

Endothelin B receptor inhibition rescues aging-dependent neuronal regenerative decline

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eLife Assessment

This **important** study examines the role of endothelin signaling in nerve regeneration, providing **convincing** evidence that it functions as a default brake on axon regrowth. Inhibiting endothelin signaling with Bosentan promotes regeneration and counteracts the decline in regenerative potential caused by aging. Since Bosentan is an FDA-approved drug, these findings could have therapeutic value in clinical settings where peripheral nerve regeneration is not adequate or seriously impaired, as is often the case in older individuals.

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Abstract

Peripheral sensory neurons regenerate their axons after injury to regain function, but this ability declines with age. The mechanisms behind this decline are not fully understood. While excessive production of endothelin 1 (ET-1), a potent vasoconstrictor, is linked to many diseases that increase with age, the role of ET-1 and its receptors in axon regeneration is unknown. Using single cell RNA sequencing, we show that satellite glial cells (SGCs), which completely envelop the sensory neuron soma residing in the dorsal root ganglia (DRG), express the endothelin B receptor (ETBR), while ET-1 is expressed by endothelial cells. Inhibition of ETBR *ex-vivo* in DRG explant cultures improves axon growth in both adult and aged conditions. *In vivo*, treatment with the FDA-approved compound, Bosentan, improves axon regeneration and reverses the age-dependent decrease in axonal regenerative capacity. Single-nuclei RNA sequencing and electron microscopy analyses reveal a decreased abundance of SGCs in aged mice compared to adult mice. Additionally, the decreased expression of connexin 43 (Cx43) in SGCs in aged mice after nerve injury is partially rescued by Bosentan treatment. These results reveal that inhibiting ETBR function enhances axon

regeneration and rescues the age-dependent decrease in axonal regenerative capacity, providing a potential avenue for future therapies.

One Sentence Summary

Inhibition of endothelin signaling improves nerve regeneration after central and peripheral injuries and counters age-related regenerative decline.

Introduction

Peripheral nerve injuries, resulting from traumatic injuries, lesions, degenerative diseases, and neuropathies, have a major impact on patient's functioning and quality of life (Maita et al., 2023 [↗](#)). While neurons in the peripheral nervous system regenerate their axons after nerve injury, functional recovery remains very limited because of the slow growth rate of axons and the often long distances that growing axons face to reconnect with their initial targets (Hoke, 2006 [↗](#)). Unfortunately, the ability of injured neurons to regenerate their axons declines with age (Pestronk et al., 1980 [↗](#); Vaughan, 1992 [↗](#); Verdu et al., 2000 [↗](#)), contributing to increases in healthcare costs and the risk of long-term disability (Pestronk et al., 1980 [↗](#); Vaughan, 1992 [↗](#); Verdu et al., 2000 [↗](#); Yun, 2015 [↗](#)). Thus, discovery of therapeutics that promote axon regeneration and counter age-related decline in regenerative capacity is vital.

Primary sensory neurons, with their cell soma located in the dorsal root ganglia (DRG), convey sensory information from peripheral tissue to the brain via the spinal cord, and represent a useful model to identify the molecular and cellular mechanisms that promote axon regeneration. After injury, successful axonal regeneration of sensory neurons requires activation of neuronal-intrinsic epigenetic, transcriptional, and translational programs (He and Jin, 2016 [↗](#); Mahar and Cavalli, 2018 [↗](#); Rishal and Fainzilber, 2014 [↗](#); Tedeschi and Bradke, 2017 [↗](#)). Additionally, non-neuronal cells at the site of injury and in the DRG play an important role in axon regeneration after injury. Satellite glial cells (SGCs) surrounding the cell soma (Avraham et al., 2020 [↗](#); Avraham et al., 2021 [↗](#); Jager et al., 2020 [↗](#)) and Schwann cells surrounding the axon (Jessen and Mirsky, 2016 [↗](#)), undergo changes in transcriptional states that support axon regeneration. Macrophages recruited to the nerve (Ydens et al., 2020 [↗](#); Zigmond and Echevarria, 2019 [↗](#)), and macrophages proliferating in the DRG (Feng et al., 2023 [↗](#)) also contribute to axon regeneration after injury.

During aging, decreases in the efficiency of the axonal transport system (Andrews et al., 2016 [↗](#); Black and Lasek, 1979 [↗](#)), mitochondrial function (Sutherland et al., 2021 [↗](#)), and epigenetic and transcriptional mechanisms (Li et al., 2010 [↗](#)) limit the regenerative capacity of neurons. Increases in pro-inflammatory cytokines and macrophage infiltration into the peripheral nerve (Buttner et al., 2018 [↗](#)), as well as an increased numbers of T cells in the DRG, have been shown to contribute to the limited regenerative capacity of neurons in aged mice (Zhou et al., 2022 [↗](#)). Additionally, age-related decline in the de-differentiation, activation, and senescence of Schwann cells hampers axonal re-growth in damaged peripheral nerves (Fuentes-Flores et al., 2023 [↗](#); Kang and Lichtman, 2013 [↗](#); Painter et al., 2014 [↗](#)). While changes in SGCs morphology (Pannese et al., 1996 [↗](#)), as well as changes in SGCs-neuronal coupling have been reported with age (Huang et al., 2006 [↗](#); Procacci et al., 2008 [↗](#)), the contribution of SGCs to age-dependent decreases in axonal regenerative capacity has not been investigated.

Endothelin B receptor (ETBR), a G-protein coupled receptor, is expressed in SGCs (Mapps et al., 2022 [↗](#); Pomonis et al., 2001 [↗](#)). One of the ligands for ETBR, Endothelin-1 (ET-1), is a potent vasoconstrictor secreted by endothelial cells and is the predominant isoform of endothelin in the human cardiovascular system (Luscher and Barton, 2000 [↗](#); Yanagisawa et al., 1988 [↗](#)). Previous

studies in trigeminal and nodose ganglia have shown that ET-1 activates SGCs through ETBR and reduces gap junction coupling *in vitro* (Feldman-Goriachnik and Hanani, 2017). Studies in astrocytes, which share many functional and molecular features with SGCs (Avraham et al., 2022; Avraham et al., 2020; Hanani and Verkhatsky, 2021) have shown that ETBR inhibition reduces reactive astrocytes and may be a beneficial target to modulate brain diseases (Koyama, 2021). Additional studies in astrocytes have shown that ET-1 signaling via ETBR inhibits the expression of Connexin 43 (Cx43) and reduces gap junction mediated coupling (Blomstrand et al., 2004; Rozyczka et al., 2005). Cx43 is the most highly expressed member of the connexin family in SGCs (Avraham et al., 2022; Huang et al., 2005) and its expression decreases during aging (Procacci et al., 2008). Notably, ET-1 production increases with age and is linked to many age-associated diseases (Barton, 2014; Jankowich and Choudhary, 2020; Stauffer et al., 2008). However, the function of ETBR in SGCs is not known, and whether endothelin signaling contributes to axon regeneration and age-dependent axon regenerative decline has not been examined.

First, to determine the role of endothelin signaling in axon regeneration, we performed unbiased single-cell RNA-sequencing of lumbar DRG from mice 3 months of age (adult). Examination of endothelins and their receptors showed enrichment of *Edn1*, the gene encoding ET-1 in endothelial cells and *Ednrb*, the gene encoding ETBR, in SGCs. Inhibition of ETBR using the selective antagonist BQ788 in DRG explant cultures enhanced axonal outgrowth in adults and restored axonal regenerative capacity in aged conditions. Treatment of adult and aged mice with Bosentan, an FDA-approved ETBR/ETAR antagonist (Clozel et al., 1994), improved axon regeneration after peripheral nerve injury. To further examine the changes in SGCs induced by aging, we performed single-nuclei RNA-sequencing (snRNA-seq) and transmission electron microscopy of DRG cells from adult and 21-month-old mice (aged). We observed decreased abundance of SGCs in aged conditions, together with extensive transcriptional reprogramming in SGCs, reflecting heightened demands for structural integrity, cell junction remodeling, and glia–neuron interactions within the aged DRG microenvironment. Mechanistically, we found that Bosentan treatment increased expression of Cx43 in SGCs after injury in adult and age mice. These results suggest that ETBR signaling limits axon regeneration after nerve injury and plays a role in age-related decline in regenerative capacity. Thus, ETBR antagonism may be a potential therapeutic avenue to enhance axon growth after nerve injury and to restore axon regenerative capacity that declines with age.

Results

Ednrb is highly expressed in SGCs

The dense vascularization of the DRG cell body area coupled with the high permeability of these capillaries in rodents and human (Godel et al., 2016; Jimenez-Andrade et al., 2008) suggests that neurons and their surrounding SGCs may be influenced by vascular-derived signals. Whole mount preparation of DRG from Lycopersicon Esculentum Lectin (LEL) injected *Fabp7^{CreER::Ai14}* mouse, which labels SGCs with tdTomato (Avraham et al., 2020) revealed the dense vascularization in the neuronal soma rich area of the DRG, as previously reported (Jimenez-Andrade et al., 2008) (Fig. 1A, Figure 1 - Movie S1). Additionally, immunofluorescence staining of neurons and SGCs in the DRG from LEL injected adult mouse demonstrated that blood vessels are closely juxtaposed to SGCs surrounding sensory neuron somas (Fig. 1B).

To understand the role of endothelin intercellular signaling on axon regeneration, we first performed single cell RNA sequencing (scRNA-seq) on lumbar DRGs from adult female mice (3 months of age) using the Chromium Single Cell Gene Expression Solution (10 X Genomics), as described previously (Avraham et al., 2022; Avraham et al., 2020; Avraham et al., 2021). We sequenced a total of 16,764 cells from two female biological replicates with an average of 3,579 genes and 12,090 transcripts detected per cell (Figure 1C, Figure 1-Figure supplement 1A-B, see

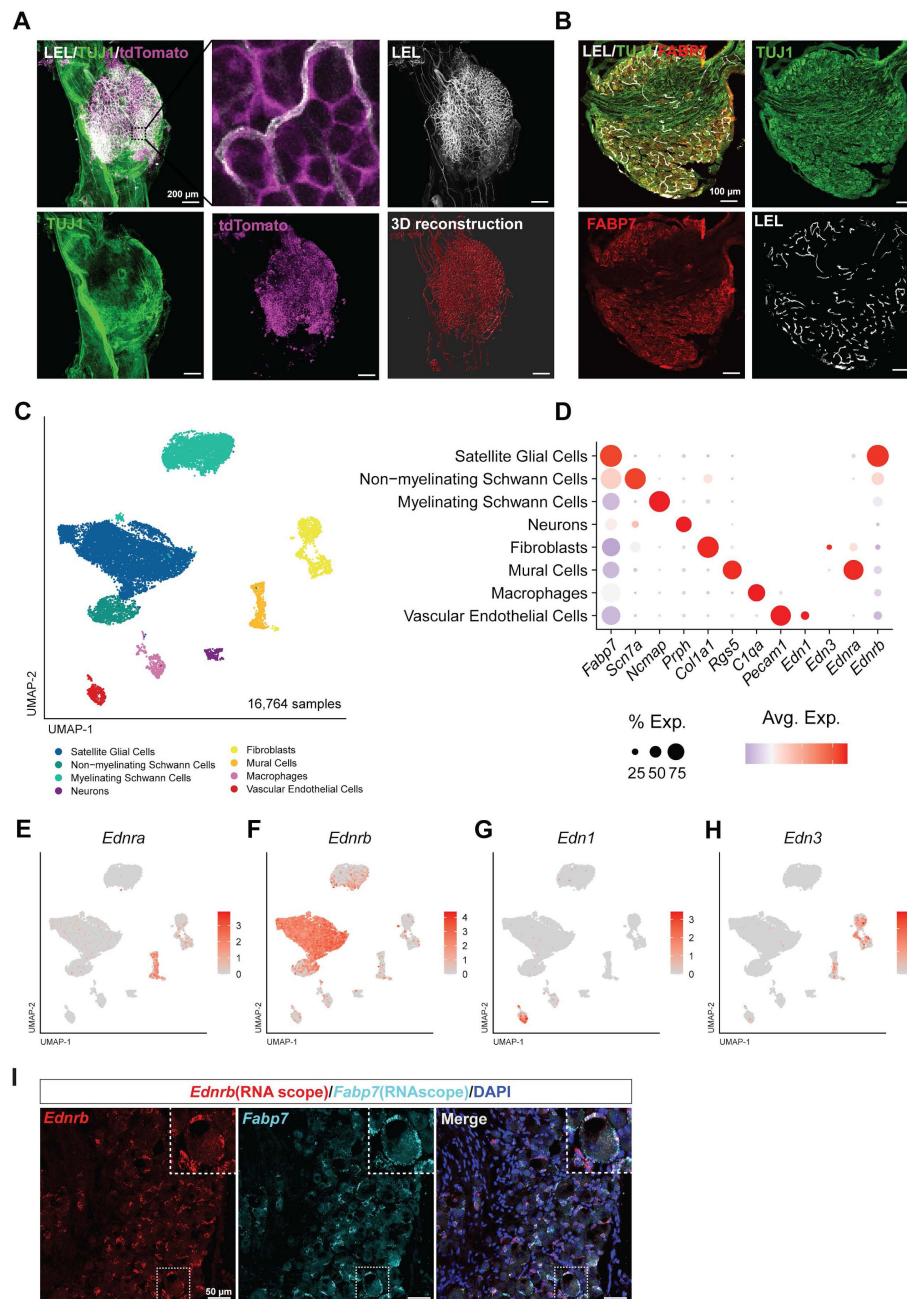


Figure 1

***Ednrb* is highly expressed in satellite glial cells.**

A. Representative whole-mount-stained images of DRG from *Lycopersicon Esculentum* Lectin (LEL) injected *Fabp7CreER::Ai14* mice, labeled for TUJ1 (green), tdTomato (magenta), and LEL (grey). 3D reconstruction of blood vessels via LEL labeling (scale bars, 200 μ m). Note that TUJ1 antibody staining is limited by penetration of the antibody in the whole mount.

