liposome suspensions. Clodronate and PBS liposomes were resuspended at room temperature before injection.

2.3 In vivo permeability assay

To quantify endoneurial capillary permeability, mice were injected with 100 μL 1% Evans blue in the tail vein, 3 h before they were sacrificed. Microscopic visualization of Evans blue extravasation was done on paraffin-embedded sciatic nerve cross-sections. Alternatively, mice were administered with 50 μL, 150 kDa, 70 kDa, or 10 FITC-Dextran (25 mg/mL) 30 min before they were sacrificed. FITC-dextran extravasation was evaluated from whole mount sciatic nerves.

2.4 Behavioural tests

Mice were placed in individual clear acrylic box with a wire mesh floor and acclimated for 20 min. Tactile responses were evaluated by quantifying the withdrawal threshold of the hind paw in response to stimulation with flexible Von Frey filaments. The mechanical withdrawal threshold was measured by applying a series of eight calibrated Von Frey filaments (Bioseb) to the mid-plantar surface of the foot.

Mice were placed in individual clear acrylic box on a glass plate and acclimated for 20 min. The hind paw withdrawal latency in response to infrared heat stimulus was determined with Plantar test (Bioseb). The baseline was set between 3 and 7 s by adjusting the heat source intensity (90 one-digit steps), and an automatic 15 s cut-off time was used to prevent tissue damage.

2.5 X-gal staining

For X-gal staining, mice were euthanized by IP injection of Pentobarbital 150 mg/kg, then perfused with LacZ fix solution (PBS containing 0.2% glutaraldehyde, 5 mmol/L EGTA, and 100 mmol/L MgCl2) before sciatic nerve were harvested. Nerves were stained overnight at 37°C in X-gal staining solution (1 mg/mL X-gal (Sigma)).

2.6 Immunostaining

ECs were identified with rat anti-CD31 antibodies (BD Biosciences, Le Pont de Claux, France). Smooth muscle cells were identified with mouse anti-smooth muscle α-actin (Sigma-Aldrich, Saint-Quentin Fallavier, France). GFP was identified with anti-GFP antibodies (Molecular Probes). Mannose receptor, C type 1 (MrC-1), and VEGFA were stained using goat-anti-Mrc-1 antibodies (R&D Systems) and rabbit anti-VEGFA antibodies (Abcam), respectively. ClodN was identified with rabbit anti-Cldn5 antibodies (Invitrogen). For immunofluorescence analyses, primary antibodies were incubated with Alexa-Fluor-conjugated secondary antibodies (Invitrogen) and nuclei were counterstained with DAPI (1/5000). For immunohistochemical analyses, primary antibodies were sequentially stained with biotin-conjugated secondary antibodies (Vector, Laboratories) and streptavidin-horseradish peroxidase (HRP) complex (Amersham), then the stain was developed with a diaminobenzidine (DAB) substrate kit (Vector); tissues were counterstained with haematoxylin.

