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ARTICLE

Modulation of p75^{NTR} prevents diabetes- and proNGF-induced retinal inflammation and blood–retina barrier breakdown in mice and rats

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Abstract
Aims/hypothesis Diabetic retinopathy is characterised by early blood–retina barrier (BRB) breakdown and neurodegeneration. Diabetes causes imbalance of nerve growth factor (NGF), leading to accumulation of the NGF precursor (proNGF), as well as the NGF receptor, p75 neurotrophin receptor (p75^{NTR}), suggesting a possible pathological role of the proNGF–p75^{NTR} axis in the diabetic retina. To date, the role of this axis in diabetes-induced retinal inflammation and BRB breakdown has not been explored. We hypothesised that modulating p75^{NTR} would prevent diabetes- and proNGF-induced retinal inflammation and BRB breakdown.
Methods Diabetes was induced by streptozotocin in wild-type and p75^{NTR} knockout (p75KO) mice. After 5 weeks, the expression of inflammatory mediators, ganglion cell loss and BRB breakdown were determined. Cleavage-resistant proNGF was overexpressed in rodent retinas with and without p75^{NTR} short hairpin RNA or with pharmacological inhibitors. In vitro, the effects of proNGF were investigated in retinal Müller glial cell line (rMC-1) and primary Müller cells.
Results Deletion of p75^{NTR} blunted the diabetes-induced decrease in retinal NGF expression and increases in proNGF, nuclear factor κB (NFκB), p-NFκB and TNF-α. Deletion of p75^{NTR} also abrogated diabetes-induced glial fibrillary acidic protein expression, ganglion cell loss and vascular permeability. Inhibited expression or cleavage of p75^{NTR} blunted proNGF-induced retinal inflammation and vascular permeability. In vitro, proNGF induced p75^{NTR}-dependent production of inflammatory mediators in primary wild-type Müller and rMC-1 cultures, but not in p75KO Müller cells.
Conclusions/interpretation The proNGF–p75^{NTR} axis contributes to retinal inflammation and vascular dysfunction in the rodent diabetic retina. These findings underscore the importance of p75^{NTR} as a novel regulator of inflammation and potential therapeutic target in diabetic retinopathy.

Keywords BRB breakdown · Diabetic retinopathy · Ganglion loss · IL-1β · Inflammation · Müller cells · NFκB · p75KO · proNGF · TNF-α

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Abbreviations

BRB	Blood–retina barrier
BRN3A	Brain-specific homeobox/POU domain protein 3A
GCL	Ganglion cell layer
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescence protein
HOC	Homozygous knockout control
HOD	Homozygous knockout diabetic
IFU	Infectious units
NFκB	Nuclear factor κB
NGF	Nerve growth factor
$p75^{ICD}$	p75 intracellular domain
p75KO	$p75^{NTR}$ knockout
$p75^{NTR}$	p75 neurotrophin receptor
pGFP	GFP plasmid
PM	Primary mouse Müller cells
proNGF	NGF precursor
RGCs	Retinal ganglion cells
rMC-1	Rat Müller glial cell line
shRNA	Short hairpin RNA
TrkA	Tyrosine receptor kinase A
VEGF	Vascular endothelial growth factor
WT	Wild-type

Introduction

Diabetic retinopathy, the leading cause of blindness in working-age adults in the USA, is a progressive and potentially devastating vascular-neurodegenerative disease [1]. A growing body of evidence reviewed by various teams [2–4] supports the concept that diabetes-induced oxidative stress disturbs retinal homeostasis by activating glial cells, reducing neurotrophic and prosurvival inputs, and increasing proinflammatory cytokines. Recent findings by us indicate that diabetes causes an imbalance of nerve growth factor (NGF), leading to accumulation of the NGF precursor (proNGF) and the NGF receptor, p75 neurotrophin receptor ($p75^{NTR}$) [5–7]. Diabetes-induced peroxynitrite also enhances $p75^{NTR}$ expression and tyrosine nitration, which inhibits prosurvival signalling of the NGF receptor, tyrosine receptor kinase A (TrkA) [6, 7]. Thus, diabetes shifts the neurotrophin balance from NGF towards proNGF and receptor activity towards $p75^{NTR}$, suggesting a possible pathological role of the proNGF–$p75^{NTR}$ axis in the diabetic retina. Although the proNGF–$p75^{NTR}$ axis has been implicated in neurodegenerative diseases [8–10], its role in diabetes-induced retinal inflammation and blood–retina barrier (BRB) breakdown has not been fully elucidated.

The $p75^{NTR}$ receptor, a member of the TNF receptor superfamily [11], has multiple and cell-specific functions, which, as previously reviewed [12–14], are dependent upon the availability of ligands and co-receptors. In the retina, $p75^{NTR}$ is expressed predominantly by Müller cells, which also express the $p75^{NTR}$ co-receptors, sortilin and TrkA [6, 15]. The $p75^{NTR}$–sortilin complex has a higher affinity for proNGF and is usually pro-apoptotic, while $p75^{NTR}$–TrkA has increased NGF affinity and is usually pro-survival [13, 16]. $p75^{NTR}$ lacks a catalytic domain [17, 18] and signals through association of effector molecules with the cytoplasmic p75 intracellular domain ($p75^{ICD}$) generated by α-secretase-catalysed cleavage of the extracellular domain followed by γ-secretase-catalysed intramembrane proteolysis [19, 20]. The outcome of $p75^{ICD}$ signalling can be beneficial or detrimental depending upon $p75^{NTR}$ co-receptor interactions and the cellular environment [21, 22]. Specifically, the $p75^{NTR}$ receptor has been involved in nuclear factor κB (NFκB) activation and production of proinflammatory mediators [15, 23, 24]. In the present work, we hypothesised that modulation of $p75^{NTR}$ would prevent diabetes- and proNGF-induced retinal inflammation and BRB breakdown. Molecular and pharmacological approaches were used to examine this hypothesis. A $p75^{NTR}$ knockout (p75KO) mouse model was used to examine diabetes-induced inflammation and preservation of BRB function. The effects of modulating $p75^{NTR}$ expression or activity were examined in a model of proNGF overexpression and in Müller cell cultures.