B. Representative images of sectioned DRG from *Lycopersicon Esculentum* Lectin (LEL) injected C57BL/6 mice, labeled for TUJ1 (green), *Fabp7* (red), and LEL (cyan) (Scale bars, 100 μ m).

C. UMAP analysis of adult DRG 10X sequencing data identified 8 cell clusters based on known marker genes.

D. Dot plot analysis showing the average gene expression (color coded) and number of expressing cells (dot size) for the marker genes.

E-H. UMAP overlay for expression of *Ednra* (**E**), *Ednrb* (**F**), *Edn1* (**G**) and *Edn3* (**H**).

I. Representative RNAScope *in situ* hybridization images showing *Ednrb* (red), *Fabp7* (cyan) and DAPI (blue) of L4 DRGs from 3-month-old mice (scale bars, 50 μ m).

methods for filtering criteria). Following unsupervised clustering, we identified eight clusters corresponding to the principal cell types of the mouse DRG. Examination of the cluster-specific marker genes verified the presence of major cellular subtypes including SGCs (*Fabp7/Kcnj10*), myelinating Schwann cells (*Ncnmap/Mag*), non-myelinating Schwann cells (*Scn7a/L1cam*), neurons (*Isl1/Prph*), endothelial cells (*Pecam1/Flt1*), fibroblasts (*Col1a1/Pdgfra*), mural cells (*Rgs5/Notch3*), and macrophages (*C1qa/Aif1*) (**Fig. 1D** [↗](#), Figure 1-Figure supplement 1C, Figure 1-supplementary Data File 1). We then examined the expression of endothelin and their receptors. Endothelin (*Edn1*) and endothelin-3 (*Edn3*) were expressed in endothelial cells and fibroblasts, respectively (**Fig. 1 D,G,H** [↗](#)). *Ednrb* was enriched in SGCs (**Fig. 1 D,F** [↗](#)), whereas *Ednra* was expressed mainly in Mural cells (**Fig. 1 D,E** [↗](#)). *Ednrb* enrichment in SGCs is consistent with prior studies (Mapps et al., 2022 [↗](#); Pomonis et al., 2001 [↗](#); Tasdemir-Yilmaz et al., 2021 [↗](#)). Using a published scRNAseq data set across species (Bhuiyan et al., 2024 [↗](#)), we observed similar enrichment of *Ednrb* expression in SGCs in humans (Figure 1-Figure supplement 1D). *Ednrb* mRNA in mouse SGCs was validated by RNA *in situ* hybridization, which showed *Ednrb* co-localized with the SGC marker *Fabp7* (Avraham et al., 2020 [↗](#)) (**Fig. 1I** [↗](#)).

Endothelin B receptor inhibition increases axonal growth *in vitro* and *ex vivo*

To investigate the role of ETBR signaling in axonal growth, we used different compounds to inhibit or activate endothelin receptors in dissociated L4-L5 DRG mixed cultures. In these DRG cultures, SGCs lose their adhesive contacts with neuronal soma and adhere to the coverslip as time progresses (Belzer et al., 2010 [↗](#); Valtcheva et al., 2016 [↗](#)). Within 24 hours in culture, most SGCs are found close to neurons and display flat morphology (**Fig. 2B** [↗](#)) (Belzer et al., 2010 [↗](#)). In these *in vitro* conditions, ET-1 or ET-3 may be released by endothelial cells or fibroblasts. DRG cells were plated and treated with an ETBR antagonist (BQ788), ETBR agonist (IRL1620), endothelin A receptor (ETAR) antagonist (BQ123), or vehicle for 24 hours (**Fig. 2A** [↗](#)). Neurons were then labeled with antibodies against beta III tubulin (TUBJ1) and axonal radial length was quantified. Antagonism of ETBR with BQ788 significantly increased axonal radial length compared to vehicle (Fig.C,D). However, agonism of ETBR with IRL1620 or antagonism of ETAR with BQ123 had no significant effects on axonal radial length compared to vehicle treatment (**Fig. 2C,D** [↗](#)). These results indicate that ETBR signaling limits axonal growth in *in vitro* mixed culture conditions. Inhibition of ETBR may alter trophic factors produced by SGCs into the culture media, leading to axon growth promoting conditions.

To further examine the role of ETBR antagonism on axonal growth, we used DRG explants in which L3-L5 DRGs from adult mice were seeded in matrigel and cultured for 7 days (Feng et al., 2023 [↗](#)). Explants were then treated with vehicle or BQ788 at days *in vitro* (DIV) 1 and radial axon growth was assessed at DIV7 by measuring the length of 50 or more axons per explant (**Fig. 2E** [↗](#)). Radial axon growth was significantly increased in explants treated with BQ788 compared to vehicle treatment (**Fig. 2F-G** [↗](#)). To confirm that the *in vivo* morphology of SGCs enveloping neuronal somas was preserved, DRG explants were immunostained with TUBJ1 for neurons and FABP7 for SGCs at DIV 7 (**Fig. 2H** [↗](#)). These results further indicate that ETBR signaling limits axon growth capacity in an *ex-vivo* model.

Bosentan treatment improves axon regeneration after nerve injury

To determine the potential role of ETBR in regulating nerve regeneration *in vivo*, we used our established *in vivo* regeneration assays (Avraham et al., 2020 [↗](#); Cho and Cavalli, 2012 [↗](#); Cho et al., 2015 [↗](#); Cho et al., 2013 [↗](#)) in combination with the FDA-approved compounds that antagonize endothelin receptors, Bosentan and Ambrisentan. These compounds have been utilized to treat pulmonary arterial hypertension, and their safety and efficacy have been demonstrated through various clinical trials (Chen et al., 2018 [↗](#); Galie et al., 2008 [↗](#); Peacock et al., 2015 [↗](#); Rubin et al.,

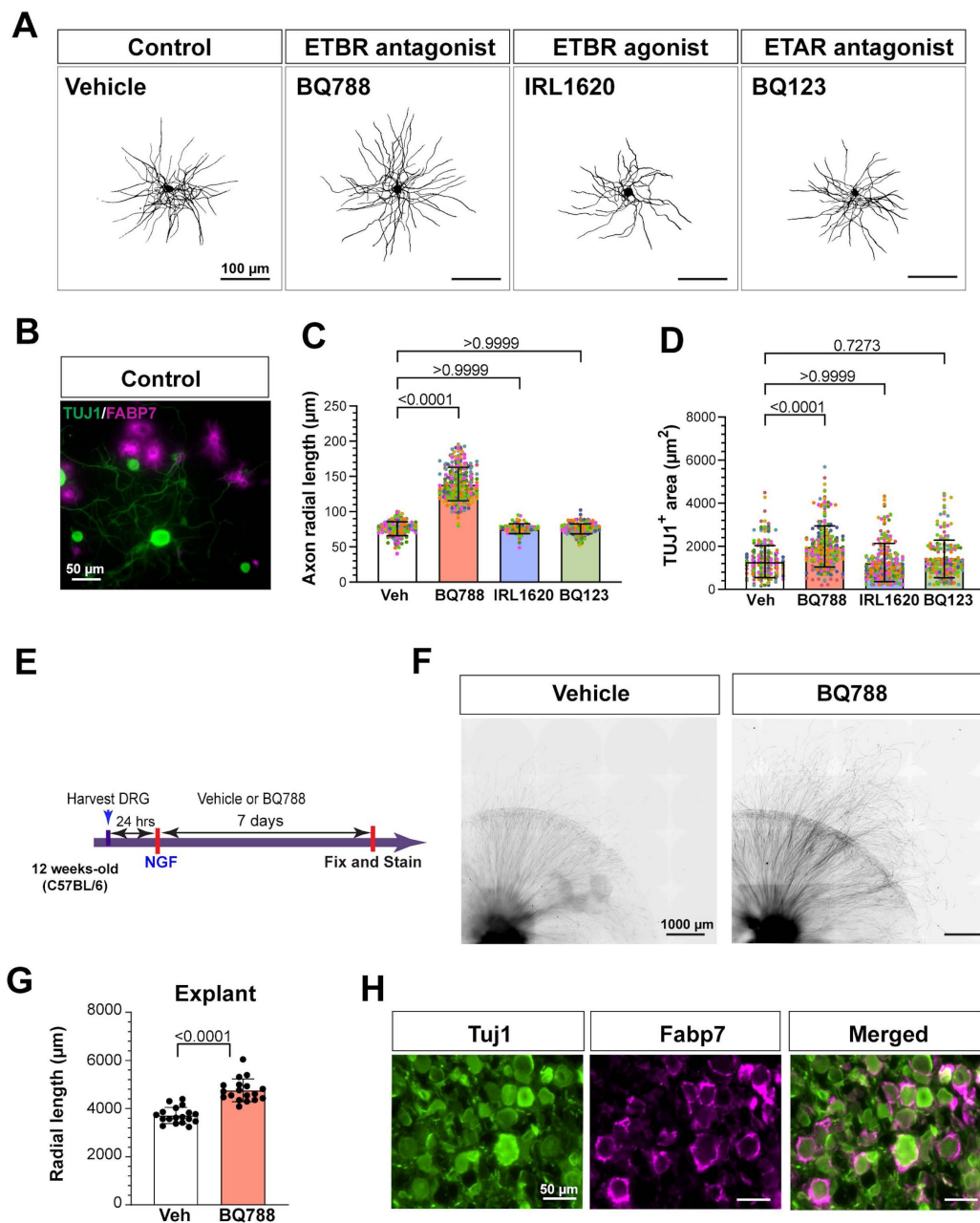


Figure 2

Endothelin B receptor inhibition increases axonal growth *in vitro* and *ex vivo*.

- A.** Representative images showing TUJ1 (black) immunostaining of neurons in DRG cultures (scale bars, 100 μ m).
- B.** Representative image of TUJ1 (green) and FABP7 (magenta) immunostaining of neurons and SGCs in control DRG cultures (scale bars, 50 μ m).
- C,D.** Quantification of axonal radial length (B) and TUJ1⁺ area (C) per neuron. Different colors indicate biological replicates. N = 246 (Veh; 8 replicates), 318 (BQ788; 8 replicates), 320 (IRL1620; 8 replicates), and 244 (BQ123; 8 replicates). Data presented as mean $\pm$ SD.
- E.** Scheme of drug treatment and DRG explant model.
- F.** Representative images of DRG explants 7 days after drug treatment, immunostained for TUJ1 (black) (scale bars, 1000 μ m).
- G.** Quantification of radial length of the 35 longest axons from DRG explants from indicated groups N=36 explants from 6 individual mice (BQ788; 18 replicates, Veh; 18 replicates).
- H.** Representative images of DRG explants immunostained for TUJ1 (green), FABP7 (magenta), and merged (scale bars, 50 μ m).

2002 [\[4\]](#)). Bosentan is an antagonist of both ETAR and ETBR (Clozel et al., 1994 [\[4\]](#)), while Ambrisentan is an antagonist selective for ETAR (Humbert et al., 2004 [\[4\]](#); Newman et al., 2007 [\[4\]](#)). Adult mice were treated orally with vehicle, Bosentan or Ambrisentan 2 hours prior to receiving a sciatic nerve crush (SNC) injury. Axon regeneration was assessed 24 hours after injury by measuring the intensity of SCG10 labeled axons, a marker for regenerating axons (Shin et al., 2014 [\[4\]](#)) (**Fig.3A-E** [\[4\]](#)). Since axon growth is relatively slow 24 hours post injury (Shin et al., 2012 [\[4\]](#)), we first determined whether ETBR inhibition could accelerate axon growth in the first 24 hours. (Shin et al., 2012 [\[4\]](#); Smith and Skene, 1997 [\[4\]](#)). SCG10 intensity was measured distal to the crush site, which was determined according to the highest SCG10 intensity along the nerve (Avraham et al., 2020 [\[4\]](#); Cho et al., 2013 [\[4\]](#); Feng et al., 2023 [\[4\]](#); Shin et al., 2014 [\[4\]](#)) and axon elongation was quantified by measurement of the 10 longest axons, as previously described (Carlin et al., 2019 [\[4\]](#)) (**Fig.3B** [\[4\]](#)). A 50% regeneration index was quantified by normalizing the average SCG10 intensity at distances away from the crush site to the SCG10 intensity at the crush site, to account for both the length and number of regenerating axons past the crush site and plotting the distance at which SCG10 intensity is half of that at the crush site (Cho et al., 2013 [\[4\]](#); Feng et al., 2023 [\[4\]](#)) (**Fig.3C** [\[4\]](#)). Mice that received Bosentan had significantly longer axons and a higher 50% regeneration index 1 day after SNC compared to mice that received vehicle, whereas mice that received Ambrisentan had no significant differences in axon regeneration compared to vehicle (**Fig.3B-D** [\[4\]](#)). Quantification of the percentage of SCG10 intensity normalized to the crush site at several distances from the injury was also greater in Bosentan treated mice compared to vehicle (**Fig.3E** [\[4\]](#)). Similarly, at 3 days post SNC, mice that received Bosentan 2 hours before SNC, and then every 24 hours, had improved axon regeneration compared to vehicle, whereas mice that received Ambrisentan had no significant differences in axon regeneration compared to vehicle (Figure 3 - Figure supplement 1A-E). Examination of *Edn1*, *Ednra*, and *Ednrb* mRNA by RT-qPCR and ETBR by western blot in non-treated mice showed no significant differences in mRNA or protein levels at 3 days post SNC compared to control (Figure 3 - Figure supplement 1F-H). These results suggest that, although ETBR levels do not change after injury, ETBR antagonism increases axon regeneration after peripheral nerve injury at both 1 and 3 days. We next sought to determine whether Bosentan treatment over the course of nerve regeneration improves target re-innervation. Regenerating axons of sciatic nerves extend to the epidermis and start to re-innervate the skin of the hind paw at ~2–3 weeks after injury. We administered Bosentan or vehicle 2 hours prior to SNC, then daily for 7 days, followed by once a week for 2 weeks (**Fig. 3F** [\[4\]](#)). Intraepidermal nerve fiber density (IENFD) in glabrous skin of the hind paw was assessed by quantification of PGP9.5 positive nerve fibers per millimeter of skin 24 days after SNC. Mice that received Bosentan had a trend towards increased IENFD compared to vehicle-treated mice (**Fig.3 G,H** [\[4\]](#)). These results suggest that Bosentan treatment may accelerate target re-innervation after injury.