2.7 Quantitative RT–PCR

RNA was isolated by using Tri Reagent® (Molecular Research Center Inc.) as instructed by the manufacturer. For quantitative RT–PCR analyses,
total RNA was reverse transcribed with M-MLV reverse transcriptase (Promega), and amplification was performed using B-R SYBER Green SuperMix (Quanta Biosciences). The relative expression of each mRNA was calculated by the comparative threshold cycle method and normalized to hypoxanthine-guanine phosphoribosyltransferase (HPRT) mRNA expression. Dhh mRNA was amplified using the following primers: 5′-C TTGGAACATACCGACGTCCTG-3′ and 5′-ATGTAATTTGCTCAAGCC T-3′, Gli1 mRNA, with 5′- GAAGAAATCTTGCTGCCATT-3′ and 5′-GCAACCTTCTGTCACACA-3′, Ptc1 mRNA, with 5′-CTC AGGCGAAACGAAACACA-3′ and 5′-GACAGAAAGCCAGTGTC AG-3′, Hhip mRNA, with 5′-AGCGCTAAAAGAGAAGGGAC-3′ while Cldn5 was immunoprecipitated with mouse anti-Cldn5 antibodies (5E1, Developmental Studies Hybridoma Bank) respectively. Phosphorylation of Akt was evaluated by performing SDS–PAGE using rabbit anti-Hh antibodies (Santa Cruz Biotechnology), while Cldn5 was immunoprecipitated with mouse anti-Cldn5 antibodies (5E1, Developmental Studies Hybridoma Bank). Prior to western blot analysis, Hh proteins were immunoprecipitated with mouse anti-Hh antibodies (Abcam), rabbit anti-VEGFA antibodies (Invitrogen), and rabbit anti-laminin antibodies (R&D systems) rabbit anti-VEGFA antibodies (Abcam), rabbit anti-Cldn-5 antibodies (Dako), respectively. Phosphorylation of Akt was evaluated by performing SDS–PAGE with anti-Akt1 and anti-phospho-Akt (Cell signaling) antibodies. Equal protein loading was confirmed using monoclonal anti-α-tubulin antibodies (Sigma-Aldrich).

2.8 Immunoprecipitation/western blot
Prior to western blot analysis, Hh proteins were immunoprecipitated with mouse anti-Hh antibodies (SE1, Developmental Studies Hybridoma Bank) while Cldn5 was immunoprecipitated with mouse anti-Cldn5 antibodies (Invitrogen).

Expression of total Hh, Dhh, VEGFA, and laminin was evaluated by SDS–PAGE using rabbit anti-Hh antibodies (Santa Cruz Biotechnology), goat-anti Dhh antibodies (R&D systems) rabbit anti-VEGFA antibodies (Abcam), rabbit anti-Cldn-5 antibodies (Invitrogen), and rabbit anti-laminin antibodies (Dako), respectively. Phosphorylation of Akt was evaluated by performing SDS–PAGE with anti-Akt1 and anti-phospho-Akt (Cell signaling) antibodies. Equal protein loading was confirmed using monoclonal anti-α-tubulin antibodies (Sigma-Aldrich).

2.9 Cell culture
In vitro experiments were performed with human umbilical vein endothelial cells (HUVECs) (Promocell). HUVECs were cultured in endothelial cell growth medium-2 (EGM-2) with EGM-2 SingleQuot Kit Supplement and growth factors (Lonza).

2.10 Lentivirus/transduction
The CDNA encoding the N terminal part of Dhh (NDhh) was digested with NheI and HpdI from pRRES-NDhh27 and cloned into a pRRL Sin.CPT MND lentiviral plasmid (Tronolab) after NheI and PmlI digestion. Lentiviral particles were produced at the ‘plateforme de vectorologie’ of Bordeaux University.

HUVECs were transduced via an overnight incubation in medium containing lentiviruses at a concentration yielding a MOI of 20. Successful transduction was verified by measuring GFP expression.

2.11 Statistics
Results are reported as mean ± S.E.M. Comparisons between two groups were analysed for significance with the two-tailed unpaired Student’s t-test (when n = 3–4) or with the Mann–Whitney test (when n ≥5) (using XLSTAT software). For comparisons between >2 groups, the Kruskal–Wallis test, followed by a Dunn’s post hoc test was made (using Anatasat software). Differences between groups were considered to be significant when P ≤0.05.

3. Results
3.1 Dhh knockdown induces sciatic nerve endoneurial vessel perturbations
We used Dhh-deficient mice (Dhh+/− and Dhh+/+) to investigate whether Dhh knockdown (see Supplementary material online, Figure S1A) is sufficient to induce microangiopathy. We first verified that Dhh+/− and Dhh−/− mice develop neuropathy as suggested by developmental studies.16 Dhh+/− and Dhh−/− mice demonstrated both hypalgesia (measured with the Plantar test) and neuropathic pain (allogednia evaluated with Von Frey filaments) which were inversely correlated with Dhh expression level (see Supplementary material online, Figure S1B and C).