Methods

Animal preparation All procedures with animals were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research, and the Charlie Norwood VA Medical Center Animal Care and Use Committee. We used 30 male Sprague–Dawley rats (~250 g body weight) from Harlan Laboratories (Indianapolis, IN, USA) and 60 male mice (~20 g body weight), including 18 p75KO mice and 42 wild-type (WT) mice. The $p75^{NTR}$, B6.129S4*Ngfr*^{tm1Jae}/J (p75KO, exon III knockout mice [25]) were obtained from Jackson Laboratories (Bar Harbor, Maine, USA) and crossed with C57BL6-J mice (Jackson Laboratories). These mice were crossed and back-crossed to establish a colony of homozygous p75KO and WT breeders that produced the mice used here.

Induction of diabetes Male 8-week-old WT and p75KO mice (20 g) were randomly assigned to four groups: WT controls, WT diabetic, homozygous knockout control (HOC), homozygous knockout diabetic (HOD). Mice were rendered diabetic by five consecutive intraperitoneal injections of streptozotocin (60 mg/kg) in 0.01 mol/l citrate

buffer. Diabetes was initially confirmed by detection of glucose in urine and later by blood glucose over 12.7 mmol/l. Deletion of $p75^{NTR}$ did not alter body weight in control (25.8±0.5 g HOC vs 25.3±0.4 g in WT control) or diabetic groups (24.1±0.6 g HOD vs 24.7±0.8 g in WT diabetic), or glucose levels in controls (7.6±0.8 mmol/l HOC vs 7.7±0.5 mmol/l in WT control) or diabetic groups (14.8±1.1 mmol/l HOD vs 14.4±0.8 mmol/l in WT diabetic).

Overexpression of proNGF in rodent retinas Stable overexpression of proNGF was achieved via intravitreal injection of proNGF123 plasmid [26] and electroporation as described previously by our group [27]. Rats were randomised into three groups: (1) control group, green fluorescence protein (GFP)-plasmid (pGFP) alone; (2) proNGF group, pGFP–proNGF plus lentiviral particles containing scrambled shRNA (5,000 infectious units (IFU)/eye); and (3) a group receiving pGFP–proNGF plus lentiviral particles containing short hairpin RNA (shRNA) against $p75^{NTR}$ (5,000 IFU/eye). Lentiviral particles were obtained from Dharmacon (Santa Cruz BioTechnology, Dallas, TX, USA). Animals were killed after 4 weeks. Additional groups of WT mice were randomised into four groups receiving: (1) (control group) pGFP-plasmid alone; (2) pGFP–proNGF; (3) pGFP–proNGF plus 40 ng C30-35, a selective antagonist for $p75^{NTR}$ receptor [28] (kind gift from Uri Saragovi, McGill University, Montreal, QC, Canada); and (4) pGFP–proNGF plus 100 ng γ-secretase inhibitor (compound E) (Calbiochem/EMD Biosciences, Billerica, MA, USA).

Antibodies The following primary antibodies were used: polyclonal anti-NGF and anti-proNGF (Alomone Labs, Jerusalem, Israel); polyclonal anti-$p75^{NTR}$ (catalogue number 07-476), polyclonal anti-vascular endothelial growth factor (VEGF) and monoclonal anti-GFP antibody (Millipore, Billerica, MA, USA); monoclonal anti-TNF-α, polyclonal anti-IL-1β and polyclonal anti-sortilin (Abcam, Cambridge, MA, USA); monoclonal IgG2 (R&D Systems, Minneapolis, MN, USA); polyclonal anti-glial fibrillary acidic protein (GFAP) and monoclonal anti-vimentin (Thermo Fisher Scientific, Rockford, IL, USA); polyclonal β-actin (Sigma-Aldrich, St Louis, MO, USA); monoclonal anti-brain-specific homeobox/POU domain protein 3A (BRN3A) and polyclonal anti-NFκB-p65 (Santa Cruz Biotechnology); and monoclonal anti-phospho-NFκB (p65 subunit) (Cell Signaling, Danvers, MA, USA). Secondary antibodies used were Texas-red- or Oregon-green-conjugated goat anti-mouse or goat anti-rabbit antibodies (Invitrogen, Grand Island, NY, USA), and horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse (Calbiochem/EMD Biosciences).

Immunolocalisation studies Retina sections were fixed using 2% wt/vol. paraformaldehyde and incubated overnight in PBS-containing primary antibody (1:100 dilution) or negative control at 4°C. Sections were blocked for 1 h with 20% vol./vol. goat serum in PBS containing 0.3% vol./vol. Triton X-100 and incubated for 2 h with secondary antibody (1:500) in PBS-containing 0.3% vol./vol. Triton X-100 and 1% wt/vol. BSA. Sections were coverslipped with Vectashield with DAPI (Vector Laboratories, Burlingame, CA, USA) and images collected by microscope (AxioObserver.Z1; Zeiss, Jena, Germany).

Quantification of retina ganglion cells Retina sections containing the optic nerve were counted. BRN3A is a POU-domain transcription factor that is expressed by retinal ganglion cells (RGCs) and compares favourably to fluorogold labelling [29]. Total cells (DAPI-positive) and ganglion cells (BRN3A- and DAPI-positive) in the ganglion cell layer (GCL) were counted and the length of the GCL measured from one ora-serrata to the other. Cell numbers were normalised to length of the GCL in mm.

Determination of blood–retina barrier function Integrity of the BRB was measured as described by our group [5, 30]. Mice received jugular vein injections of 10 mg/kg BSA-conjugated fluorescein (Invitrogen, Grand Island, NY, USA). After 20 min, animals were killed and blood and retinas collected. Serial imaging of fluorescence in six sections was quantified by microscope (Zeiss). Contralateral retinas were homogenised in RIPA-buffer to detect the fluorescence of retinal lysates using a fluorescent plate reader (BioTek Synergy2, Winooski, VT, USA) (excitation 370 nm, emission 460 nm). Retina fluorescence was normalised to that of serum.

Retinal protein extraction and western blot analysis Retinas and cells were isolated and homogenised in RIPA buffer [7]. Samples (30 μg protein) were separated by SDS-PAGE. Membranes were probed with primary antibodies in 5% wt/vol. milk or 0.5% wt/vol. BSA, in PBST followed by secondary antibody (1:5,000 in 5% wt/vol. milk in PBST). Blots were developed using Pierce-ECL Western Blot (Thermo Fisher) or Immobilon Western Chemiluminescent HRP (Millipore) substrates. Membranes were reprobed with β-actin to confirm equal loading. Films were scanned. Band intensities were quantified using densitometry software (alphaEaseFC, Proteinsimple, Santa Clara, CA, USA) and expressed as relative optical density to β-actin normalised to controls.