We next tested if inhibition of ETBR influences the conditioning injury paradigm, in which a prior nerve injury increases the growth capacity of neurons (Smith and Skene, 1997 [\[4\]](#)). DRG neurons from mice that received Bosentan or Ambrisentan 2 hours before SNC, and then every 24 hours for three days, were cultured and axon growth capacity was quantified by measuring the axon radial length and TUJ1 positive area (**Fig.3J-M** [\[4\]](#)). In uninjured condition, neurons extend short neurites, whereas a prior nerve injury leads to neurons growing longer neurites (**Fig. 3J-M** [\[4\]](#)), as expected (Smith and Skene, 1997 [\[4\]](#)). Bosentan, but not Ambrisentan, enhanced the axon radial length of DRG neurons cultured from uninjured adult mice, partially mimicking the conditioning injury, and also increased the growth capacity of injured neurons (**Fig.3J-M** [\[4\]](#)). Since the selective ETAR antagonists, BQ123 and Ambrisentan, had no significant effects on axon regeneration after nerve injury (**Fig. 2A-C** [\[4\]](#), **3A-E** [\[4\]](#)), our results indicate that ETBR signaling limits axon regenerative capacity.

To determine the effects of Bosentan treatment on axon regeneration in a model with lower regenerative capacity, axon growth after dorsal root crush (DRC) injury was assessed (Figure 3 - Figure supplement 2A). Following DRC injury, axon growth occurs at about half the rate of peripheral axons (Oblinger and Lasek, 1984 [\[4\]](#); Wujek and Lasek, 1983 [\[4\]](#)) and our previous work

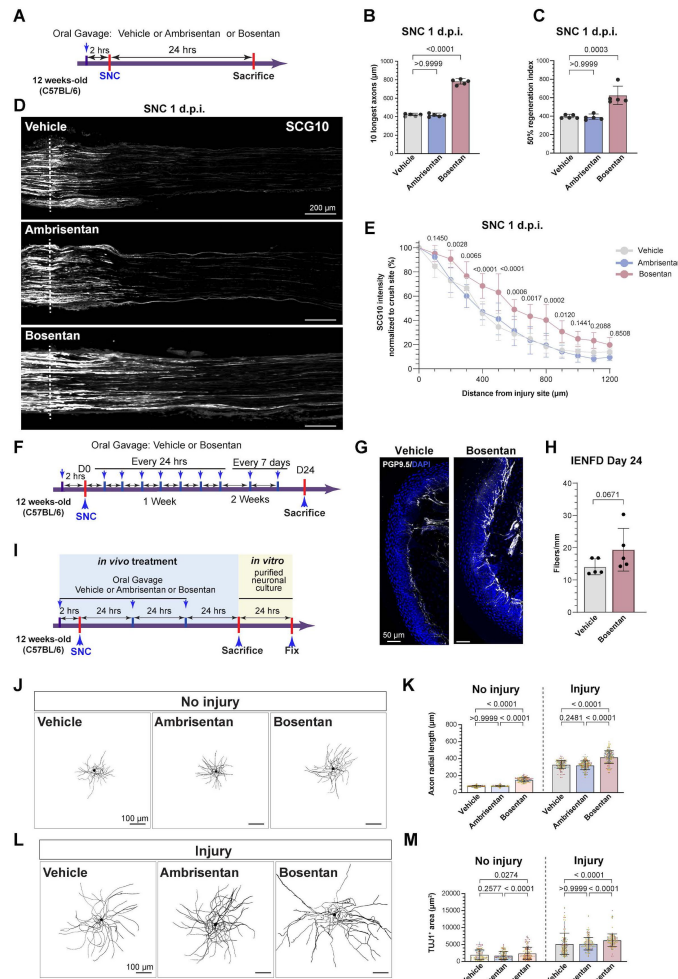


Figure 3

Bosentan treatment improves axon regeneration after peripheral nerve injury in adult mice.

- A.** Scheme of drug treatment and peripheral nerve injury model.
- B.** Quantification of the length of the 10 longest axons in indicated conditions.
- C.** Quantification of the regeneration index, calculated as the distance along the nerve where the SGC10 intensity is 50% of the SGC10 intensity at crush site.
- D.** Representative longitudinal sections of sciatic nerves 24h after SNC, immunostained for SGC10, from mice with the indicated treatment. Dotted line indicates the crush site, determined as the maximal SGC10 intensity (scale bars, 200 μ m).
- E.** Quantification of SGC10 intensity at the indicated distance normalized to the intensity at the crush site for each condition. N = 5 mice/condition.
- F.** Scheme of long-term Bosentan treatment.
- G.** Representative images of hindpaw skin after long-term Bosentan treatment immunostained for PGP9.5 (white) and DAPI (blue) (scale bars, 50 μ m).
- H.** Quantification of intraepidermal nerve fiber density (IENFD) 24 days after sciatic nerve crush from the indicated groups. N=5 mice/condition.
- I.** Scheme of adult DRG neuronal culture and treatments.
- J,L.** Representative images showing TUJ1 (black) immunostaining of neurons in DRG cultures (scale bars, 100 μ m).
- K,M.** Quantification of axonal radial length (K) and total TUJ1⁺ area (L). Different colors represent different biological replicates. N (neuron number) = 177 (vehicle; three biological replicates, naïve mice), 168 (Ambrisentan, three biological replicates, naïve mice), 183 (Bosentan; three biological replicates, naïve mice), 186 (vehicle; three biological replicates, injured mice), 204 (Ambrisentan, three biological replicates, injured mice) and 210 (Bosentan; three biological replicates, injured mice), respectively. The data are presented as mean $\pm$ SD.

has shown that manipulation of SGCs after DRC improves axon regeneration (Avraham et al., 2021). Axon regeneration at 3 days post DRC was assessed by measuring SCG10 intensity, as described above. Mice that received Bosentan had significantly more axon regeneration along the dorsal root compared to vehicle (Figure 3 - Figure supplement 2B-E). Together, these results indicate that ETBR inhibition improves axon regeneration after both peripheral and central axon injury in DRG neurons.

Inhibiting ETBR rescues aging-dependent axonal regenerative decline

It is known that elevated tissue or plasma concentrations of ET-1 occurs with age (Jankowich and Choudhary, 2020). We thus evaluated the expression levels of ET-1 and ETBR in DRG of adult and aged mice by western blot. For loading controls, we used both ponceau staining for total protein and GAPDH, as we observed lower levels of GAPDH in aged mice. Regardless of the normalization method, we found that ET-1 levels were significantly elevated in aged mice compared to adult (Figure 4 - Figure supplement 1A). ETBR levels were only elevated in aged mice when normalized to the GAPDH control (Figure 4 - Figure supplement 1BB). These results confirm that ET-1 levels are elevated in DRG tissue in aged mice.

To determine the role of ETBR signaling on axon growth during aging, DRG explants from aged (21-month-old) mice were treated with BQ788 or vehicle (Fig. 4A). Aged DRG explants treated with BQ788 had significantly increased radial axon length compared to vehicle (Fig. 4B-C). In fact, aged explants treated with BQ788 had significantly more radial axon growth than to that of adult (3-month old) vehicle explants (Fig. 4C). *In-vivo* Bosentan pre-treatment in aged mice also increased radial axon growth in DRG explants (Figure 4 - Figure supplement 1C-E). Axon regeneration after peripheral nerve injury in aged mice *in vivo* was assessed by measuring SCG10 intensity in adult and aged mice 3 days post SNC. As expected, aged vehicle-treated mice had significantly less axon regeneration compared to adult vehicle-treated mice (Fig. 4D-G) (Zhou et al., 2022). However, aged mice treated with Bosentan had significantly increased axon regeneration compared to vehicle-treated aged mice. Indeed, treatment with Bosentan increased axon regeneration in aged mice to levels similar to vehicle-treated adult mice (Fig. 4D-G). These results indicate that inhibition of ETBR rescues the age-dependent decline in axon regeneration.

Aging affects SGCs abundance, transcriptional profile, and morphology

To examine the effect of age on the transcriptional profiles of SGCs, we isolated lumbar DRG from adult and aged mice and performed single nucleus RNA-seq using the Illumina Single Cell 3' RNA Prep (Illumina). We chose this microfluidics-free system for single-nuclei mRNA capture, barcoding, and library prep because it has been shown to be gentler and capture rarer cell populations in the brain (Frazel et al., 2023), and our initial attempt with 10X Genomics on aged tissue yielded insufficient amount of viable cells. After quality control and filtering, we recovered 17,852 high-quality single-nucleus transcriptomes (10,717 adult, 7,135 aged) across three biological replicates (one adult, two aged), with an average of 2,854 genes and 9,899 transcripts detected per cell (Figure 5 - Figure supplement 1A-B). Following RPCA-based integration by age, unsupervised clustering identified 16 distinct cell types (Fig. 5A). These included four principal neuronal classes (PEP, NF, NP, TH), three glial cell types (SGCs, NMSC, MSC), three previously described fibroblast subsets (EF, EM, PM) (Zhao et al., 2022) vascular endothelial cells, mural cells, and several immune-related populations (MYEL, TC, BC, HSC) (Fig. 5A). Each cluster was distinguished by multiple unique marker genes, consistent with prior DRG single-cell studies (Avraham et al., 2020; Avraham et al., 2021; Jung et al., 2023; Mapps et al., 2022; Renthal et al., 2020; Usoskin et al., 2015) (Fig. 5B).



G. Quantification of the SCG10 intensity at the indicated distance normalized to the intensity at the crush site for each condition. N (mouse number) = 4(adult, vehicle), 3 (Aged, vehicle) and 3 (Bosentan+Age), respectively. The data are presented as mean $\pm$ SD.

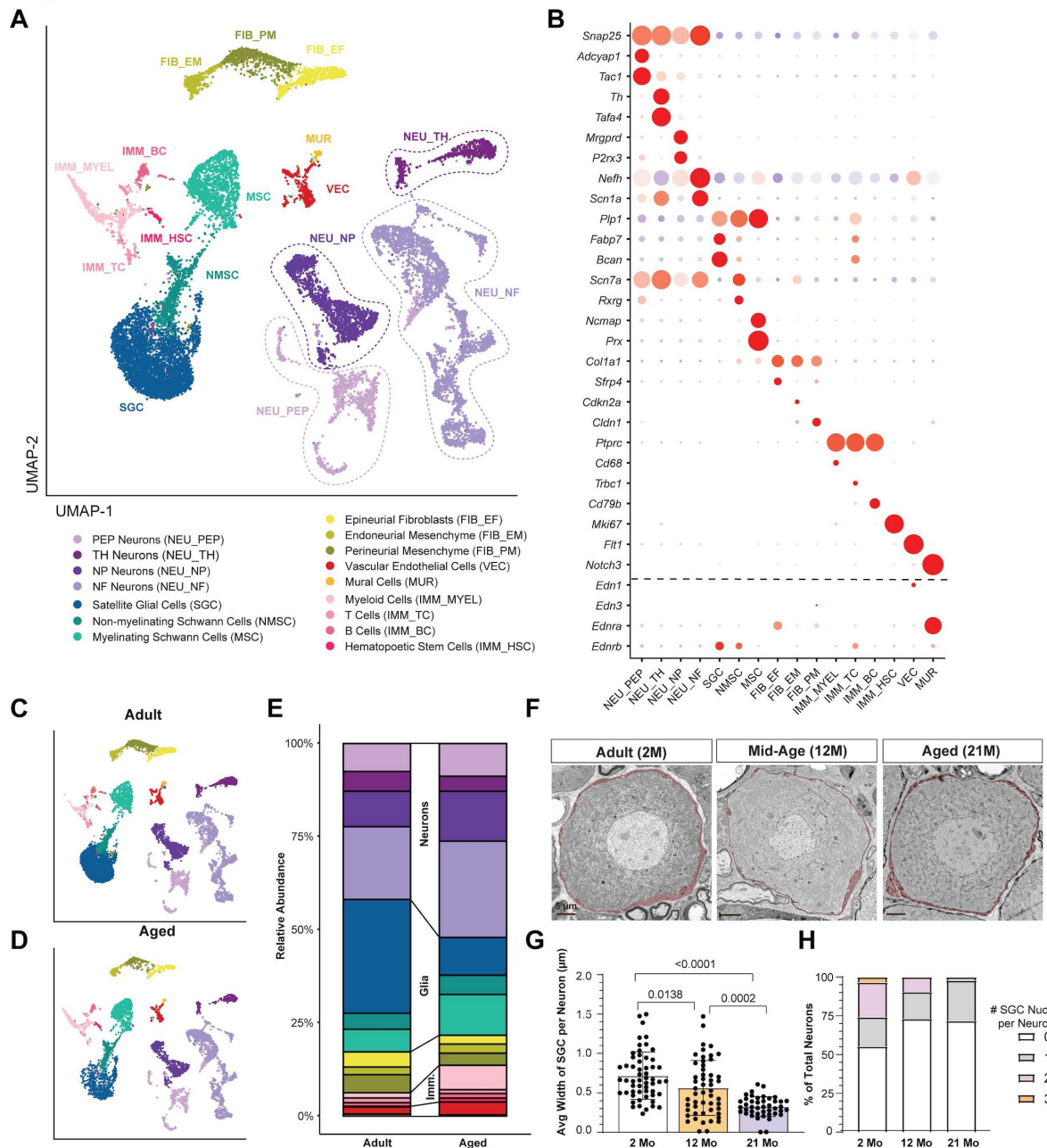


Figure 5

Aging alters SGC abundance and morphology.