The blood vessel network of Dhh-deficient nerves was examined after both in tato CD31 staining of vasa nervorum (Figure 1A) and CD31 staining of sciatic nerve cross-sections (Figure 1B). In Dhh+/− mice, endoneurial microvessel density was significantly increased while it did not differ significantly between WT and Dhh−/− mice (Figure 1C). Furthermore, the average endoneurial lumen vessel diameter was larger in Dhh+/− mice compared with Dhh+/+ and WT mice (Figure 1B and D).

We quantified endoneurial vessel permeability by measuring Evans Blue extravasation in the sciatic nerve of Dhh-deficient mice and of their WT control littermates. Endoneurial microvessel permeability was increased in both Dhh+/− and Dhh−/− mice compared with WT mice, and vessel permeability was inversely correlated with Dhh expression level (Figure 1E and F).

These data demonstrate that Dhh deficiency is sufficient to induce endoneurial blood vessel network anomalies, including increased permeability, vasodilatation, and abnormal vessel density, demonstrating that Dhh is necessary for vasa nervorum homeostasis.

3.2 Dhh signalling to ECs is necessary to maintain BNB integrity
Because Hh ligands were shown to regulate EC function both in vitro31,34 and in vivo,23 we investigated whether the vasa nervorum phenotype of Dhh-deficient mice was due to impaired Hh signalling in ECs. The direct action of Dhh on ECs was investigated using Pdgfb-CreERT2;SmoFlox/Flox mice in which Hh signalling is specifically disrupted in ECs. The activity of the Cre recombinase in the endothelium of vasa nervorum was verified by X-gal whole mount staining of Pdgfb-CreERT2; Rosa26R sciatic nerves (see Supplementary material online, Figure S2A) while the endothelial specificity of the Pdgfb promoter was verified via GFP staining of Pdgfb-CreERT2 nerve cross-sections (see Supplementary material online, Figure S2B–D).

Vasa nervorum of Pdgfb-CreERT2;SmoFlox/Flox mice exhibit an endoneurial vascular phenotype which present no modification in vascular density (Figure 2A and B) and no perturbation of the average endoneurial microvessel diameter (Figure 2C). However, we found that endoneurial Evans Blue extravasation was increased in Pdgfb-CreERT2;SmoFlox/Flox nerve compared with their SmoFlox/Flox control littermates (Figure 2D). Furthermore, we found that endoneurial Evans Blue extravasation was increased in Pdgfb-CreERT2;SmoFlox/Flox nerve with compared with their SmoFlox/Flox control littermates (Figure 2D). Furthermore, we found that endoneurial Evans Blue extravasation was increased in Pdgfb-CreERT2;SmoFlox/Flox nerve compared with their SmoFlox/Flox control littermates (Figure 2D). Furthermore, we found that endoneurial Evans Blue extravasation was increased in Pdgfb-CreERT2;SmoFlox/Flox nerve compared with their SmoFlox/Flox control littermates (Figure 2D).

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We further characterized the phenotype of Smo-deficient endoneurial endothelium by measuring its permeability to different sized tracers. To this aim, Pdgfb-CreERT2;SmoFlox/Flox mice and their control littermates were administered 150, 70, or 10 kDa FITC-Dextran intravenously. As shown in Figure 2F, endoneurial capillaries of Pdgfb-CreERT2;SmoFlox/Flox mice were permeable to molecules up to 150 kDa.

3.3 BNB permeability is associated with Cldn5 down-regulation
BNB permeability is dependent on the expression of cell–cell junction proteins including tight junction proteins Cldn5 and Occludin that have been shown to be regulated by Shh in the brain.23 We measured the expression of Cldn5 in the sciatic nerve of Pdgfb-CreERT2;SmoFlox/Flox and SmoFlox/Flox mice by immunostaining of nerves and
immunoprecipitation assays from nerve total protein extracts. As shown in Figure 3A and B, Cldn5 expression (in green) was weaker in endoneurial vessels of Pdgfb-CreERT2; Smoflox/flox compared with control mice; Cldn5 down-regulation in the nerves of Pdgfb-CreERT2; Smoflox/flox was confirmed by immunoprecipitation assays followed by western blot analyses (Figure 3C and D).