Quantitative real-time PCR A one-step quantitative RT-PCR kit (Invitrogen) was used to amplify 10 ng retinal mRNA as described [6, 7]. PCR primers (electronic supplementary material [ESM] Fig. 1d) were purchased from Integrated DNA Technologies (Coralville, IA, USA). Quantitative

PCR was performed (Realplex Master Cycler; Eppendorf, Hamburg, Germany). The expression of TNF-α, NGF or VEGF was normalised to 18S and expressed relative to WT control.

Tissue culture studies with rat Müller cell line The rat retinal Müller glial cell line (rMC-1), obtained from V. Sarthy (Department of Ophthalmology, Northwestern University, Chicago, IL, USA), has been previously characterised [31]. Cells were grown to confluence for 48 h in normal glucose (5 mmol/l) or high glucose (25 mmol/l) as described previously [6] and treated overnight with 50 ng/ml cleavage-resistant proNGF (Alomone Labs) in the presence or absence of the $p75^{NTR}$ receptor antagonist (20 μmol/l) [28] or 10 μmol/l compound E (γ-secretase inhibitor).

Primary mouse Müller cells Primary mouse Müller cells (PM) were cultured as described previously [32]. Briefly, eyes from mice at postnatal day 7 were incubated overnight in serum-free DMEM with 0.1% vol./vol. penicillin/streptomycin. Retinas were removed by dissection and plated in 5 mmol/l glucose growth medium (DMEM, 10% vol./vol. FBS, 1% vol./vol. penicillin/streptomycin). The purity of the cultures was verified by immunostaining for the Müller cell marker vimentin. PM were switched to low serum medium (0.5% vol./vol. FBS) overnight, followed by treatment with 100 μmol/l peroxynitrite or 50 ng/ml cleavage-resistant proNGF, or both for 6 h. For long-term studies, cells from WT were treated with the antagonist for $p75^{NTR}$ receptor (20 μmol/l) and compared with untreated WT or p75KO cells. Cells were grown in low-serum medium, which was changed every 2 days, and collected after 6 days.

Determination of TNF-α by ELISA Levels of TNF-α in PM cell supernatant fractions were determined using ELISA following the manufacturer's protocol (Catalogue number 88-7013-22; eBioscience, San Diego, CA, USA,). Briefly, ELISA plates were coated with the capture antibody and left overnight at 4°C, followed by samples with avidin-horseradish peroxidase. Absorbance was measured at 450 nm (microplate reader; Synergy2-BioTeck).

Data analysis Results are expressed as mean ± SEM. Data normality was verified by evaluation of the histogram and the Shapiro–Wilk test. For normal data with equal variances, differences between experimental groups were evaluated by ANOVA, followed by the Tukey–Kramer multiple comparison test. Non-parametric data were evaluated by Wilcoxon's (Mann–Whitney) test, followed by Steel–Dwass all-pairs comparisons. Data analysis was performed using JMP Pro 10.0.1 release 2 software (SAS Institute, Cary, NC) Significance was defined as $p<0.05$.

Results

Deletion of $p75^{NTR}$ does not cause diabetes-induced changes in sortilin or TrkA expression The p75KO mouse model lacks Exon III, which encodes the receptor-binding region [25], and was used to examine to what extent $p75^{NTR}$ modulates diabetes-induced inflammation. Expression of $p75^{NTR}$ and the co-receptors sortilin and TrkA was examined in WT control, WT diabetic, HOC and HOD mice after 4 to 5 weeks of streptozotocin-induced diabetes (Fig. 1a). Diabetes induced a significant 1.5-fold increase in $p75^{NTR}$ expression in WT diabetic compared with WT control mice. $p75^{NTR}$ expression was not detected in HOC and HOD mice (Fig. 1b). No significant differences were found in expression patterns of the $p75^{NTR}$ co-receptors, sortilin or TrkA, in any of the groups.

Deletion of $p75^{NTR}$ eliminates the diabetes-induced imbalance in proNGF and NGF expression We examined whether deletion of $p75^{NTR}$ eliminates the diabetes-induced proNGF and NGF imbalance observed in experimental and clinical diabetes [5]. Diabetes induced a significant 2.5-fold increase in proNGF (Fig. 2a) and a 0.6-fold decrease in NGF expression (Fig. 2b) in retinas of WT diabetic vs WT control mice, changes not seen in HOD vs HOC mice (Fig. 2a, b). ESM Figure 1a shows a diabetes-induced increase in *Ngf* mRNA in WT diabetic, but not in HOD or HOC mice, where *Ngf* expression was similar to that in WT control retinas. Interestingly, basal proNGF protein levels were elevated and NGF levels decreased in p75KO mice vs WT controls (Fig. 2a, b), suggesting that regulation of proNGF levels may involve proteolytic events related to $p75^{NTR}$ signalling, rather than a direct effect on pro*Ngf* gene expression. As shown in ESM Fig. 2a–c, deletion or inhibition of $p75^{NTR}$ decreased proNGF expression in cell lysate, but did not alter NGF levels. Deletion of $p75^{NTR}$, but not inhibition modestly increased proNGF release and decreased NGF release in cell

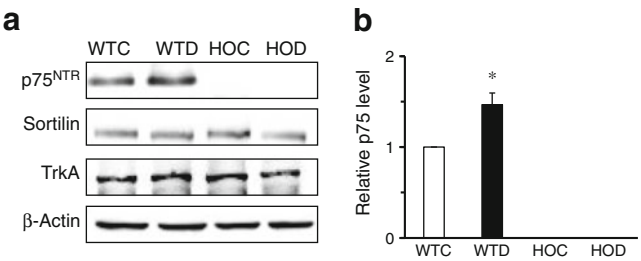

Fig. 1 Deletion of $p75^{NTR}$ does not cause diabetes-induced changes in sortilin or TrkA expression. (**a**) Representative blots and (**b**) densitometric analysis of $p75^{NTR}$ protein levels in retinas of WT control (WTC), WT diabetic (WTD), HOC and HOD mice after 4 to 5 weeks of streptozotocin-induced diabetes. Note (**b**) that $P75^{NTR}$ was absent in HOC and HOD mice. Protein levels were normalised to β-actin and respective WT controls; $n=4$, $^*p<0.05$