- A.** UMAP plot of adult and aged snRNA-seq identified 16 cell clusters based on known marker genes.
- B.** Dot plot analysis showing the average gene expression (color coded) and number of expressing cells (dot size) for the marker genes.
- C,D.** UMAP plot of DRG cells from adult (**C**) and aged (**D**) mice.
- E.** Bar plot of cell proportions in DRGs of adult and aged mice.
- F.** Representative TEM images of DRG sections from adult (2M), middle aged (12M) and aged (21M) mice showing neuronal cell bodies and the enveloping SGCs (SGCs are pseudo-colored in red) (scale bars, 5 μ m).
- G.** Quantification of the average width of SGCs sheath per neuron soma.
- H.** Frequency of neurons soma in TEM images with 0, 1, 2, or 3 SGCs nuclei in 2M, 12M, and 21M old mice.

To further investigate aging-associated transcriptional changes in SGCs, we performed differential expression analyses followed by GO (gene ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment (Figure 5 - Figure supplement 1C,D). Many genes were upregulated in aged SGCs, including those involved in cell junction assembly, adherens and tight junction pathways, and focal adhesion, indicating significant alterations in cell-cell contacts. In addition, we observed changes in pathways regulating cell projection organization, synapse organization and structure, and supramolecular fiber organization, suggesting widespread remodeling of SGC architecture. Several signaling pathways implicated in structural and morphogenetic processes, such as axon guidance and Wnt signaling, were also enriched in aged SGCs. Genes related to cellular senescence were also upregulated in aged SGCs (Figure 5 - Figure supplement 1D). Collectively, these results suggest that aging triggers extensive transcriptional reprogramming in SGCs, reflecting heightened demands for structural integrity, cell junction remodeling, and glia–neuron interactions within the aged DRG microenvironment.

Comparative analysis of relative cell-type abundances between adult and aged DRG revealed a marked decrease in SGC representation and a corresponding rise in immune cell and neuronal populations in aged samples (**Fig. 5C–E**). Changes in morphology of SGCs have been reported with age in rabbit, with a decrease in SGCs number and SGCs retracting and leaving the neuronal soma exposed to the extra cellular DRG environment (Pannese et al., 1997; Pannese et al., 1996). In the DRG of aged mice, neurons appeared to be enveloped by SGCs similarly to adults (Huang et al., 2006), but quantification of SGCs number were not performed. We thus examined the impact of age on SGCs morphology and number by transmission electron microscopy (TEM). In adult mice, each sensory neuron was enveloped by its own SGCs sheath as described previously (Pannese, 1981; Pannese, 2010). In aged mice however, SGCs appeared thinner compared to adult mice (**Fig 5F**). Quantification of the average width of SGCs per neuron demonstrated a significant decrease during aging (**Fig.5G**). Quantification of the number of SGC nuclei per neuronal soma in TEM images revealed that 21-month-old mice had fewer SGCs per neuron compared to 2-month-old mice (**Fig.5H**), indicating a decrease in SGC number with age. These results suggest that, in addition to significant transcriptional changes, SGCs may undergo atrophy during aging.

ETBR inhibition increases the expression of Cx43 in SGCs in adult and aged mice

Cx43 is a member of the connexin family and can form hemichannels and gap junction channels (Mazaud et al., 2021). Several studies suggest that both Cx43 gap junctions and hemichannels operate in SGCs and have an important role in communication between SGCs and between SGCs and sensory neurons (Hanani and Spray, 2020; Retamal et al., 2017). Cx43 in human SGCs was proposed to play a key role in the protection and maintenance of neurons in spiral ganglia (Liu et al., 2014). Past studies in mice have shown that Cx43 expression in SGCs decreases during aging (Procacci et al., 2008). In cultured SGCs and astrocytes, endothelin signaling reduces the expression of Cx43 (Blomstrand et al., 2004; Feldman-Goriachnik and Hanani, 2017; Rozyczka et al., 2005). We thus investigated the role of endothelin signaling and aging in Cx43 expression in SGCs. *Gja1*, the gene for Cx43, is enriched in SGCs in both adult and aged mice (**Fig.6A**), as expected (Procacci et al., 2008). Changes in *Gja1* expression level with age were statistically difficult to assess because there were less SGCs in aged mice compared to adult, and *Gja1* is only detected in 20% of SGCs. We therefore quantified Cx43 protein expression level in SGCs by immunofluorescence (**Figure 6** - Movie S2). The level of Cx43 expression, measured by both the Cx43 expression area and the number of Cx43 puncta in Fabp7 positive SGCs surrounding neuronal soma, was significantly lower in aged mice compared to adult mice (**Fig.6C–D**), consistent with prior studies (Ohara et al., 2008; Procacci et al., 2008). Nerve injury increased Cx43 levels in both adult and aged vehicle-treated mice and Bosentan treatment led to an increase in Cx43 expression in both adult and aged mice (**Fig.6C–E**). Examination of Cx43 expression in SGCs after DRC in adult mice showed no increase in Cx43 levels compared to uninjured mice, but

Bosentan-treated mice increased Cx43 levels compared to vehicle (Figure 6 - Figure supplement 1A-C). These results reveal a correlation between Cx43 levels in SGCs and axon regenerative capacity, providing a potential mechanism by which ETBR inhibition with Bosentan may promote axon regeneration after injury (**Fig. 6F**). Future experiments will determine if Cx43 acts downstream of ETBR and how Cx43 regulates axon regeneration.

Discussion

Peripheral nerve injuries have a major impact on patient's functioning and quality of life (Maita et al., 2023). The ability of injured neurons to regenerate their axons declines with age (Geoffroy et al., 2016; Geoffroy et al., 2017; Pestronk et al., 1980; Vaughan, 1992; Verdu et al., 2000), contributing to an increased risk of long-term disability (Pestronk et al., 1980; Vaughan, 1992; Verdu et al., 2000; Yun, 2015). Our study shows that ETBR functions in part to limit axonal regenerative capacity and that Bosentan, an FDA-approved ETBR/ETAR antagonist, increases axonal regeneration after peripheral and central axon nerve injury. Furthermore, Bosentan rescues the age-dependent decrease in axonal regenerative capacity. These results suggest that ETBR inhibition may be a beneficial therapeutic to promote axon regeneration after injury.

Our results demonstrate that *Ednrb* is highly enriched in SGCs in the DRG, and selective antagonism of ETBR in DRG mixed cultures and DRG explants increases axon outgrowth. Furthermore, we show that Bosentan treatment *in vivo* increases axon growth 1 and 3 days after nerve injury, and long-term Bosentan treatment may improve target reinnervation 3 weeks after injury. It is important to note that in the DRG mixed cultures, SGCs, while still present, do not envelop the neuronal soma as they do in the explant culture and *in vivo*. However, antagonism of ETBR in DRG mixed cultures still increases axon growth. Thus, it is possible that inhibition of ETBR alters the release of trophic factors from SGCs, stimulating the growth potential of neurons and accelerating axon growth. Future studies will be required to unravel how ETBR signaling influences the SGC secretome and its influence on axon growth. Additionally, orchestration of axon regeneration in the nerve depends on multiple cell types. Polarized vascularization in the nerve precedes Schwann cell migration (Bhat et al., 2024; Cattin et al., 2015). Since ETBR is expressed in Schwann cells and has been shown to play a role in Schwann cell generation (Brennan et al., 2000), we cannot rule out the possibility that Bosentan treatment *in vivo* may affect axon regeneration through ETBR in Schwann cells in addition to SGCs. However, given our results demonstrating that DRG explants maintain the SGC-sensory neuron morphology, and axon outgrowth in this model does not rely on repair Schwann cells, we can conclude that blocking ETBR signaling in SGCs contributes to increasing axon outgrowth, at least in the initial axon growth phase. Together, these results suggest a novel molecular mechanism in peripheral nerve regeneration.

We hypothesize that the major ligand acting on ETBR in the DRG is ET-1. This is based on our scRNA-seq data in the DRG showing *Edn1*/ET-1 enrichment in endothelial cells, as well as the dense vascularization in DRG. Additionally, the observation that elevated tissue or plasma concentrations of ET-1 occurs with age (Jankowich and Choudhary, 2020) and our results showing that ET-1 levels increase in the aged DRG support the notion that ETBR signaling in SGCs limits axon regenerative capacity in aged mice. However, other studies have suggested that ET-1 is also expressed by small diameter neurons in human DRG (Giaid et al., 1989), thus it is possible that neuronally derived ET-1 may act on ETBR in SGCs. Additionally, our scRNA-seq data shows that another ligand for ETBR, *Edn3*/ET-3, is enriched in fibroblasts in the DRG. While ET-1 binds to ETAR and ETBR with the same affinity, ET-3 shows a higher affinity to ETBR than to ETAR (Davenport et al., 2016). It also has been shown in cultured cortical astrocytes that application of either ET-1 or ET-3 leads to a robust inhibition of connexin Cx43 expression (Rozyczka et al., 2005). Thus, we cannot exclude the possibility that ET-3 may act on ETBR in SGCs to regulate axon growth. Lastly, as our scRNA-seq data shows that *Ednra* is enriched in mural cells (Chen et al.,

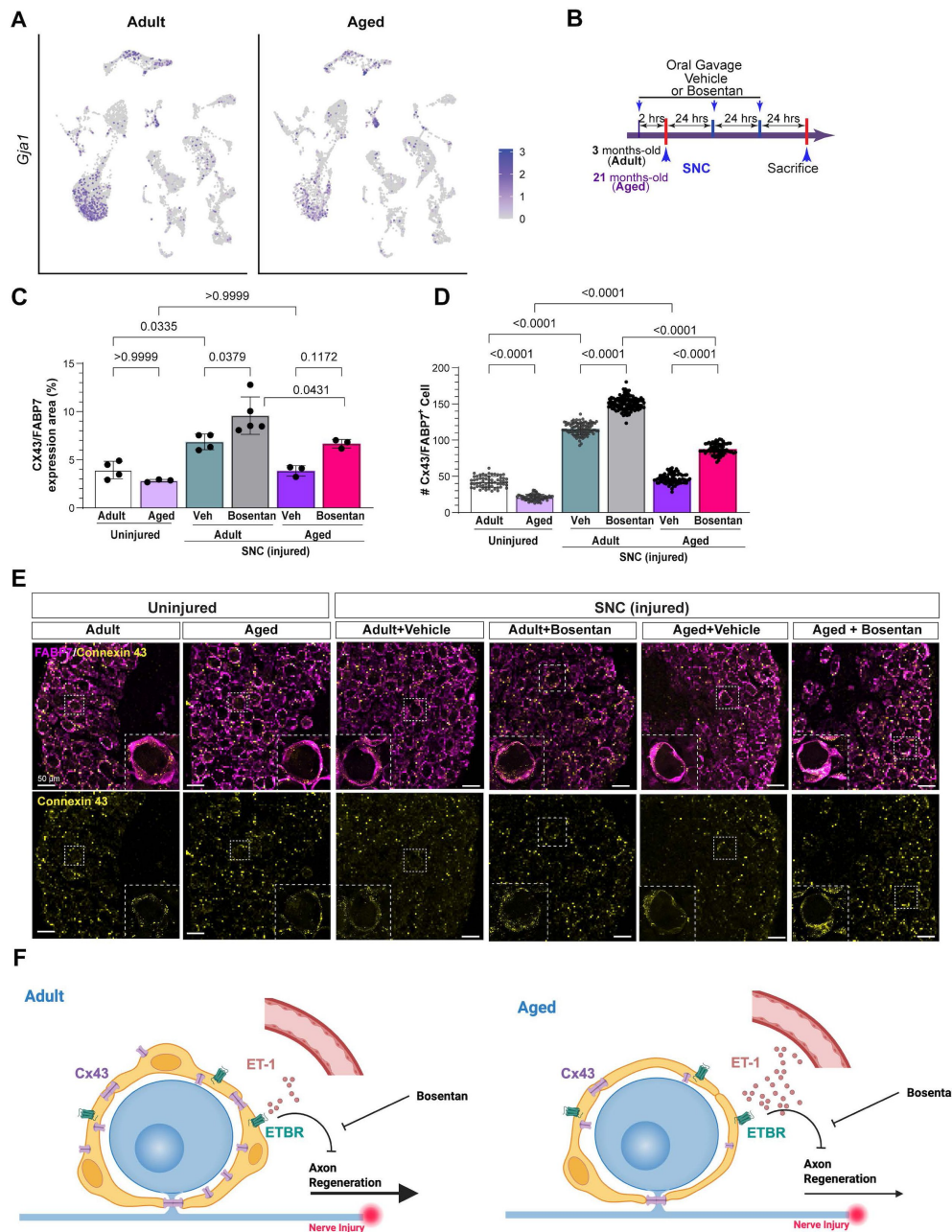


Figure 6

ETBR inhibition increases the expression of Cx43 in SGCs in adult and aged mice.

- A.** UMAP overlay for expression of *Gja1* in adult and aged mouse DRG.
- B.** Scheme of drug treatment and peripheral nerve injury model.
- C.** Quantification of the percentage of the Cx43/FABP7 expression area.
- D.** Quantification of the average number of Connexin 43 (Cx43) puncta per FABP7+ cell. The ratio of total Cx43 puncta to the number of FABP7+ cells surrounding a TUJ1+ neuron was measured. N(cell number) = 60(adult, uninjured), 62(aged, uninjured), 96(vehicle, adult, SNC), 117(bosentan, adult, SNC), 74(vehicle, aged, SNC) and 74(bosentan, aged, SNC), respectively.
- E.** Representative immunostaining images showing Connexin 43 (Cx43), FABP7 and TUJ1 in L4 DRGs from the indicated condition (scale bars, 50 μ m).
- F.** Proposed model for the role of ETBR in age-dependent decline in axon regenerative capacity

2000 [\[10\]](#)), it is also possible that endothelin's impact on vascular permeability through ETAR activity contributes to axon regeneration. Future experiments will be required to test how injury and age affects vascular permeability in the DRG. Nonetheless, whether via ET-1 or ET-3, our results highlight that ETBR acts to limit spontaneous sensory axon regeneration, and that inhibition of ETBR promotes axon regeneration.