Altogether those results show that BNB permeability induced by the loss of Hh signalling in ECs is associated with Cldn5 down-regulation. To investigate the mechanisms by which Dhh regulates Cldn5 expression, we first evaluated whether the loss of Dhh or the loss of Hh signalling in ECs affected Hh canonical signalling in the sciatic nerve. As shown in Supplementary material online, Figure S3A–F, the Hh transcriptional targets Gli1 and Hhip were modulated neither in Dhh2/2 nerves nor in Pdgfb-CreERT2; Smoflox/flox nerves strongly suggesting that Dhh does not activate Hh canonical signalling in adult sciatic nerves. Of note, Ptc1 mRNA was significantly down-regulated in Dhh-deficient nerves compared with WT nerves. This likely reflects the absence of perinerve as a consequence of the characteristic developmental defects of Dhh KO mice. Additionally, we found that Smo KO in ECs leads to a significant decrease in phosphorylated Akt levels (see Supplementary material online, Figure S3G).

To investigate the role of the PI3K/Akt pathway in Dhh regulation of Cldn5, an in vitro approach was taken. HUVECs overexpressing Dhh were treated with GDC-0449, a Smo inhibitor, Wortmanin, a PI3K inhibitor, or vehicle. As shown in Supplementary material online, Figure S4, both GDC-0449 and Wortmannin decreased Cldn-5 expression in Dhh-overexpressing HUVECs demonstrating that Dhh promotes Cldn5 expression in ECs through Smo and the PI3K/Akt pathway. Contrary to differential expression of Cldn5, ZO-1 expression was not affected by Dhh overexpression.

3.4 The increased capillary density and diameter observed in Dhh−/− nerve is due to massive nerve inflammation

Developmental studies revealed that in the absence of Dhh, the impaired development of nerve sheets is accompanied by a massive infiltration of

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**Figure 1** Dhh knockdown is sufficient to induce sciatic nerve microangiopathy. (A) Whole mount sciatic nerves were immunostained with anti-CD31 antibodies (in green) to visualize blood vessels (n = 8 per group). (B) Sciatic nerve cross-sections were immunostained with anti-CD31 antibodies (in brown) (n = 10 per group). (C) Capillary density was quantified as the number of CD31+ blood vessels/mm². (D) The average lumen diameter of endoneurial capillaries was measured. (E and F) Sciatic nerves were harvested from 12-week-old Dhh+/+, Dhh+/−, and Dhh−/− mice injected with Evans Blue (n = 6 per group). (E) Evans Blue fluorescence (in red) of sciatic nerve cross-sections was imaged and (F) quantified using SigmaScan pro5 software. *P ≤ 0.05, **P ≤ 0.01, NS, not significant, Kruskal–Wallis test.
inflammatory cells including macrophages and neutrophils.\textsuperscript{16} We validated these results in adult mice (data not shown) and found that the macrophages infiltrating Dhh\textsuperscript{−/−} nerves are Mrc-1\textsuperscript{+} M2 macrophages (Figure 4A–C) expressing VEGFA (Figure 4D). As a consequence, the overall VEGFA protein expression level is strongly increased in the sciatic nerve of Dhh\textsuperscript{−/−} mice (Figure 4E).

The effect of macrophage infiltration on the endoneurial vascular network was evaluated after mice were depleted from macrophages by clodronate liposome or vehicle administration. As shown in Figure 4F and G, both capillary density and capillary diameter, that were specifically increased in Dhh\textsuperscript{−/−}, returned to baseline following clodronate treatment demonstrating that these effects are not directly due to the absence of Dhh but are the consequence of nerve inflammation.