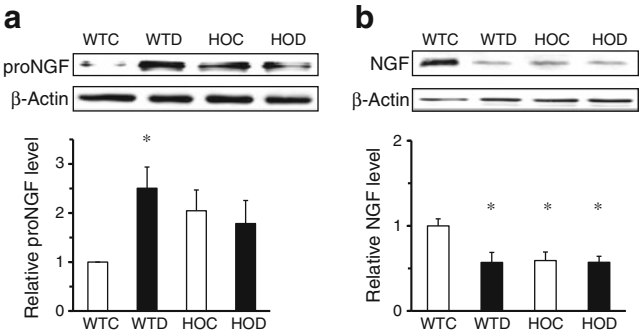

Fig. 2 Deletion of p75^{NTR} eliminates the diabetes-induced imbalance in proNGF and NGF expression. (**a**) Representative blots and statistical analysis of proNGF and (**b**) NGF protein levels in retinas of WT control (WTC), WT diabetic (WTD), HOC and HOD mice after 4 to 5 weeks of streptozotocin-induced diabetes. Protein expression is presented as relative optical density of western blot bands normalised to β-actin and respective WT controls; $n=6$, $^{*}p<0.05$

soup, without reaching statistical significance, probably due to the small sample size.

Deletion of p75^{NTR} eliminates the diabetes-induced upregulation of inflammatory mediators We next examined the effect of deleting p75^{NTR} on the production of diabetes-induced inflammatory mediators. A 3.4-fold increase in 26 kDa membrane-bound TNF-α (Fig. 3a, b), a twofold increase in p-NFκB (Fig. 3a, c) and a 1.9-fold increase in NFκB (Fig. 3a, d) were observed in retinas from WT diabetic compared with WT control mice. These effects were absent in retinas from HOD compared with WT control and HOC mice (Fig. 3b, c). Interestingly, basal levels of TNF-α and NFκB were markedly higher in HOC than in WT control mice (Fig. 3b, d), but not at the mRNA level (ESM Fig. 1b). As shown in ESM Fig. 2a, d, deleting or antagonising the p75^{NTR} receptor increased membrane-bound TNF-α (26 kDa) while decreasing soluble TNF-α (17 kDa) vs untreated WT primary Müller cells. Analysis of VEGF expression in WT diabetic mice showed a modest, but not significant 1.36-fold increase (Fig. 3e) and a 1.27-fold increase in mRNA (ESM Fig. 1c) compared with WT controls. Deletion of p75^{NTR} reduced VEGF in some HOC and HOD retinas, bit did not significantly alter protein or gene expression in these mice.

Silencing of p75^{NTR} expression blunts proNGF-induced glial activation and inflammation The above results suggested that deletion of p75^{NTR} eliminates the diabetes-induced imbalance in proNGF. To evaluate to which extent proNGF–p75^{NTR} stimulation induces retinal inflammation independently of the diabetic milieu, overexpression of cleavage-resistant proNGF and knockdown of p75^{NTR} using shRNA were performed in healthy rat retinas. Representative blots (Fig. 4a) and statistical analysis show that overexpression of cleavage-resistant proNGF plus scrambled shRNA induced a 2.5-fold increase in proNGF (Fig. 4b), a 1.8-fold increase in p75^{NTR} (Fig. 4c), a 3.6-fold increase in NFκB (Fig. 4d), a 1.7-fold increase in TNF-α (Fig. 4e) and a 2.3-fold increase in IL-1β (Fig. 4f). Knockdown of p75^{NTR} reduced proNGF-induced inflammatory responses to control levels. Immunohistochemical analysis showed that, relative to GFP controls (Fig. 5a–e), overexpression of proNGF induced expression of proNGF, p75^{NTR}, TNF-α, IL-1β and GFAP (Fig. 5f–j). This was localised to inner layers of the retina and the outer limiting membrane, which are areas occupied by Müller glial cells. GFAP staining was typical of activated Müller cells, appearing robustly in the GCL and extending radially into the retina. Knockdown of p75^{NTR} by p75^{NTR} shRNA reduced the increased expression of proNGF, p75^{NTR}, TNF-α, IL-1β and GFAP (Fig. 5k–o) to GFP control levels.

Deletion of p75^{NTR} eliminates diabetes-induced glial activation and ganglion cell loss To confirm that deletion of p75^{NTR} rendered p75KO mice insensitive to diabetes-induced inflammation, we next examined GFAP expression and ganglion cell numbers after 4 to 5 weeks of streptozotocin-induced diabetes. Representative micrographs (Fig. 6a–d) show that elevated GFAP (red) was present in WT diabetic (Fig. 6b), but not in WT control (Fig. 6a), HOC or HOD mice (Fig. 6c, d). Neuronal death, especially ganglion cell loss, is also characteristic of the diabetic retina [33]. RGCs were identified as BRN3A-positive cells (Fig. 6f) that co-localised with DAPI-positive nuclei (Fig. 6g). Mouse IgG2 as negative isotype control showed minimal staining (Fig. 6h). Subsequent analysis of these counts showed no difference in total cell number in the GCLs of WT control, WT diabetic, HOC or HOD mice (Fig. 6i), but a significant 25% decrease in BRN3A-positive ganglion cells in WT diabetic compared with WT control retinas (Fig. 6j). No difference in BRN3A-positive ganglion cell numbers was observed between HOC and HOD retinas (Fig. 6j). Although basal numbers of BRN3A ganglion cells were reduced in HOC and HOD mice, this difference was not significant.