Although a biologically intrinsic mechanism to inhibit axon regeneration appears counterintuitive, other mechanisms limiting axon regenerative capacity have been reported. Axonally synthesized proteins typically support nerve regeneration through retrograde signaling and local growth mechanisms ([Sahoo et al., 2018 \[11\]](#)). RNA binding proteins (RBP) are needed for this process and other aspects of post-transcriptional regulation of neuronal mRNAs. One such RBP, the RNA binding protein KHSRP is locally translated following nerve injury, promotes decay of other axonal mRNAs and slows axon regeneration ([Patel et al., 2022 \[12\]](#)). Additionally, Rho signaling pathway is an inhibitory regulatory mechanism that slows the growth of spiral ganglion neurite in culture ([Lie et al., 2010 \[13\]](#)). These mechanisms may function to limit plasticity and prevent maladaptive neural rewiring that can happen after injury ([Costigan et al., 2009 \[14\]](#)), but can also hinder beneficial recovery after injury. Whether ETBR inhibition over a longer time period has consequences on target innervation and functional recovery will require further investigation.

Investigation of the effects of aging on sensory ganglia have shown that the number of neurons decrease with age in different species including human, cats and rats ([Aldskogius and Risling, 1989 \[15\]](#); [Nagashima and Oota, 1974 \[16\]](#); [Schmalbruch, 1987 \[17\]](#)). The effect of age on SGCs has received less attention, but we know that the number of SGCs decrease with age in the rabbit ([Pannese et al., 1997 \[18\]](#); [Pannese et al., 1996 \[19\]](#)). In agreement with these prior studies, our snRNA-seq and morphology data reveal a marked decrease in SGC representation in the DRG of aged mice. Our EM data indicate that the number of SGCs surrounding each neuronal soma decreases with age and appear thinner, suggesting SGC atrophy in addition to SGC loss. KEGG pathway analysis also suggests that aged SGCs transition into a senescent phenotype during aging, a state where cells no longer divide but remain viable. SGCs have the capacity to divide, although this process is very slow ([Maniglier et al., 2022 \[20\]](#)). It was proposed that SGC self-renewal maintained the SGC population through adulthood, ensuring neuronal survival throughout life ([Maniglier et al., 2022 \[20\]](#)). This balance may become dysfunctional with age, leading to the accumulation of senescent SGCs. In the nerve, senescence of Schwann cells in aged mice was shown to undermine axonal regeneration, and systemic elimination of senescent cells with senolytic drugs improves axonal regeneration ([Fuentes-Flores et al., 2023 \[21\]](#)), raising the interesting possibility that senescent SGCs also contribute to limit axon regeneration in aged mice. Future studies are needed to determine if aged SGCs, similarly to aged Schwann cells, secrete inhibitory factors that limit axon regeneration. We also observed changes in pathways regulating cell projection organization, synapse organization and structure, and supramolecular fiber organization, suggesting widespread remodeling of SGC architecture. Several signaling pathways implicated in structural and morphogenetic processes, such as axon guidance and Wnt signaling, were also enriched in aged SGCs. Collectively, these results suggest that aging triggers extensive transcriptional reprogramming in SGCs, reflecting heightened demands for structural integrity, cell junction remodeling, and glia–neuron interactions within the aged DRG microenvironment.

Cx43 is a transmembrane protein that performs canonical gap junction functions, such as connecting adjacent cells and allowing the diffusion of ions and small molecules. Cx43 can also form hemichannels that may have an important role in communication between SGCs and sensory neurons ([Retamal et al., 2017 \[22\]](#)). Emerging evidence suggests that connexins can perform non-channel functions, such as protein interaction, cell adhesion, and intracellular signaling ([Mazaud et al., 2021 \[23\]](#)). Using our snRNA-seq data to compare *Gja1* levels in adult vs aged SGCs was statistically difficult to interpret, as there were significantly fewer SGCs in aged mice. The histology examining Cx43 protein levels was normalized to total SGCs, and showed a significant

decrease in Cx43 puncta/SGC in aged mice, consistent with previous studies and the hypothesis that a prominent decrease in Cx43 is a marker of senescence (Procacci et al., 2008 [DOI](#)). It has also been shown in SGCs of aged mice that there is an increase in gap junctions and dye coupling (Huang et al., 2006 [DOI](#)). This apparent discrepancy could be explained by expression changes in connexin types other than Cx43 that may contribute to the gap junctions. Indeed, our snRNA-seq data revealed changes in other connexins with age, but the percentage of cells expressing these genes and the level of expression was not robust enough to draw meaningful conclusions. Deeper sequencing would likely be required to verify this hypothesis. It is also important to note that, while sequencing is a strong tool, aging has profound effects on post-translational regulation, such as altered protein half-life, defects in protein trafficking, and increased protein degradation (Santos and Lindner, 2017 [DOI](#); Skariah and Todd, 2021 [DOI](#)). Thus, in conjunction with sequencing, examination of protein levels of various connexins in SGCs will be necessary.

Our results show that Cx43 in SGCs from both adult and aged injured mice increased with Bosentan treatment compared to vehicle treatment. Studies in cultured SGCs and astrocytes have demonstrated that endothelin signaling reduces gap junctions coupling (Blomstrand et al., 2004 [DOI](#); Feldman-Goriachnik and Hanani, 2017 [DOI](#); Rozyczka et al., 2005 [DOI](#)). Thus, we hypothesize that ETBR inhibition in SGCs contributes to axonal regeneration by increasing Cx43 levels, gap junction coupling or hemichannels, facilitating SGC-neuron communication. However, it is also possible that other mechanisms downstream of ETBR signaling in SGCs contribute to axon regeneration. For example, glutamate uptake by SGCs is regulated by glutamate transporters GLT-1 and GLAST and is crucial for neuronal function (Berger and Hediger, 2000 [DOI](#); Hanani and Verkhratsky, 2021 [DOI](#); Ohara et al., 2008 [DOI](#)). Studies in astrocytes suggest that endothelin exposure reduces glutamate uptake by decreasing transporter expression. One of the functions of GLAST in astrocyte is related to lesion-induced plasticity in the developing brain (Takasaki et al., 2008 [DOI](#)), suggesting that ETBR might limit axon regeneration by influencing SGCs' glutamate reuptake. As ETBR is a G protein-coupled receptor which interacts with Gi and Gq (but not Gs) (Ham et al., 2024 [DOI](#); Tani et al., 2024 [DOI](#)) its inhibition could have numerous effects on intracellular signaling pathways and transcriptional response in SGCs. Further studies are needed to determine the detailed molecular mechanisms by which ETBR signaling in SGCs limits axon regeneration.

Taken together, our results suggest that ETBR signaling limits axon regeneration after nerve injury and contributes to age-related decreases in neuronal regenerative capacity. Bosentan, an FDA approved ETBR/ETAR antagonist, significantly increased axon regeneration in both adult and aged mice, suggesting a potential therapeutic avenue that may be used to enhance axonal regeneration after nerve injury.

Materials and methods

Study design

The main goals of this study were to investigate the role of ETBR in axonal regeneration, explore its impact on SGCs in the DRG, and evaluate the therapeutic potential of Bosentan, an FDA-approved drug, on axon regeneration and age-related decline capacity for nerve repair. We performed scRNA-seq and snRNAseq analysis on DRG samples from adult and aged mice. We analyzed samples from mice subjected to various treatments and performed *in vitro* assays including immunofluorescence (IF), Western blotting, quantitative real-time polymerase chain reaction (qPCR), RNA *in situ* hybridization (RNAscope) and TEM. For *in vitro* cell assays, data were collected from at least three independent cultures, as indicated in figure legends. *In vivo* and *ex-vivo* experiments were conducted using biological replicates, as denoted by the 'n' values in the figure legends. The sample sizes were determined based on previous experience for each experiment, and mice were randomly assigned to the experimental groups whenever possible. No

mice, outliers, or other data points were excluded. Details on animal assignment, randomization, and blinding in different experiments are found in the corresponding sections describing each experiment in Materials and Methods.

Animals

Mice of different age groups were included in the study, specifically 2-3-month-old (adult, female and male), 12-month-old (mid-age, female), and 21-month-old (aged, female). Wild-type C57BL/6 mice were purchased from Envigo (Envigo #027) and Jackson Laboratory (Stock No: 000664). Ai14 (B6.Cg-Gt(ROSA)26Sortm14(CAG-tdTomato)Hze/J, JAX Stock No: 007914) mice was obtained from The Jackson Laboratory (Madisen et al., 2010 [DOI](#)). The *Blbp^{CreER}* (*Fabp7^{CreER}*) mouse line was a generous gift from Dr. Toshihiko Hosoya (Maruoka et al., 2011 [DOI](#)). Ai14 mice were crossed with *Fabp7^{CreER}* mice to obtain *Fabp7^{CreER}::Ai14* mice. Mice were housed in the animal facility at Washington University in St. Louis, where temperature (64-79°F) and humidity (30%-70%) were carefully controlled. They were socially housed in individually ventilated cages, with 1-5 mice per cage, and subjected to a 12-hour light/dark cycle (6 am/6 pm). Mice had unrestricted access to food and water throughout the study. All experimental procedures were conducted following the approved protocol (21-0104) by the Institutional Animal Care and Use Committees of Washington University in St. Louis. All experiments adhered to relevant guidelines and regulations. The study obtained approval from the Washington University School of Medicine Institutional Animal Care and Use Committee (IACUC) under protocol A-3381-01. The mice were housed and cared for in the animal care facility at Washington University School of Medicine, which is accredited by the Association for Assessment & Accreditation of Laboratory Animal Care (AALAC) and complies with the PHS guidelines for Animal Care. The facility has been accredited since 7/18/97, and its USDA Accreditation Registration number is 43-R-008.

Primary adult DRG culture

L4 and L5 DRGs were collected from 12-week-old mice and placed in cold dissection medium composed of HBSS (Thermo Fisher, Gibco; Catalog#: 14175-079) with 10% 1M HEPES (Thermo Fisher, Gibco; Catalog#: 15630080). The DRGs were then transferred to freshly prepared pre-warmed dissociation medium containing 15U/mL Papain suspension (Worthington Biochemical; Catalog#: LS003126), 0.3mg/mL L-cysteine (Sigma; Catalog#: C7352), 0.1mg/mL Deoxyribonuclease I (Worthington Biochemical; Catalog#: LS002139), and 10% 1M HEPES in HBSS. The samples were incubated at 37°C for 20 minutes. After washing the samples twice with pre-warmed HBSS, collagenase (150µg/mL; Sigma; Catalog#: C6885) was added, and the samples were incubated at 37°C for another 20 minutes. Following two additional washes with pre-warmed HBSS, the resulting single-cell suspension was gently triturated and resuspended in complete medium consisting of Neurobasal™-A Medium (Thermo Fisher, Gibco; Catalog#: 12349015) supplemented with B-27™ Plus Supplement (Thermo Fisher, Gibco; Catalog#: A3582801) and GlutaMAX™ Supplement (Thermo Fisher, Gibco; Catalog#: 35050061). The cell suspension was then passed through 70-micron cell strainers. The single-cell suspension was then centrifuged at 500 × rpm at 4°C for 5 minutes, and the cell pellet was resuspended in complete Neurobasal medium. Cells were seeded on poly-D-lysine (PDL)-coated 18mm coverslips at a density of 1.0×10^3 cells per coverslip. For drug treatment, BQ788 (Sigma; Catalog #B157) 1µM, BQ123 (R&D system; Catalog #: 1188) 1mM, IRL620 (Sigma; Catalog #: SCP0135) 100nM or DMSO as vehicle control were added directly to the complete medium just before suspending the cells and seeding them into separate culture plates and incubated for the entire 24-hour culture period. The animals were randomized into groups and the researcher was not blinded during the analysis.

DRG Explant culture

L3-L5 DRGs from male and female 8-wk- or 21-months- old mice were collected into cold explant media [Putrescine Dihydrochloride (Sigma Aldrich, Catalog# p7505), L-Glutamine (Thermofisher, Catalog# A2916801), Insulin Transferrin Selenium (Sigma Aldrich, Catalog# A4034), Glucose (Sigma

Aldrich, Catalog# G7021), Basal Medium Eagle (ThermoFisher, Catalog# 35050061), Bovine Serum Albumin (Sigma Aldrich, Catalog# A9430), Ham's F-12 Nutrient Media (ThermoFisher, Catalog# 11765-054)]; DRG roots were trimmed to 2mm with a sharp scalpel, then placed into a 15ul drop of a 1:1 ratio of explant media and ECM Gel from Engelbreth-Holm-Swarm murine sarcoma (Sigma Aldrich, Catalog# E1270) in cell culture plates. 24 hours after plating, Nerve Growth Factor (NGF) (Alomone, Catalog# N-240) was added to the media. Note that NGF does not promote significant effects on axon growth in DRG explants, but stimulates glial cell migration (Klimovich et al., 2020 [DOI](#)). We opted to include NGF in our explant assay to increase the potential of stimulating axon regeneration with pharmacological manipulations of ETBR. Seven days after plating, explants were fixed and stained for TUJ1 to quantify axon length.

Sciatic nerve and dorsal root injuries

Sciatic nerve crush injuries were performed following established protocols (Avraham et al., 2021 [DOI](#)). Briefly, 12-week-old mice were anesthetized using 1.5% inhaled isoflurane. A small skin incision was made to expose the sciatic nerve at mid-thigh level, approximately 1.2 cm from the L4 dorsal root ganglion (DRG). The sciatic nerve was fully crushed for 10 seconds using 0.1mm forceps (#55). The wound was closed with wound clips, and the mice were placed on a warming pad until fully awake. At the designated time points post-surgery, L4 and L5 dorsal root ganglia were dissected for further analysis. The animals were randomized into groups.