As shown in Supplementary material online, Figure S5, Mrc-1\textsuperscript{+} macrophage infiltration and VEGFA levels were also increased in Dhh\textsuperscript{−/−} and in Pdgfb-Cre\textsuperscript{ERT2, Smo\textsuperscript{Flox/Flox}} nerves; however, both M2 macrophage density and VEGFA levels remained significantly lower than the ones observed in Dhh\textsuperscript{−/−} nerves. Accordingly, M2 macrophage infiltration was not sufficient to promote angiogenesis and to increase capillary density in Dhh\textsuperscript{−/−} and in Pdgfb-Cre\textsuperscript{ERT2, Smo\textsuperscript{Flox/Flox}} nerves (Figures 1 and 2, respectively).

### 3.5 Impaired Hh signalling in ECs is sufficient to induce neuropathy

Finally, we measured the functional consequences of impaired Hh signalling in endoneurial ECs. Peripheral neuropathy was assessed in Pdgfb-Cre\textsuperscript{ERT2, Smo\textsuperscript{Flox/Flox}} and in their Smo\textsuperscript{Flox/Flox} control littermates using behavioural tests: hypoalgesia was measured with the Plantar test while neuropathic pain (allodynia) was evaluated with Von Frey filaments. The paw withdrawal latency to thermal heat was significantly greater in Pdgfb-Cre\textsuperscript{ERT2, Smo\textsuperscript{Flox/Flox}} compared with Smo\textsuperscript{Flox/Flox} demonstrating hypoalgesia in those mice (Figure 5A). Moreover, Pdgfb-Cre\textsuperscript{ERT2, Smo\textsuperscript{Flox/Flox}} mice were more sensitive to Von Frey filaments showing that these mice experienced neuropathic pain (Figure 5B). These tests demonstrate, for the first time, that an EC defect is sufficient to induce neuropathy.

### 3.6 Dhh expression is strongly decreased in the sciatic nerves of diabetic mice

Type 2 diabetic mice (Lepr\textsuperscript{db/db}) were shown to develop diabetic neuropathy.\textsuperscript{3} They exhibit an endoneurial vascular phenotype characterized by decreased endoneurial microvessel lumen diameter (see Supplementary material online, Figure S6A and B), an endothelium thickening...
To begin, we quantified Dhh mRNA expression in the sciatic nerves of 12-week-old Lepr db/db and in their control non-diabetic Lepr db/+ littermates. Dhh mRNA levels were down-regulated by 87% in Lepr db/db sciatic nerves (Figure 6A) compared with Lepr db/+ sciatic nerves. The resulting decrease in Dhh and total Hh protein expression in diabetic sciatic nerves was verified, by immunoprecipitation assays (Figure 6B–D). To investigate mechanisms leading to the down-regulation of Dhh in the setting of diabetes, we tested whether Schwann cell Dhh mRNA expression was modulated by high glucose levels, hypoxia, or advanced glycation endproducts (AGEs) and found that AGEs significantly reduced Dhh mRNA expression in primary cultured mouse Schwann cells (see Supplementary material online, Figure S6C) strongly suggesting that the increased level of glycated proteins related to hyperglycaemia is responsible for the down-regulation of Dhh.

To demonstrate the essential role of Dhh in preserving nerve function in adult mice, Lepr db/db mice were administered with the Smoothened agonist SAG or vehicle for 7 days. Paw withdrawal latency to thermal heat of SAG-treated Lepr db/db mice was significantly reduced (Figure 6E). Additionally, the sensitivity of SAG-treated Lepr db/db mice to Von Frey filaments, indicative of neuropathic pain, was significantly decreased compared to PBS liposome-treated Lepr db/db mice and similar to Lepr db/+ mice (Figure 6F) demonstrating that the loss of Dhh expression observed in diabetic animals contributes to the development of neuropathy.

These data further confirm that Dhh down-regulation is a feature of diabetic nerves in the setting of both type 2 and type 1 diabetes.18

### 3.7 Cldn5 is down-regulated in diabetic sciatic nerves

In parallel, we verified whether Dhh down-regulation in diabetic sciatic nerves was associated with a reduction in Cldn5 expression. Consistently with what we found in Pdgfb-CreERT2; SmoFlox/Flox mice, Cldn5 expression was significantly diminished in the sciatic nerves of Lepr db/db mice (Figure 7A and B).