Modulation of p75^{NTR} prevents diabetes- and proNGF-induced BRB breakdown Representative micrographs (Fig. 7a–d) show increased extravasation of BSA-conjugated green fluorescence in WT diabetic (Fig. 7b), but not in WT control (Fig. 7a), HOC (Fig. 7c) or HOD retinas (Fig. 7d). This effect was associated with a twofold increase in fluorescence of retinal lysate from WT diabetic compared with WT controls, with no increase in HOC or HOD mice (Fig. 7e). In an alternative model, overexpression of cleavage-resistant proNGF in WT mice also caused a twofold increase in BRB breakdown as assessed by extravasation of BSA-fluorescein (Fig. 7f). This effect was abrogated by the selective antagonist

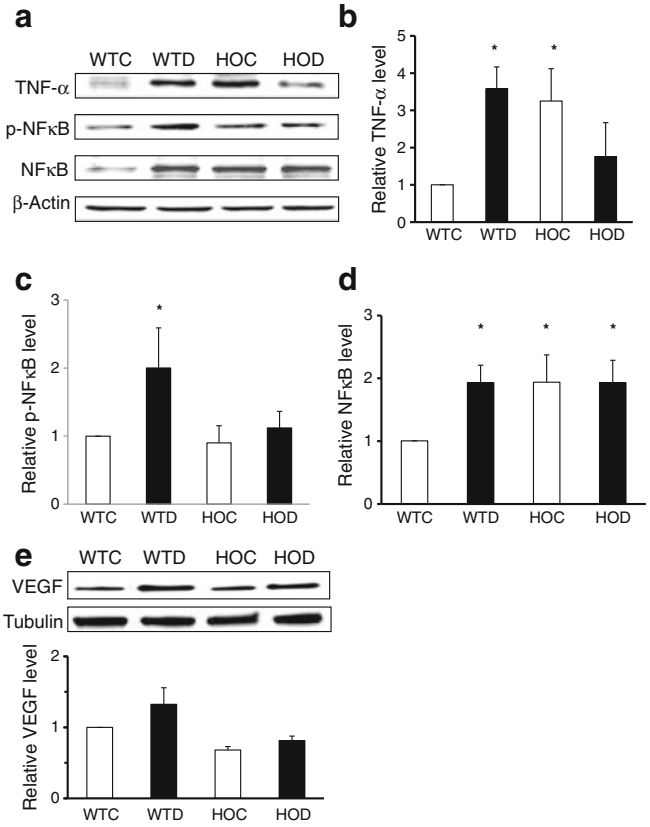

Fig. 3 Deletion of p75^{NTR} eliminates diabetes-induced upregulation of inflammatory mediators after 4 to 5 weeks of streptozotocin-induced diabetes. (**a**, **e**) Representative blots and protein expression of TNF-α (**b**), p-NFκB (**c**), NFκB (**d**) and VEGF (**e**) in retinas of WT control (WTC), WT diabetic (WTD), HOC and HOD mice at the diabetes duration mentioned. Protein expression is presented as relative optical density of western blot bands normalised to β-actin and respective WT controls; n=4–6, *p<0.05

for p75^{NTR} receptor (40 ng/eye) or by the selective γ-secretase inhibitor (100 ng/eye).

Blocking of p75^{NTR} action and p75^{NTR} deletion prevent proNGF-mediated inflammation in Müller cells We and others have demonstrated p75^{NTR} co-localisation of proNGF in Müller cells [5, 6, 15]. Previously we reported that high glucose significantly stimulates p75^{NTR} expression in rMC-1 [6]. Here, blocking the action or cleavage of p75^{NTR} was evaluated in rMC-1 cultures maintained in high glucose plus mutant-proNGF. Representative blots (Fig. 8a) and statistical analyses (Fig. 8b–d) show that exposure of rMC-1 cells to proNGF further induced p75^{NTR} expression (1.6-fold) compared with high glucose cultures alone (Fig. 8d). The proNGF-induced elevation of p75^{NTR} was accompanied by a significant 1.6-fold increase in TNF-α (Fig. 8b) and a small, but significant 1.3-fold increase in p-NFκB and NFκB (Fig. 8c). These effects were markedly reduced by co-treatment with compound E (γ-secretase inhibitor). Interestingly, exposure of cells to high glucose and proNGF plus the p75^{NTR} antagonist or compound E also resulted in upregulation of p75^{NTR} expression vs controls (Fig. 8d). Finally we investigated whether deletion of p75^{NTR} rendered Müller cells insensitive to the proinflammatory effects of proNGF. Cultures of primary Müller cells were isolated [32] and characterised by robust expression of the Müller cell marker vimentin (Fig. 8e). Western blot confirmed the lack of p75^{NTR} expression in p75KO Müller cells (Fig. 8f). To mimic the pro-oxidative milieu of the diabetic retina, primary Müller cells were cultured in 100 μmol/l peroxynitrite and stimulated with mutant proNGF. Treatment of WT Müller cells with peroxynitrite alone or in combination with proNGF induced release of TNF-α into the supernatant fraction (Fig. 8g). In contrast, treatment of p75KO-Müller cells with peroxynitrite alone or in combination with proNGF showed no significant change in secreted TNF-α compared with p75KO controls (Fig. 8h).

Discussion

Retinal inflammation, as reviewed by others [34, 35], is increasingly recognised as an important factor in the pathogenesis of diabetic retinopathy. The present results demonstrate the importance of p75^{NTR} in diabetes- and proNGF-induced retinal inflammation and BRB breakdown. First, deletion of p75^{NTR} blunted diabetes-induced retinal inflammation, glial activation, ganglion cell loss and vascular permeability. Second, inhibition of p75^{NTR} or its cleavage blocked proNGF-induced expression of inflammatory mediators both in vivo in a rodent model that overexpressed cleavage-resistant proNGF and in vitro in Müller cells.

Diabetic retinopathy is characterised by early BRB breakdown, which causes loss of vision through macular oedema and/or vitreoretinal neovascularisation. However, as previously reviewed [36], current treatments to prevent macular oedema are invasive and of limited efficacy. Understanding the mechanisms behind early increases in diabetes-induced vascular permeability is essential for developing novel and effective therapeutic targets for diabetic retinopathy. A large body of evidence demonstrates a clear role for diabetes-induced oxidative stress, resulting in glial activation and release of proinflammatory cytokines including VEGF, TNF-α and IL-1β [30, 34, 37–40]. In addition to these known inflammatory mediators, the present study provides compelling support for an upstream role of the proNGF–p75^{NTR} axis in diabetes-induced retinal inflammation. Our studies show that silencing p75^{NTR} expression abrogated proNGF-induced increases in TNF-α, NFκB, IL-1β and glial activation (Figs 4, 5), and that modulation of p75^{NTR} activity by a p75^{NTR} antagonist or its cleavage using the γ-secretase inhibitor prevented proNGF-induced BRB