Dorsal root crush injuries were performed as previously described (Avraham et al., 2021 [DOI](#)). Surgery was conducted on 12-week-old mice under 1.5% inhaled isoflurane anesthesia. A small midline skin incision (~1 cm) was made over the thoracic vertebrae at L2-L3, followed by paraspinal muscle release and stabilization of the vertebral column with metal clamps under the L2-L3 transverse processes. Dorsal laminectomy was performed using forceps at the L2-L3 level, and the right L3-L5 dorsal roots were crushed simultaneously for 5 seconds. The proximity between L3-L5 roots resulted in a crush distance of 1-2 mm to L3 DRG, 4-5 mm to L4 DRG, and 7-8 mm to L5 DRG. The crushing process exerted force on the roots, causing disruption of nerve fibers without interrupting the endoneurial tube. Paraspinal muscles were sutured using 6-0 sutures (Ethicon, Catalog#: J212H), and the wound was closed with clips. Mice were placed on a warming pad until fully awake, and L4 dorsal root ganglia and L4 dorsal roots were dissected at 3 days post injury. The animals were randomized into groups.

Drug Administration

To assess the *in vivo* functions of endothelin receptors, pharmacological testing was conducted. For the analysis of nerve regeneration at one day post-injury, oral gavage administration of Ambrisentan (Tocris, Catalog# 5828, 10 mg/kg body weight) (Kappes et al., 2020 [DOI](#)) and Bosentan (Sigma, Catalog# PHR2708, 10 mg/kg body weight) (Pinho-Ribeiro et al., 2014 [DOI](#)) was performed 2 hours before the injury, with sample collection taking place 24 hours after the injury. For the analysis of nerve regeneration after 3 days post-injury, Ambrisentan (10 mg/kg body weight) and Bosentan (10 mg/kg body weight) were administered via oral gavage 2 hours before the injury and once daily after the injury. The treatments were randomized within the surgery mice group.

Western Blotting

L4-L5 DRGs were collected and lysed in RIPA Buffer (Cell Signaling, catalog #9806) and heated at 99°C for 10 min. The protein lysates were then loaded onto a 10% Ready Gel® Tris-HCl Precast Gels (BioRad, catalog#1611119) in running Buffer (BioRad, Catalog#1610744) and transferred to 0.2 µm PVDF membranes (BioRad, Catalog#1620216). The membranes were blocked in 5% non-fat dry milk (BioRad, Catalog#1706404XTU) Tris-Buffered Saline (TBS) containing 0.1% Tween-20 at pH 7.6 for 1 h. Following blocking, the membranes were incubated overnight at 4°C with primary antibodies Rabbit-anti-ETBR (Abcam, Catalog# ab117529, 1:500) Mouse-anti-ET-1 1:500 (Invitrogen, Catalog# MA3-005) Rabbit-anti-GAPDH 1:5000 (Cell Signaling, Catalog# 5174s) in blocking buffer.

Afterward, the membranes were washed three times with TBST (Tris-buffered saline with 0.1% Tween-20) and then incubated with horseradish peroxidase-conjugated anti-rabbit (Invitrogen, Catalog# 31460,1:5000) or anti-mouse (Invitrogen, Catalog # 31430,1:5000) antibodies in blocking buffer for 1 h at room temperature. Subsequently, the membranes were washed three times with TBST and developed using SuperSignal™ West Dura (Thermo Scientific, Catalog# 34075). The ChemiDoc System (Bio-Rad) was used to image the membranes. For subsequent analysis, the membranes were stripped using a mild stripping buffer composed of (1.5% glycine, 0.1% SDS, and 1% Tween -20, pH2.2 in ddH₂O). Following stripping, the protocol was repeated for GADPH staining (Santa Cruz Biotechnology, Catalog #sc-51907, 1:8000) as the loading control. The resulting images were analyzed using Fiji software. To quantify the protein levels, the intensity of ET-1 or ETBR was normalized to the intensity of GADPH. The researcher was not blinded during the analysis.

Quantitative real time PCR

For qRT-PCR, L4 and L5 DRGs of each mouse were collected and total RNA was extracted using RNeasy Mini Kit (QIAGEN, Cat# 74104). For cDNA synthesis, 500 ng of RNA was converted into cDNA with the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher, Catalog# 4368814) according to manufacturer's specifications. Quantitative PCR was completed using the PowerUp™ SYBR™ Green Master Mix (ThermoFisher, Cat# A25780) using gene-specific primers (resource table) from Primerbank (<https://pga.mgh.harvard.edu/primerbank/>). qRT-PCR was performed on a QuantStudio 6 Flex System. Expression fold change for each gene of interest was calculated using the ΔC_q method and normalized to the expression fold change of *Gadph* expression compared to controls. The researcher was not blinded during the analysis. The detail for each primer was listed in Supplementary Table 1.

Immunohistochemistry

Mice were euthanized with CO₂ asphyxiation and transcardially perfused with PBS followed by 4% paraformaldehyde (PFA). PFA-fixed tissues were incubated in 30% sucrose in phosphate-buffered saline (PBS) overnight at 4 °C, specimens were embedded in optimal cutting temperature compound (OCT) (Tissue-Tek), stored at -80 °C until further processing. Transverse sections of DRG (L4) and longitudinal sections of sciatic nerve were cut on a cryostat at 10μm, and stored at -20 °C until processed. Before staining, sections were warmed to room temperature and dried on a 60 °C slide warmer for 5 mins. Sections were treated with a blocking solution containing 4% normal donkey serum (NDS) (LAMPIRE; Catalog#7332100) with 0.5% Triton X-100 in PBS for 1h at room temperature. Then samples were incubated in the primary antibodies, which were diluted in 1% NDS with 0.3% Triton X-100 in PBS overnight at 4°C. After three PBS rinses, samples were incubated with Alexa Fluor-conjugated secondary antibodies in PBS with 0.3% Triton X-100 in 1h, followed by incubation in 300nM 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, Catalog# D9542) at room temperature for 10 mins. Samples were rinsed before mounting with ProLong™ Gold Antifade Mountant (Invitrogen, Catalog#:P36930). For co-staining of Cx43 and Fabp7, both of which are rabbit antibodies, the tissue sections were first blocked with 10% NDS in PBS containing 0.3% Triton X-100 at room temperature for 1h. Subsequently, the sections were incubated overnight at 4°C with rabbit anti-Cx43 antibody diluted in 0.1% Triton X-100 in PBS. After three rinses with PBS, the samples were incubated with an excess of conjugated Fab Fragment secondary antibody in PBS with 0.3% Triton X-100 for one hour. Following three washes with PBS, the sections were incubated with rabbit anti-Fabp7 antibody, diluted in 0.1% Triton X-100 in PBS, at room temperature for 2h. After three PBS washes, the samples were incubated with Alexa Fluor-conjugated secondary antibodies in PBS with 0.3% Triton X-100 for one hour. Finally, the sections were incubated with DAPI at room temperature for 10 minutes, followed by rinsing and mounting with ProLong™ Gold Antifade Mountant. DRG sections were imaged with a confocal laser-scanning microscope (Zeiss LSM880). Figures showing large longitudinal sciatic nerve sections were produced using EVOS™ M7000 Imaging System with image stitching and/or stack software. For adult DRG culture staining, neurons were fixed in pre-warmed 1%PFA in 7.5%

sucrose at 37 °C for 15 mins. Then post-fixed in prewarmed 2%PFA in 15% sucrose in 30 mins. Rise with PBS 3 times, the fixed neurons were directly for immunostaining following same staining protocol described above. Cultured neurons were imaged with acquired using the ECLIPSE Ti2 inverted microscope.

Whole-mount DRG immunostaining was performed by injecting 0.1 μ L of Lycopersicon Esculentum Lectin (Vector, Catalog# DL-1178-1) through the tail vein to label the blood vessels (Robertson et al., 2015 [\[4\]](#)). L4 DRGs were dissected after 20 minutes and fixed overnight in 4% PFA at 4°C. Following a previously established protocol (Yang et al., 2017 [\[4\]](#)), DRGs underwent a series of washing and permeabilization steps with 0.3% Triton X-100 in PBS, repeated every hour for 5 hours. Subsequently, the tissues were incubated with primary antibodies in a blocking solution consisting of 75% 0.3% PBST, 20% DMSO, and 5% Donkey Serum for 4 days, followed by further washing with 0.3% Triton X-100 in PBS every hour for 5 hours. The tissues were then incubated with secondary antibodies in the blocking solution for 2 days, with subsequent washing using 0.3% Triton X-100 in PBS every hour for 5 hours. All of these washing and incubation procedures were carried out on a rocking platform at room temperature. The tissues were dehydrated in methanol for 1 hour and cleared using a 1:2 mixture of Benzyl Alcohol to Benzyl Benzoate. Imaging was carried out using an LSM880 confocal microscope, and blood vessel reconstruction was performed with Imaris 9.7.

Primary antibodies included rabbit anti-FABP7 (Invitrogen, Catalog#:PA5-24949, 1:1000); rabbit anti-STMN2(SCG10) (Novus a bio-technie, Catalog#:NBP1-49461, 1:1000); rabbit anti-Connexin-43 (Cell Signaling, Catalog#: 3512s, 1:200); mouse anti TUJ1 (β -III tubulin) (Biolegend, Catalog#:801202, 1:1000). Secondary antibodies conjugated to Alexa Fluor 488, Alexa Fluor 594 and Alexa Fluor 647 (Invitrogen), conjugated Fab Fragment Donkey anti Rabbit Alexa Fluor (Jackson ImmunoResearch Labs) were diluted 1:500.

For intraepidermal nerve fibers density measurements, fixed, free floating 50- μ m thick sections of footpads from the hind limbs were stained with rabbit anti-Protein Gene Product 9.5 (1:500, LSBIO, Cat# LS-B5981-50), followed by AF594 conjugated secondary antibody (1:500, Invitrogen, Cat# A-21207). Sections were mounted and coverslipped using VECTASHIELD anti-fade mounting media with DAPI (Vector Laboratory, Cat# H-2000). PGP 9.5 positive intraepidermal nerve fibers (IENFs) crossing into the epidermis were counted from Z-stack images using the 20x objective on a Nikon W1 CSU SoRa. IENF densities were averaged from three to four sections for each animal. Counting was performed with the observer blinded to the treatment group.

RNAscope® in situ hybridization

The RNAscope® fluorescent multiplex reagent kit (Advanced Cell Diagnostics, ACD) was used according to the manufacturer's instructions. Slides were retrieved from a -80°C freezer, washed with PBS to remove OCT, and dried on a 60°C slide warmer for 5 minutes. Post-fixation was performed by immersing the slides in cold (4°C) 4% paraformaldehyde (PFA) for 15 minutes. Tissues were dehydrated using a series of ethanol washes (50% ethanol for 5 minutes, 70% ethanol for 5 minutes, and 100% ethanol for 10 minutes) at room temperature. Slides were air dried briefly and then incubated in RNAscope® Target Retrieval buffer (ACD; Catalog# 322000) at 98-100°C for 5 minutes. After rinsing in 100% ethanol, hydrophobic boundaries were drawn around each section using a hydrophobic pen (ImmEdge PAP pen; Vector Labs). Once the boundaries were dry, protease III reagent was added to each section and incubated for 15 minutes. Slides were briefly washed in PBS at room temperature. Each slide was placed in a prewarmed humidity control tray (ACD) with dampened filter paper, and a mixture of probes was pipetted onto each section until fully submerged. The slides were then incubated in a HyBEZ oven (ACD) at 40°C for 2 hours. Following the probe incubation, the slides were washed twice with 1x RNAscope wash buffer and returned to the oven for 30 minutes after submersion in AMP-1 reagent. This washing and amplification process was repeated using AMP-2, AMP-3, and AMP-4 reagents with incubation periods of 15 minutes, 30 minutes, and 15 minutes, respectively. For all mouse experiments, AMP-4

ALT A (Channel 1 = Atto 488, Channel 2 = Alexa 550, Channel 3 = Atto 647) was used. Slides were then washed twice with 0.1M phosphate buffer (PB, pH 7.4). Subsequently, the slides were processed with 300nM DAPI at room temperature for 10 minutes and cover-slipped using Prolong Gold Antifade mounting medium. DRG sections were imaged using a confocal laser-scanning microscope (Zeiss LSM880).

Transmission electron microscopy

For transmission electron microscopy (TEM), mice were perfused PBS and then with the fixative (2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M Cacodylate buffer), followed by post-fixation in same fixation 24hrs at 4°C. For secondary post fixation, the samples were rinsed in 0.15 M cacodylate buffer containing 2mM calcium chloride 3 times for 10 minutes each followed by a secondary fixation in 1% osmium tetroxide and 1.5% potassium ferrocyanide in 0.15 M cacodylate buffer containing 2 mM calcium chloride for 1 hour in the dark. The samples were then rinsed 3 times for 10 minutes each in ultrapure water and en bloc stained with 2% aqueous uranyl acetate overnight at 4 °C in the dark. After 4 washes for 10 minutes each in ultrapure water, the samples were dehydrated in a graded acetone series (10%, 30%, 50%, 70%, 90%, 100% x3) for 10 minutes each step, infiltrated with Spurr's resin (Electron Microscopy Sciences) and embedded and polymerized at 60 °C for 72 hours. The samples from adult mice were processed as above except they were dehydrated in a graded ethanol series (10%, 30%, 50%, 70%, 90%, 100% x3) for 10 minutes each step, infiltrated with Epon resin (Electron Microscopy Sciences) and embedded and polymerized at 60 °C for 48 hours. Post curing, 70 nm thin sections were cut, post-stained with 2% aqueous uranyl acetate and Sato's lead and imaged on a TEM (Jeol JEM-1400 Plus) at 120 kV.