These results reveal, for the first time, that the increase in endoneurial capillary permeability in diabetic neuropathic nerves is associated with a Cldn5 down-regulation. Moreover, these results are fully consistent with our in vitro and in vivo data demonstrating that Dhh regulates Cldn5 expression. Furthermore, we verified that SAG therapy increases Cldn5 protein levels in the vasa nervorum of Lepr db/db mice (Figure 7C and D) and found that Cldn5 up-regulation in SAG-treated Lepr db/db mice was associated with a significantly decreased endoneurial capillary permeability to Evans Blue (Figure 7E and F).

Altogether those results confirm an essential role of Hh signalling in maintenance of Cldn5 expression and endoneurial capillary integrity.

### 4. Discussion

Microangiopathy has long been suggested to be responsible for diabetic neuropathy yet, this has never been fully verified. Through utilization of endothelium-specific KO mice, the present paper demonstrates for the first time that endothelial dysfunction is sufficient to cause neuropathy. More specifically, it elucidates the crucial role of Dhh in the maintenance of BNB and its likely implication in diabetes-associated microangiopathy. First, this study confirms that Dhh expression is decreased in diabetic sciatic nerves and then demonstrates that specific inhibition of Hh signalling in ECs is sufficient to induce BNB breakdown and subsequent neuropathy. Altogether, these data identify an original mechanism, which may actively contribute to the development of diabetic neuropathy.

Recent findings from peripheral nerve injury models have revealed that exogenous Shh protein administration enhances nerve recovery.18,19 Additionally, Calcutt et al.18 found a quantitative decrease in the amount of Dhh mRNA in the sciatic nerves of streptozotocin-treated rats. In accordance with these studies, we found that Dhh mRNA and protein levels are both down-regulated in Lepr db/db sciatic nerves.
Figure 4  The increased capillary density and diameter in Dhh\(^{-/-}\) nerves are due to M2 macrophage infiltration. Sciatic nerves were harvested from 12-week-old Dhh\(^{-/-}\) and Dhh\(^{+/+}\) mice. (A) Whole mount sciatic nerves were co-immunostained with anti-F4/80 antibodies to identify total macrophages (in green) and with anti-Mrc-1 antibodies (in red) to detect M2 macrophages. Nuclei were stained with DAPI. (B and C) M2 macrophage infiltration was quantified as the number of Mrc-1\(^+\) macrophages (in green) per high power fields (HPF) (\(n = 8\) per group). (D) Whole mount sciatic nerve were co-immunostained with anti-VEGFA antibody (in red) and anti-Mrc-1 antibody (in green). (E) VEGFA protein expression levels in whole sciatic nerve extract were evaluated by western blot analysis (\(n = 3\) per group). (F and G) 12-week-old Dhh\(^{-/-}\) and Dhh\(^{+/+}\) mice were treated with Clodronate liposomes or PBS liposomes (\(n = 6\) per group). Sciatic nerve cross-sections were immunostained with anti-CD31 antibodies. (F) Capillary density was quantified as the number of CD31\(^+\) blood vessels/mm\(^2\). (G) The average lumen diameter of endoneurial capillaries was measured. *\(P \leq 0.05\), **\(P \leq 0.01\), ***\(P \leq 0.001\), NS, not significant, Mann–Whitney test (C) or Kruskal–Wallis test (F and G).