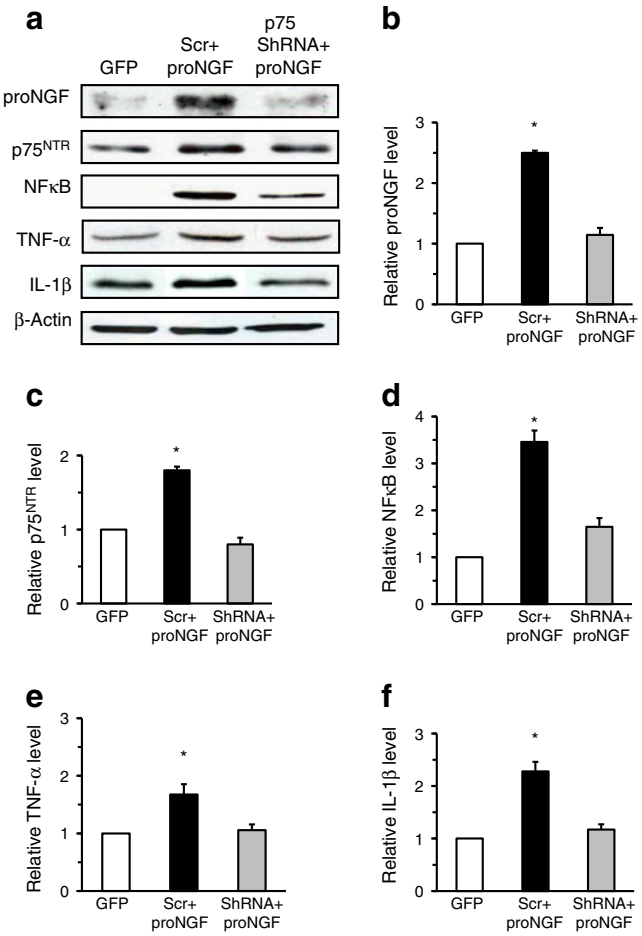

Fig. 4 Silencing p75^{NTR} expression blunts proNGF-induced inflammation. (**a**) Representative blots and protein expression levels of proNGF (**b**), p75^{NTR} (**c**), NFκB (**d**), TNF-α (**e**) and IL-1β (**f**) in retinas removed 4 weeks after intravitreal injections of pGFP (control), scrambled shRNA plus cleavage-resistant proNGF (Scr+proNGF), and p75^{NTR} shRNA plus cleavage-resistant proNGF (p75shRNA+proNGF). Protein expression is presented as relative optical density of western blot bands normalised to β-actin and GFP controls; n=4, *p<0.05

breakdown in vivo (Fig. 7). Deletion of p75^{NTR} blunted diabetes-induced alterations in proNGF and NGF protein expression (Fig. 2), and diabetes-induced increases in TNF-α, p-NFκB and VEGF (Fig. 3). Interestingly, deletion of p75^{NTR} caused basal alterations in levels of proNGF, TNF-α and total NFκB (Fig. 3), but not in the corresponding mRNA levels, suggesting that protein alteration could be attributed to post-translational protein processing in these KO mice. Moreover, our results show that deleting or antagonising the p75^{NTR} receptor in primary Müller cells increased membrane-bound TNF-α (26 kDa) while decreasing soluble TNF-α (17 kDa) compared with WT. Deletion of p75^{NTR} tended to increase release of proNGF and decrease release of NGF in cell soup compared with WT Müller cells. Thus the p75^{NTR} receptor was shown to play an important role in the regulation of proNGF and NGF levels, and in the relationship between membrane-bound and soluble TNF-α. Most importantly, the differences in TNF-α expression patterns may explain the dichotomy between the insensitivity of p75KO retinas to diabetes-induced vascular permeability and the occurrence of cell death, despite the higher levels of membrane-bound TNF-α and altered proNGF and NGF expression. The notion that deletion of p75^{NTR} alters proteolytic activity, resulting in accumulation of proNGF and membrane-bound TNF-α is supported by recent research on Alzheimer's disease showing that interaction of the p75^{NTR} extracellular tail domain with β-amyloid inhibited the accumulation of aggregates and subsequent protein expression patterns without altering gene expression [41].

Although NGF-induced upregulation of p75^{NTR} has been shown to occur through TrkA signalling pathways [42], to our knowledge, p75^{NTR} regulation of proNGF has not been fully elucidated. Our previous studies showed a positive correlation between proNGF and p75^{NTR} levels in human and experimental models of diabetes [5, 6], and in a model of

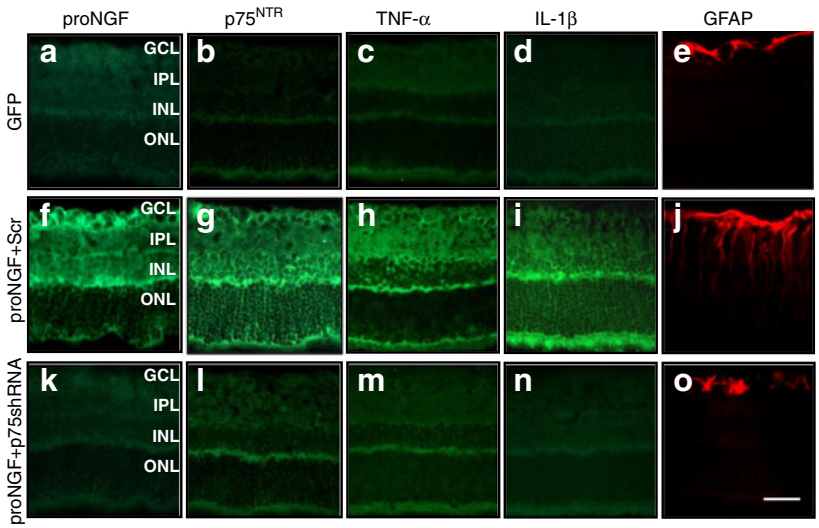

Fig. 5 Overexpression of proNGF induced glial Müller activation and inflammation. Immunohistochemical analysis of retinas was performed 4 weeks after intravitreal injections of pGFP alone (control) (**a**–**e**), proNGF plus scrambled p75^{NTR} shRNA (proNGF+Scr) (**f**–**j**) and proNGF plus p75^{NTR} shRNA (proNGF+p75shRNA) (**k**–**o**). Retinal sections were examined from four different rats in each group. Scale bar 50 μm. INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer

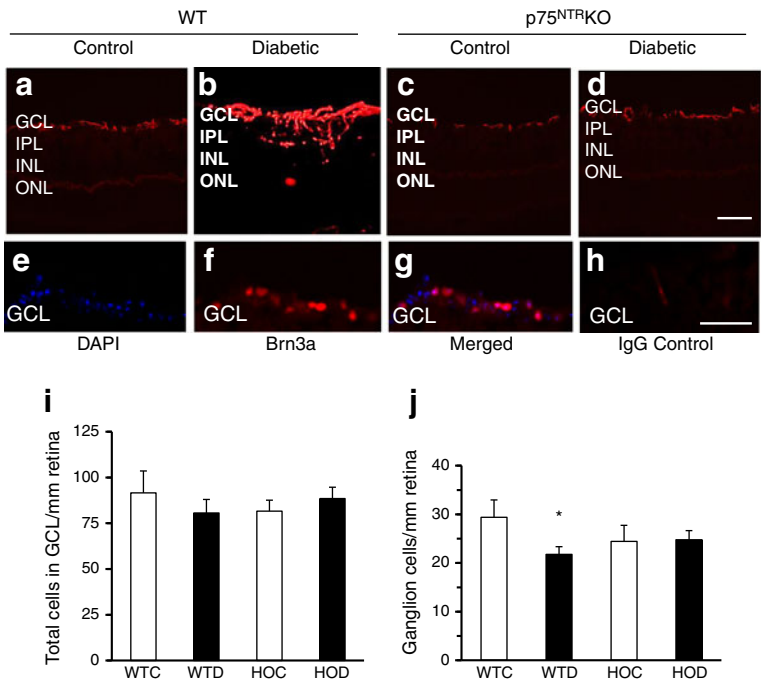

Fig. 6 Deletion of $p75^{NTR}$ eliminates diabetes-induced glial activation and ganglion cell loss. (**a**–**d**) Representative micrographs (scale bars 50 μm) showing GFAP expression (red) and (**e**–**h**) BRN3A expression (red), total nuclei (blue), with statistical analysis of total cells in (**i**) the GCL and (**j**) ganglion cells in retinas of WT control (WTC), WT diabetic (WTD), HOC and HOD mice after 4 to 5 weeks of streptozotocin-induced diabetes; $n=6–8$, $^*p<0.05$. INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer

proNGF overexpression [27]. Here, we show that knocking-down expression of $p75^{NTR}$ downregulates proNGF expression, supporting the positive link between the ligand and receptor. In contrast to the elevated $p75^{NTR}$ expression in WT diabetic mice, sortilin levels were similar in all four groups. These results are consistent with our previous findings in diabetic rat retinas [6] and in a model of proNGF overexpression [27]. Although approximately 10% of sortilin protein is located at the plasma membrane where it can act as a $p75^{NTR}$ co-receptor, most sortilin is located in the cytoplasm, where it aids trafficking of proteins to the plasma membrane [16]. Thus, increased sortilin at the plasma membrane could participate in proNGF–$p75^{NTR}$ signalling without a detectable change in total sortilin expression.

Despite altered basal protein levels in p75KO mice, our data confirm previous findings that breakdown of BRB can occur early on in streptozotocin-induced diabetes [5, 30, 39, 43]. Deletion of $p75^{NTR}$ rendered p75KO mice insensitive to glial activation, increased vascular permeability and diabetes-induced ganglion cell loss, all of which are hallmarks of early diabetic retinopathy. Although the 25% loss of BRN3A-positive ganglion cells in WT diabetic mice is higher than the 7% loss reported by others [44] after 6 weeks of streptozotocin-induced diabetic in C57BL6 mice, our total GCL counts and percentage of BRN3A-positive cells in WT control mice are comparable to published reports [45, 46]. Diabetes-induced ganglion cell loss also varies with the duration of diabetes, insulin supplementation and counting method. Thus Martin et al report a 25% loss of total cells in the GCL after 14 weeks of streptozotocin-induced diabetes in C57BL6 mice without insulin supplementation [47], while Barber and co-workers report a significant 10% loss of neuronal cells in the GCL layer in mice after 7.5 months of streptozotocin-induced diabetes with insulin supplementation [33]. Although p75KO mice appear to have fewer BRN3A cells in the GCL than WT controls, this difference was not statistically significant.

Our current findings suggest a pathway by which proNGF-stimulated cleavage of $p75^{NTR}$ activates NFκB with subsequent production of inflammatory mediators such as TNF-α. These results are consistent with work by others, in which $p75^{NTR}$-induced expression of IL-1β and TNF-α was attributed to activation and nuclear translocation of NFκB [15, 19, 23]. The $p75^{NTR}$ receptor, similar to Notch and amyloid precursor protein, undergoes γ-secretase catalysed intramembrane proteolysis [17] to liberate the intracellular domain ($p75^{ICD}$), which can recruit several intracellular binding proteins that dictate specific signalling pathways [19]. The $p75^{NTR}$ intracellular domain can recruit a complex of proteins, including IκB-kinase-β, which, as previously reviewed [48], phosphorylates IκB, resulting in release and activation of NFκB. While the involvement of the $p75^{ICD}$ in NFκB activation has been reported by others [18], the exact molecular mechanisms are still under study. One possibility is that $p75^{ICD}$ associates with TNF receptor-associated factor-6 [49] to mediate activation of NFκB. Alternatively, $p75^{ICD}$ may regulate NFκB activation directly by translocating to the nucleus, where it can act as a transcription enhancer [17–20]. The protective effects of inhibiting $p75^{NTR}$ or its cleavage ($p75^{NTR}$ receptor antagonist and compound E) could be attributed, partly, to decreased retinal inflammation, as demonstrated by their abrogation of increased TNF-α and