Single Cell RNA sequencing

L4 and L5 DRGs from mice were collected and placed into cold dissection medium consisting of Hank's Balanced Salt Solution (HBSS; ThermoFisher, Gibco; Catalog#: 14175-079) supplemented with 10% 1M HEPES (ThermoFisher, Gibco; Catalog#: 15630080). Subsequently, the ganglia were transferred to freshly prepared pre-warmed dissociation medium composed of 15 U/mL Papain suspension (Worthington Biochemical; Catalog#: LS003126), 0.3 mg/mL L-cysteine (Sigma; Catalog#: C7352), 0.1 mg/mL Deoxyribonuclease I (Worthington Biochemical; Catalog#: LS002139), and 10% 1M HEPES in HBSS. The samples were then incubated at 37°C for 20 minutes. After two washes with pre-warmed HBSS, the ganglia were incubated with collagenase (150 µg/mL; Sigma; Catalog#: C6885) at 37°C for 20 minutes. Following an additional two washes with pre-warmed HBSS, the resulting single cell suspension was resuspended by gently triturating in complete medium consisting of Neurobasal™-A Medium (ThermoFisher, Gibco; Catalog#: 12349015) supplemented with B-27™ Plus Supplement (ThermoFisher, Gibco; Catalog#: A3582801) and GlutaMAX™ Supplement (ThermoFisher, Gibco; Catalog#: 35050061). The cell suspension was then passed through 70-micron cell strainers. The single cell suspension was further resuspended in HBSS containing 10% 1M HEPES and 0.1% Fetal Bovine Serum (FBS; ThermoFisher; Catalog#: A3160401). The cells were stained with the LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (ThermoFisher; Catalog#: L34965) to identify live cells. Live single cells were sorted using the MoFlo cell sorter (Beckman Coulter, Indianapolis, IN). The sorted cells were washed with a PBS+0.04% BSA solution and manually counted using a hemocytometer. The cell solution was adjusted to a concentration of 700-1000 cells/µL and loaded onto the 10X Chromium system.

Single-cell RNA-Seq libraries were prepared using the Chromium Next GEM Single Cell 3' (v3.1 Kit) from 10X Genomics. Read alignment and transcript counting were performed with the Cell Ranger (v7.1.0) package from 10X Genomics and 19,356 cells with >300 detected genes were retained for downstream analysis. Sample QC, principal component analysis, clustering, DGE, quantification, and statistical analysis were performed using the Seurat package (v5.1.0). Following normalization with SCTransform, principal component analysis, and graph-based clustering, four low quality and/or doublet clusters were identified and removed based on the following characteristics: statistical enrichment (DGE $p < 0.05$) of mixed major cell type marker genes or mitochondria-

specific transcripts, and/or low average genes per cell (<1000). After removal of low-quality clusters, cells with disproportionately high mitochondrial transcript levels (calculated as the fraction of reads mapping to mitochondrial genes) or UMI counts were filtered by setting a cutoff at the median plus five median absolute deviations (MAD) for each metric. The remaining 16,764 high-quality cells were visualized using UMAP (Uniform Manifold Approximation and Projection). Differential gene expression (DGE) analyses were performed in the Seurat package with a Wilcoxon Rank Sum test, and genes with Bonferroni-adjusted p-values < 0.05 were considered for downstream analysis and visualization.

Single Nucleus RNA sequencing

L3, L4 and L5 DRGs were collected from mice and immediately placed on dry ice. DRG were stored in -80 until processing. Nuclei were isolated using the Illumina Nuclei Isolation Kit (Illumina; Catalog# 20132795). The DRG were dissociated using a KIMBLE 2mL Dounce homogenizer (Sigma; Catalog# D8938) in Nuclei Extraction Buffer (NEB) and passed through a 40-micron cell strainer. The nuclei were briefly centrifuged and then passed through a 10-micron cell strainer. The nuclei were resuspended in Nuclei Suspension Buffer (NSB) and counted for intact nuclei using 7-AAD stain (Sigma; Catalog# SML1633). Nuclei were processed using the Illumina Single Cell 3' RNA Prep, T20 Kit (Illumina; Catalog# FB0005366, FB0005373, FB0005362, FB0005382)

Single-nuclei RNA-Seq libraries were prepared using the Illumina V Library Prep Kit (Illumina; Catalog# 20132789, FB0005374, FB0005373). Read alignment and transcript counting were performed with the Pipeseeker (v3.3.0) package from Fluent BioSciences and 19,270 cells with >1000 genes were retained for downstream analysis (11,521 adult, 7,749 aged). Sample QC, integration, clustering, DGE, quantification, and statistical analysis were performed using the Seurat package (v5.1.0). Following normalization with SCTransform, RPCA batch integration, and graph-based clustering, five low quality and/or doublet clusters were identified and removed based on the following characteristics: statistical enrichment (DGE $p < 0.05$) of mixed major cell type marker genes or mitochondria-specific transcripts, and/or low total cell count (<50). The remaining 17,852 high-quality cells were visualized using UMAP. Differential gene expression (DGE) analyses were performed in the Seurat package with a Wilcoxon Rank Sum test, and genes with Bonferroni-adjusted p-values < 0.05 were considered for downstream analysis and visualization. KEGG pathway and GO enrichment were performed using the clusterProfiler package in R. The researcher was not blinded during analysis.

Images acquisition and quantification

Confocal images were captured under the LSM880 confocal microscope (Carl Zeiss) unless otherwise specified. The images were captured under following parameters: 1 × optical zoom, scan speed 6, averaged 2 times, a pinhole of 1 AU, 1024 × 1024 pixel size. The z-stack images were projected into overlay images using the “Maximum Intensity Projection” function in Zen Black software (Carl Zeiss). Sciatic nerve longitudinal images, the spinal cord and brain images were captured under the EVOS™ M7000 Imaging System (Invitrogen). The images were captured under 10x objective in 1980 × 1080 pixel size. The stitched nerve images generated by the EVOS imaging system. The neuronal culture images were captured under the ECLIPSE Ti2 inverted microscope (Nikon). The images were captured under 10x objective in 2304 × 2304 pixel size. Three tissue sections were analyzed on average for each independent mouse. The researcher performing this analysis was not blinded during analysis.

For quantifying Cx43 expression area in the DRG, the CX43⁺ and Fabp7⁺ area was measured using “Analysis Particles” function in Fiji. To quantify CX43, confocal imaging with Z-stacks (10 μm) was performed using an LSM880 microscope. The acquired images were subsequently used for 3D reconstruction using Imaris 9.7 software. The staining area of FABP7 underwent initial surface reconstruction, while the CX43 puncta were isolated through spot reconstruction at a resolution of 0.1 μm x 0.2 μm. The average number of CX43 puncta was determined for each FABP7⁺ cell

(Supplementary Video S1). For quantifying the regeneration index in the injured sciatic nerve section, the highest SCG10 intensity along the nerve was defined as the crush site, as described (Cho et al., 2013 [DOI](#); Feng et al., 2023 [DOI](#)). The average SCG10 intensity at distances away from the crush site was normalized to the SCG10 intensity at the crush site using “measure” function in Fiji. For quantifying the neuron regenerative capacity in cultured DRG neurons, TUJ1 stained neurons were segmented by thresholding and subjected to neurite tracing using “Simple Neurite Tracer” plug-in in Fiji. The researcher was not blinded during analysis.

DRG explant analysis was performed by creation of an ROI around each explant. The ROI was removed for organoid contrast and the radial length was measured from the outer peripheral section of the ROI. The 35 longest axons in each image were measured (μm) and averaged.

For TEM, SGCs width around the neuron was measured by averaging 4 individual points that did not contain an SGCs nucleus around the neuron using Fiji/ImageJ software. Frequency of neurons with 0,1,2, or 3 SGCs nuclei per neuron was quantified by counting each image by eye.

Statistical analysis

All statistical analyses and graphs were conducted, organized and generated in GraphPad Prism 9. Numerical data were presented as mean $\pm$ SD from at least three independent animals. Group mean difference was analyzed by either two-tailed unpaired Student's t-test or one-way ANOVA with Bonferroni post-hoc test. For experiments including groups and multiple time-point measurements, data were analyzed by two-way ANOVA with Bonferroni post-hoc test. P values below 0.05 were considered as significant difference. The number of animals in each group is presented in figure legends. No a priori exclusion criteria or statistical power calculations were used.

Data and materials availability

All data associated with this study are presented in the paper or the Supplementary Materials. scRNA and snRNA sequencing data have been deposited at NCBI Gene Expression Omnibus (GEO) under accession number GSE298413 and GSE298412.

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Author contributions

R.F. and V.C. conceptualized the study, C.G and V.C. provided study supervision. R.F., S.F.R, S.J., I.A. and O.A performed experiments and analyzed data. R.F., S.F.R, S.J. and I.A. generated figures and visualizations. M.B.T performed bioinformatics analyses. R.F., S.F.R and V.C. wrote the manuscript and all authors edited the manuscript.

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Additional files

Supp Material and Figures [↗](#)

Supp Data 1. Marker genes for 10x scRNA-seq analysis. [↗](#)

Supp Data 2. Marker genes for Illumina snRNA-seq analysis. [↗](#)

Supp Data 3. DGE for SGCs in aged vs adult DRG. [↗](#)

Supp Data 4. GO pathway analysis for SGC in aged vs adult DRG. [↗](#)

Supp Data 5. KEGG pathway analysis for SGC is aged vs adult DRG. [↗](#)

Movie S1. Z-stack video of whole mount preparation of DRG from *Lycopersicon Esculentum* Lectin (LEL) injected *Fabp7CreER::Ai14* mouse, which labels SGCs with tdTomato, immunostained for TUJ1 (neurons). [↗](#)

Movie S2. Z-stack video of DRG section immunostained for FABP7 and CX43. [↗](#)

Supp Table 1. List of qPCR Primers. [↗](#)

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Reviewer #1 (Public review):

The manuscript by Feng et al. reported that Endothelin B receptor (ETBR) expressed by the satellite glial cells (SGCs) in the dorsal root ganglions (DRG) acted to inhibit sensory axon regeneration in both adult and aged mice. Thus, pharmacological inhibition of ETBR with specific inhibitors resulted in enhanced sensory axon regeneration in vitro and in vivo. In addition, sensory axon regeneration significantly reduces in aged mice and inhibition of ETBR could restore such defect in aged mice. Moreover, the study provided some evidence that the reduced level of gap junction protein connexin 43 might act downstream of ETBR to suppress axon regeneration in aged mice. Overall, the study revealed an interesting SGC-derived signal in the DRG microenvironment to regulate sensory axon regeneration. It provided additional evidence that non-neuronal cell types in the microenvironment function to regulate axon regeneration via cell-cell interaction.

However, the molecular mechanisms by which ETBR regulates axon regeneration are unclear, and the structure of the manuscript is relatively not well organized, especially the last section. Some discussion and explanation about the data interpretation are needed to improve the manuscript.

(1) The result showed that the level of ETBR was not changed after the peripheral nerve injury. Does it mean that its endogenous function is to limit the spontaneous sensory axon regeneration? In other words, the results suggest that SGCs expressing ETBR or vascular endothelial cells expressing its ligand ET-1 act to suppress sensory axon regeneration. Some explanation or discussion about this are necessary. Moreover, does the protein level of ETBR or its ligand change during aging?

(2) In ex vivo experiments, NGF was added in the culture medium. Previous studies have shown that adult sensory neurons could initiate fast axon growth in response to NGF within 24 hours. In addition, dissociated sensory neurons could also initiate spontaneous regenerative axon growth without NGF after 48 hours. Some discussion or rationale is needed to explain the difference between NGF-induced or spontaneous axon growth of culture adult sensory neurons and the roles of ETBR and SGCs.

(3) In cultured dissociated sensory neurons, inhibiting ETBR also enhanced axon growth, which meant the presence of SGCs surrounding the sensory neurons. Some direct evidence is needed to show the cellular relationship between them in culture.

(4) In Figure 3, the in vivo regeneration experiments first showed enhanced axon regeneration either at 1 day or 3 days after the nerve injury. The study then showed that inhibiting ETBR could enhance sensory axon growth in vitro from uninjured naïve neurons or conditioning lesioned neurons. To my knowledge, in vivo sensory axon regeneration is relatively slow during the first 2 days after the nerve injury and then enter the fast regeneration mode in the 3rd day, representing the conditioning lesion effect in vivo. Some discussion is needed to compare the in vitro and the in vivo model of axon regeneration.

(5) In Figure 5, the study showed that the level of connexin 43 increased after ETBR inhibition in either adult or aged mice, proposing an important role of connexin 43 in mediating the enhancing effect of ETBR inhibition on axon regeneration. However, in the study there was no direct evidence supporting that ETBR directly regulate connexin 43 expression in SGCs. Moreover, there was no functional evidence that connexin 43 acted downstream of ETBR to regulate axon regeneration.

In the revised manuscript, most comments have been addressed with some new experiments or text revisions in the results or discussion. For representative images showing in vitro cultured DRG neurons, it would be much more convincing if several neurons in the same imaging field are shown, rather than a single neuron (Figure 2A, 3J).

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Reviewer #2 (Public review):

Summary:

Feng and colleagues set out to investigate the effect of manipulating endothelin signaling on nerve regeneration, focusing on the crosstalk between endothelial cells (ECs) in dorsal root ganglia (DRG), which secrete ET-1, and satellite glial cells (SGCs), which express the ETBR receptor. ETBR signaling limits axon growth. Using in vitro explant assays coupled with pharmacological inhibition in mouse models of nerve injury, the authors demonstrate that the ETAR/ETBR antagonist Bosentan promotes axon regeneration, and that this effect is maintained in aged mice. Although Bosentan inhibits both endothelin receptors A and B, comparison with an ETAR-specific antagonist suggests primary involvement of the ET-1/ETBR pathway. In the DRG, ETBR is mostly expressed by SGCs, a cell type implicated in nerve regeneration. SGCs ensheath and couple with DRG neurons through gap junctions formed by Cx43. The pro-regenerative effects of ETBR inhibition are attributed in part to an increase in Cx43 levels, which are expected to enhance neuron-SGC coupling. snRNA sequencing and TEM analysis reveal a decline in SGC numbers, morphological changes, and transcriptional reprogramming that may impair their pro-regenerative capacity.