Figure 5  Impaired Hh signalling in ECs is sufficient to induce neuropathy. (A) Hind paw latency to hot stimulation was measured in 12-week-old Smo\(^{Flox/Flox}\) and Pdgfb-Cre\(^{ERT2}\), Smo\(^{Flox/Flox}\) mice using the Plantar test (\(n = 6\) per group, three stimulations per mouse). (B) Response to Von Frey’s hair paw application was evaluated in Smo\(^{Flox/Flox}\) and Pdgfb-Cre\(^{ERT2}\), Smo\(^{Flox/Flox}\) mice (\(n = 6\) per group, seven applications per mouse). *\(P \leq 0.05\), NS, not significant, Mann–Whitney test.
Diabetic microangiopathies are characterized by a vessel wall thickening\(^2\)–\(^6\) together with an increased vasoconstriction\(^7\) leading to a deleterious decreased tissue perfusion. In this study, we used Dhh-deficient mice to specifically investigate the role of Dhh on vascular complications in the setting of diabetic neuropathy. As expected, basement membrane thickening, endothelium thickening, and the resulting decrease in blood vessel lumen diameter are suggested to be the direct effect of hyperglycaemia on ECs;\(^35,36\) however, they were not observed in Dhh-deficient mice. Conversely, this study reveals for the first time that BNB rupture is common between Lepr\(^{db/db}\) mice and Dhh-deficient mice (Dhh\(^{+/-}\) and Dhh\(^{-/-}\)) suggesting that abnormal permeability observed in diabetic nerve is due to Dhh down-regulation. Nerve oedema has been documented in both human and experimental diabetic neuropathy,\(^37\) and endoneurial capillaries of human neuropathic nerves were shown to be permeable to high molecular weight molecules such as albumin;\(^38\) results obtained in rodents, however, remain controversial. One study performed in 1978 reported that the BNB of alloxan- and streptozotocin-diabetic rat, galactose intoxicated rat, and Lepr\(^{db/db}\) mice was impermeable to both albumin and HRP.\(^39\) Conversely, another study has shown that extravascular

Figure 6 Dhh expression is strongly decreased in the sciatic nerve of Lepr\(^{db/db}\) mice (A–D). Sciatic nerves were harvested from 12-week-old Lepr\(^{+/+}\) and Lepr\(^{db/db}\) mice (n = 8 per group). (A) Dhh mRNA expression was measured via real-time RT–PCR and normalized to HPRT mRNA. (B) Hh protein expression was evaluated after immunoprecipitation followed by western blot analysis. (C and D) Dhh precursor and total active Hh ligand expression levels were quantified using SigmaScan Pro5 software (n = 3 per group). (E and F) Lepr\(^{+/+}\) and Lepr\(^{db/db}\) mice were treated with 5 mg/kg/day SAG or vehicle for 7 days (n = 5 mice per group). (E) Hind paw latency to hot stimulation was measured using the Plantar test (three stimulations per mouse). (F) Response to Von Frey’s hair paw application was evaluated (seven applications per mouse). *P ≤ 0.05, **P ≤ 0.01, Mann–Whitney (A), Student’s t-test (C and D) or Kruskal–Wallis test (E and F).
Albumin was markedly increased in the sciatic nerves of streptozotocin-diabetic rats, the effect of which being apparent at 3 weeks of diabetes. Consistent with this last study, we found extravasation of Evans blue, which is known to be mainly associated with albumin, in the sciatic nerve of Leprdb/db mice.

Few studies have reported that more advanced stages of neuropathy-associated microangiopathy were characterized by an increased capillary density in nerves and inflammation. Similarly, we found that vasodilatation and abnormal vessel density observed in the sciatic nerves of Dhh knockout mice are indirectly due to VEGFA secretion by M2 macrophage infiltration in the endoneurium (data not shown). VEGFA is a pro-angiogenic factor known to be responsible for microvascular permeability and to participate in retinopathy and nephropathy-associated microangiopathy. Our data agreeably demonstrate that VEGFA further enhances BNB permeability induced by Dhh deficiency as well as the severity of microangiopathy in favour of the argument for a deleterious role of VEGFA in the physiopathology of diabetic neuropathy. This may explain the non-conclusive results obtained by Ropper et al. with VEGF gene transfer therapy in diabetic neuropathy.