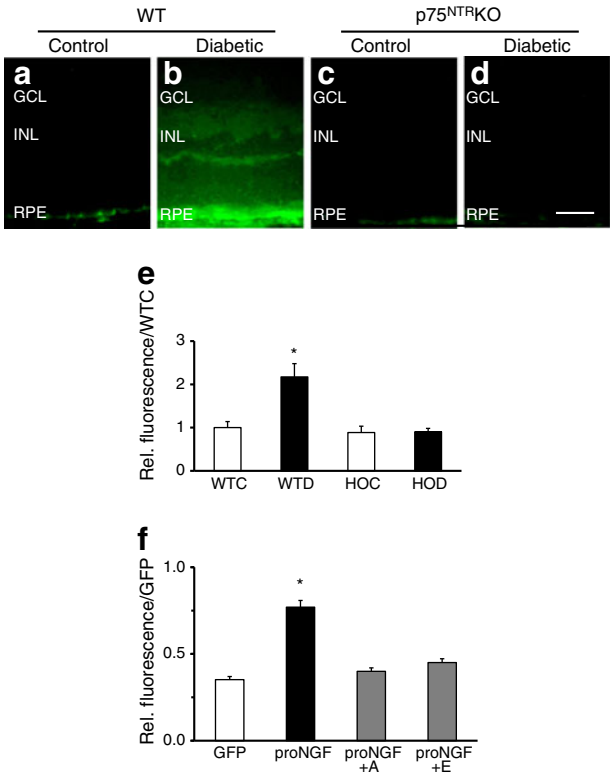

Fig. 7 Modulation of p75^{NTR} prevents diabetes- and proNGF-induced BRB breakdown. (**a**–**d**) Representative micrographs (scale bar 50 μm) and statistical analysis (**e**) of retinal vascular permeability assessed by extravasation of BSA-conjugated fluorescein after 4 to 5 weeks of streptozotocin-induced diabetes in WT control (WTC), WT diabetic (WTD), HOC and HOD mice. (**f**) Statistical analysis of proNGF-induced retinal vascular permeability assessed after 1 week in the presence of the p75^{NTR}-specific antagonist (A) (40 ng) or the γ-secretase inhibitor compound E (E) (100 ng) compared with GFP controls; n=4–6, *p<0.05. INL, inner nuclear layer; RPE, retinal pigmented epithelial layer

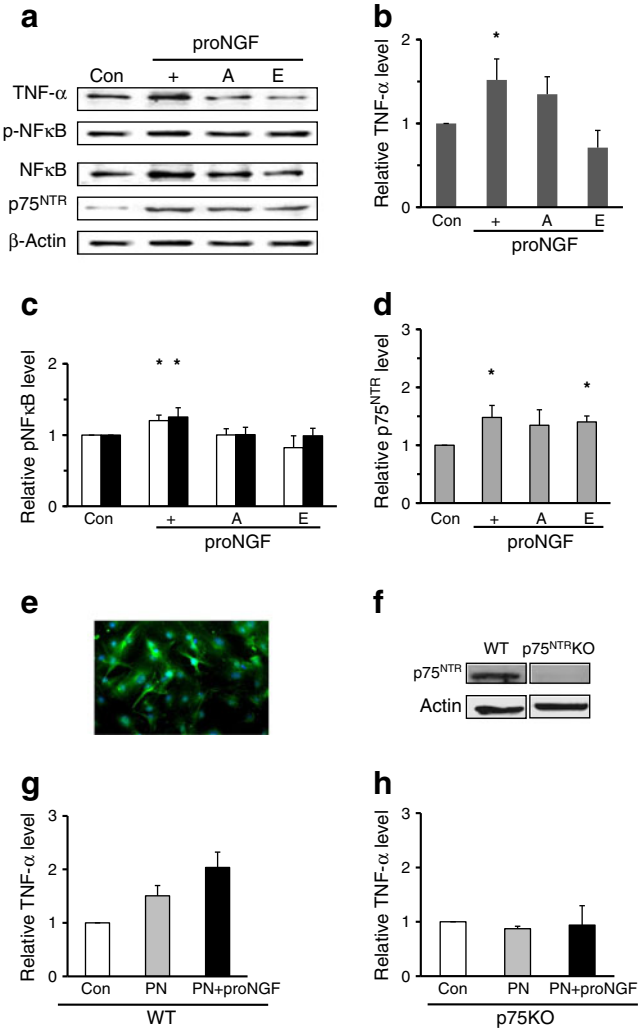

Fig. 8 Modulation of p75^{NTR} action or expression prevents proNGF-mediated inflammation in Müller cells. (**a**) Representative blots and densitometric analysis of TNF-α (**b**), p-NFκB (black columns) and NFκB (white columns) (**c**), and p75^{NTR} (**d**) protein levels in rMC-1 cells grown for 2 days in 25 mmol/l glucose (control) and treated for 16 h with cleavage-resistant proNGF (50 ng/ml) (+) in the presence or absence of the p75^{NTR} antagonist (A) (20 μmol/l) or compound E (E) (10 μmol/l), which is an inhibitor of γ-secretase-catalysed p75^{NTR} cleavage. (n=4–7, *p<0.05) (**e**) Primary Müller cells showed robust expression of the Müller cell marker vimentin (green) in most cells. (**f**) Expression of p75^{NTR} in cultures of WT Müller cells was absent in p75KO Müller cells. (**g**) Statistical analysis of TNF-α in supernatant fractions from primary WT Müller cells and (**h**) p75KO Müller cells exposed to 100 μmol/l peroxynitrite (PN) alone and in combination with 50 ng/ml cleavage-resistant pro-NGF, compared with control cells in 5 mmol/l glucose media alone (n=3)

NFκB protein expression in proNGF-stimulated rMC-1 cells (Fig. 8b, c). Our finding that diabetic insults, including peroxynitrite and proNGF, increased secretion of TNF-α in cultures of WT primary Müller, but not in p75KO Müller cells (Fig. 8g, h) also demonstrates Müller cell involvement in the p75^{NTR}-mediated inflammatory response. In summary, the deletion of p75^{NTR} not only eliminated diabetes-induced glial activation and inflammation, but also prevented ganglion cell loss and BRB breakdown, suggesting that p75^{NTR} inhibition could be a therapeutic target for the early stages of diabetic retinopathy.

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Contribution statement BAM, MMHA, SM, MFE and MAA performed experiments, analysed data and reviewed the manuscript. HUS provided p75NTR antagonist and technical expertise that was necessary for data acquisition, and critically edited the manuscript. ABE and BAM conceived the hypothesis and wrote/revised the manuscript. All authors approved the final version of the manuscript.

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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