The possible regulation of EC function by Hh ligands has long been controversial. In vitro, Hh ligands were shown to promote EC migration and survival and to induce capillary morphogenesis on MatrigelTM while, in vivo, Hh signalling in ECs was shown not to be necessary for lung vasculature development. Consistently, we found that Dhh does not promote ischaemia-induced angiogenesis by directly regulating EC function. Recently, Alvarez et al. demonstrated, for the first time, that Shh do regulate EC function in vivo by maintaining EC tight junctions in the brain. The present study demonstrates that Dhh regulates Cldn5 expression in EC and is necessary to maintain EC tight junction in the peripheral nervous system. Taken together, it appears that Hh ligands do indeed regulate EC function; nevertheless, they are not involved in the regulation of blood vessel growth, but rather in the regulation of blood vessel integrity.

**Figure 7** The increased permeability of Leprdb/db endoneurial capillaries is associated with Cldn5 down-regulation. (A and B) Sciatic nerves were harvested from 12-week-old Lepr/db+ and Lepr/dbdb mice (n = 3 per group). (A) Cldn5 protein expression in whole sciatic nerve extracts was evaluated after immunoprecipitation by western blot analysis and (B) quantified using SigmaScan proS software. (C and D) Lepr/dbdb mice were treated with 5 mg/kg/day SAG or vehicle for 7 days. (n = 6 mice per group). (C) Sciatic nerve cross-sections were co-immunostained with anti-CD31 (in red) and anti-Cldn5 (in green) antibodies. Nuclei were stained with DAPI (in blue). (D) Cldn5 protein expression was quantified as the intensity of green fluorescence using ImageJ software. (E and F) SAG- or vehicle-treated Lepr/dbdb mice were injected with Evans Blue (n = 6 per group). (E) Evans Blue fluorescence (in red) of sciatic nerve cross-sections was imaged and (F) quantified using ImageJ software. *P ≤ 0.05, **P ≤ 0.01, Student’s t-test (B) or Mann–Whitney test (D and F).
Hh ligands and more particularly Shh are proposed to regulate angiogenesis indirectly by promoting the expression of pro-angiogenic factors including VEGFA and Angpt1. Unexpectedly, we found that VEGFA levels are increased in Dhh−/− nerves, suggesting that Hh ligands are not necessary for VEGFA expression in the nervous system. This result is consistent with Nagase et al.’s results. Additionally, our observations of VEGFA overexpression due to a massive nerve inflammation may have masked a possible slight down-regulation of innate VEGFA expression in nerve cells or fibroblasts following impaired Dhh signalling. According to previous investigations, this study shows that neuropathic pain is due to endoneurial inflammatory cytokine secretion by macrophages such as IL-1β which has been shown to strongly participate in the physiopathology of neuropathic pain. Analogously, we demonstrated that clodronate liposome administration prevents neuropathic pain in streptozotocin-induced diabetic rats. On the contrary, hypoaesthesia is independent of inflammation as clodronate treatment of Dhh−/− or Pdgfb-CreERT2; SmoFlox/Flox mice did not prevent hypoaesthesia (see Supplementary material online, Figure S8). Endoneurial oedema indeed has deleterious consequences consisting of an elevation of endoneurial hydrostatic pressure that may compromise the intrascleral microcirculation leading to ischaemia and its accompanying pathology. Additionally, modification of the endoneurial microenvironment may be toxic for Schwann cells and/or axons.

Altogether our data demonstrate for the first time that Dhh signalling is necessary for vasa nervorum homeostasis in adults and that Dhh knockdown, observed in diabetic nerves, is sufficient to induce blood nerve barrier breakdown and subsequent neuropathy. Most importantly, the present work demonstrates that microangiopathy induced by Dhh knockdown is sufficient to bring about neuropathy. In conclusion, the present study gives some clues regarding the natural history of diabetic neuropathy-associated microangiopathy in the setting of type 2 diabetes. This study demonstrates that diabetes-associated microangiopathy and more specifically abnormal BNB permeability is sufficient to induce neuropathy. With this work, we propose novel therapeutic avenues for the treatment of diabetic neuropathy through identification of new possible targets, Dhh and Cldn5, which limit endoneurial capillary permeability.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

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