Strengths:

The study is well-executed, and the main conclusion (that ETBR signaling inhibits axon regeneration after nerve injury and contributes to the age-related decline in regenerative capacity) is well supported by the data. In addition, the study highlights the importance of vascular signals in nerve regeneration, a topic that has gained traction in recent years. Importantly, these results further emphasize the contribution of long-neglected SGCs to nerve tissue homeostasis and repair. Although the study does not provide a complete mechanistic understanding, the findings are robust and are likely to attract the interest of a broad readership.

Weaknesses:

While certain aspects could have been further addressed experimentally, these points were either technically challenging or considered beyond the scope of the current study, and are appropriately addressed in the Discussion.

(1) It remains to be determined whether the accelerated axon regrowth observed after nerve injury depends on cellular crosstalk mediated by ET-1 at the lesion site. Are ECs along the nerve secreting ET-1? What cells are present in the nerve stroma that could respond and participate in the repair process? Would these interactions be sensitive to Bosentan?

Dissecting these contributions would require cell-specific manipulations. The potential roles of ECs, fibroblast and SCs in the nerve are discussed.

(2) It is suggested that the permeability of DRG vessels may facilitate the release of vascular-derived signals. The possibility that the ET-1/ETBR pathway modulates vascular permeability, and that this in turn contributes to the observed effects on regeneration, is discussed.

(3) It cannot be excluded that ET-3 in fibroblasts is relevant for controlling SGC responses. The possibility that both ET-1 and ET-3 participate in ETBR- dependent effect on axon regeneration is discussed.

(4) The discovery that ET-1/ETBR signaling in SGC curtails the growth capacity of axons at baseline raises questions about the physiological role of this pathway. This remains to be elucidated with cell type-specific knockout approaches.

(5) The modulation of Cx43 expression by ET-1/ETBR is examined by immunostaining, but a complementary analysis by quantitative RT-PCR on sorted SGCs would have been a valuable addition. However, quantifying Cx43 on purified SGCs was not attainable due to technical complications.

(6) The conclusion "that ETBR inhibition in SGCs contributes to axonal regeneration by increasing Cx43 levels, gap junction coupling or hemichannels and facilitating SGC-neuron communication" are consistent with previous studies (Procacci et al., 2008) but in apparent discrepancy with increased gap junctions and dye coupling in SGCs of aged mice (Huang et al., 2006). More experiments are required to clarify what distinguishes a beneficial increase in coupling after ETBR inhibition, from what is observed in aging.

(7) The effect of Bosentan likely extends beyond the modulation of Cx43 levels. Cell type-specific knockout of Cx43 and ETBR, studies of SGCs-neuron coupling, and biochemical analysis of Cx43 functions would clarify the link between ETBR, Cx43 regulation, and axon regeneration. A discussion of alternative mechanisms is provided.

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Author response:

The following is the authors' response to the original reviews.

Reviewer #1 (Public Review):

The manuscript by Feng et al. reported that the Endothelin B receptor (ETBR) expressed by the satellite glial cells (SGCs) in the dorsal root ganglions (DRG) acted to inhibit sensory axon regeneration in both adult and aged mice. Thus, pharmacological inhibition of ETBR with specific inhibitors resulted in enhanced sensory axon regeneration in vitro and in vivo. In addition, sensory axon regeneration significantly reduces in aged mice and inhibition of ETBR could restore such defect in aged mice. Moreover, the study provided some evidence that the reduced level of gap junction protein connexin 43 might act downstream of ETBR to suppress axon regeneration in aged mice. Overall, the study revealed an interesting SGC-derived signal in the DRG microenvironment to regulate sensory axon regeneration. It provided additional evidence that non-neuronal cell types in the microenvironment function to regulate axon regeneration via cell-cell interaction.

However, the molecular mechanisms by which ETBR regulates axon regeneration are unclear, and the manuscript's structure is not well organized, especially in the last

section. Some discussion and explanation about the data interpretation are needed to improve the manuscript.

We thank the reviewer for the positive comments. We agree that the mechanisms by which ETBR signaling functions as a brake on axon growth and regeneration remain to be elucidated. We believe that unraveling the detailed molecular pathways downstream of ETBR signaling in SGCs that promote axon regeneration is beyond the scope of this manuscript. Answering these questions would first require cell specific KO of ETBR and Cx43 to confirm that this pathway is operating in SGCs to control axon regeneration. We would also need to identify how SGCs communicate with neurons to regulate axon regeneration, which is a large area of ongoing research that remains poorly understood. Our data showing that pharmacological inhibition of ETBR with specific FDA-approved inhibitors enhances sensory axon regeneration provide not only new evidence for non-neuronal mechanisms in nerve repair, but also a new potential clinical avenue for therapeutic intervention.

As suggested by the reviewer, we have extensively revised the organization of the manuscript, especially the last section of results. We have performed additional snRNAseq experiments to establish the impact of aging in DRG. We have also performed additional experiments to determine if blocking ETBR improves target tissue reinnervation. Following the reviewer's suggestion, we have also expanded the Discussion section to discuss alternative mechanisms and offer additional interpretation of our data. Below we describe how we address each point in detail.

(1) The result showed that the level of ETBR did not change after the peripheral nerve injury. Does this mean that its endogenous function is to limit spontaneous sensory axon regeneration? In other words, the results suggest that SGCs expressing ETBR or vascular endothelial cells expressing its ligand ET-1 act to suppress sensory axon regeneration. Some explanation or discussion about this is necessary. Moreover, does the protein level of ETBR or its ligand change during aging?

We thank the reviewer for this point. Our results indeed indicate that one endogenous function of ETBR is to limit the extent of sensory axon regeneration. This may be a part of a mechanism to limit spontaneous sensory axon growth or plasticity and maladaptive neural rewiring after nerve injury. While the increased growth capacity of damaged peripheral axons can lead to reconnection with their targets and functional recovery, the increased growth capacity can also lead to axonal sprouting of the central axon terminals of injured neurons in the spinal cord, and to pain (see for example Costigan et al 2010, PMID: 19400724). In the context of aging that we describe here, this protective mechanism may hinder beneficial recovery. Other mechanisms that slow axon regeneration have been reported, and include, for example, axonally synthesized proteins, which typically support nerve regeneration through retrograde signaling and local growth mechanisms. RNA binding proteins (RBP) are needed for this process. One such RBP, the RNA binding protein KHSRP is locally translated following nerve injury. Rather than promoting axon regeneration, KHSRP promotes decay of other axonal mRNAs and slows axon regeneration. Another example includes the Rho signaling pathway, which was shown to function as an inhibitory mechanism that slows the growth of spiral ganglion neurites in culture. We have now included these examples in the Discussion section.

To address the reviewer's second question, we have checked protein levels of ETBR and ET-1 in adult and aged DRG tissue. We observed a robust increase in ET-1 in aged DRG, while the levels of ETBR did not appear to change significantly. These results are now presented in Figure 4- Figure Supplement 1, and further support the notion that in aging, activation of the ETBR signaling hinders axon regeneration.

(2) In ex vivo experiments, NGF was added to the culture medium. Previous studies have shown that adult sensory neurons could initiate fast axon growth in response to NGF within 24 hours. In addition, dissociated sensory neurons could also initiate spontaneous regenerative axon growth without NGF after 48 hours. Some discussion or rationale is needed to explain the difference between NGF-induced or spontaneous axon growth of culture adult sensory neurons and the roles of ETBR and SGCs.

We appreciate the reviewer's suggestion. In adult DRG explant or dissociated cultures, NGF is not typically required for survival or axon outgrowth. However, in dissociated culture, the addition of NGF to the medium stimulates growth from more neurons compared to controls (Smith and Skene 1997). In the DRG explant, NGF does not promote significant effects on axon growth, but stimulates glial cell migration (Klimovich et al 2020). We opted to include NGF in our explant assay to increase the potential of stimulating axon regeneration with pharmacological manipulations of ETBR. We have now clarified these considerations in the Method section.

(3) In cultured dissociated sensory neurons, inhibiting ETBR also enhanced axon growth, which meant the presence of SGCs surrounding the sensory neurons. Some direct evidence is needed to show the cellular relationship between them in culture.

We thank the reviewer for raising this point and have added new data, now presented in Figure 2B, to show that in mixed DRG cultures, SGCs labeled with Fabp7 are present in the culture in proximity to neurons labeled with TUJ1, but they do not fully wrap the neuronal soma. These results are consistent with prior findings reporting that as time in culture progresses, SGCs lose their adhesive contacts with neuronal soma and adhere to the coverslip (PMID: 22032231, PMID: 27606776). While in some cases SGCs can maintain their association with neuronal soma in the first day in culture after plating, in our hands, most SGCs have left the soma at the 24h time point we examined.

(4) In Figure 3, the in vivo regeneration experiments first showed enhanced axon regeneration either 1 day or 3 days after the nerve injury. The study then showed that inhibiting ETBR could enhance sensory axon growth in vitro from uninjured naïve neurons or conditioning lesioned neurons. To my knowledge, in vivo sensory axon regeneration is relatively slow during the first 2 days after the nerve injury and then enters the fast regeneration mode on the 3rd day, representing the conditioning lesion effect in vivo. Some discussion is needed to compare the in vitro and the in vivo model of axon regeneration.

We agree that axon growth is relatively slow the first 2 days and enters a fast growth mode on day 3. This has been elegantly demonstrated in Shin et al Neuron 2012 (PMID: 22726832), where an in vivo conditioning injury 3 days prior increases axon growth one day after injury. In vitro, similar effects have been described: a prior in vivo injury accelerates growth capacity within the first day in culture, but a similar growth mode occurs in naïve adult neurons after 2-3 days in vitro (Smith and Skene 1996). We also know that the neurite growth in culture is stimulated by higher cell density, likely because non-neuronal cells can secrete trophic factors (Smith and Skene 1996). Our in vitro results thus suggest that blocking ETBR in SGCs in these mixed cultures may alter the media towards a more growth promoting state. In vivo, our data show that Bosentan treatment for 3 days partially mimics the conditioning injury and potentiates the effect of the conditioning injury. One possible interpretation is that inhibition of ETBR alters the release of trophic factors from SGCs. Future studies will be required to unravel how ETBR signaling influence the SGCs secretome and its influence on axon growth. We have now included these discussion points in the Results and Discussion Section.

(5) In Figure 5, the study showed that the level of connexin 43 increased after ETBR inhibition in either adult or aged mice, proposing an important role of connexin 43 in mediating the enhancing effect of ETBR inhibition on axon regeneration. However, in the study, there was no direct evidence supporting that ETBR directly regulates connexin 43 expression in SGCs. Moreover, there was no functional evidence that connexin 43 acted downstream of ETBR to regulate axon regeneration.

We thank the reviewer for this point and agree that we do not provide direct evidence that connexin 43 acts downstream of ETBR to regulate axon regeneration. To obtain such functional evidence would require selective KO of ETBR and Cx43 in SGCs, which we believe is beyond the scope of the current study. We have revised the Results and Discussion sections to emphasize that while we observe that ETBR inhibition increases Cx43 levels and Cx43 levels correlates with axon regeneration, whether Cx43 directly mediates the effect on axon regeneration remains to be established. We also discuss potential alternative mechanisms downstream of ETBR in SGCs that could contribute to the observed effects on axon regeneration. Specifically, we discuss the possibility that ETBR signaling may limit axon regeneration via regulating SGCs glutamate reuptake functions, because of the following reasons: 1) Similarly to astrocytes, glutamate uptake by SGCs is important to regulate neuronal function, 2) exposure of cultured cortical astrocytes to endothelin results in a decrease in glutamate uptake that correlates with a major loss of basal glutamate transporter expression (GLT-1 and 1), 3) Both glutamate transporters are expressed in SGCs in sensory ganglia 4) GLAST and glutamate reuptake function is important for lesion-induced plasticity in the developing somatosensory cortex.

Reviewer #2 (Public Review):

Summary:

In this interesting and original study, Feng and colleagues set out to address the effect of manipulating endothelin signaling on nerve regeneration, focusing on the crosstalk between endothelial cells (ECs) in dorsal root ganglia (DRG), which secrete ET-1 and satellite glial cells (SGCs) expressing ETBR receptor. The main finding is that ETBR signaling is a default brake on axon growth, and inhibiting this pathway promotes axon regeneration after nerve injury and counters the decline in regenerative capacity that occurs during aging. ET-1 and ETBR are mapped in ECs and SGCs, respectively, using scRNA-seq of DRGs from adult or aged mice. Although their expression does not change upon injury, it is modulated during aging, with a reported increase in plasma levels of ET-1 (a potent vasoconstrictive signal). Using in vitro explant assays coupled with pharmacological inhibition in mouse models of nerve injury, the authors demonstrate that ET-1/ETBR curbs axonal growth, and the ETAR/ETBR antagonist Bosentan boosts regrowth during the early phase of repair. In addition, Bosentan restores the ability of aged DRG neurons to regrow after nerve lesions. Despite Bosentan inhibiting both endothelin receptors A and B, comparison with an ETAR-specific antagonist indicates that the effects can be attributed to the ET-1/ETBR pathway. In the DRGs, ETBR is mostly expressed by SGCs (and a subset of Schwann cells) a cell type that previous studies, including work from this group, have implicated in nerve regeneration. SGCs ensheath and couple with DRG neurons through gap junctions formed by Cx43. Based on their own findings and evidence from the literature, the pro-regenerative effects of ETBR inhibition are in part attributed to an increase in Cx43 levels, which are expected to enhance neuron-SGC coupling. Finally, gene expression analysis in adult vs aged DRGs predicts a decrease in fatty acid and cholesterol metabolism, for which previous work by the authors has shown a requirement in SGCs to promote axon regeneration.

Strengths:

The study is well-executed and the main conclusion that "ETBR signaling inhibits axon regeneration after nerve injury and plays a role in age-related decline in regenerative capacity" (line 77) is supported by the data. Given that Bosentan is an FDA-approved drug, the findings may have therapeutic value in clinical settings where peripheral nerve regeneration is suboptimal or largely impaired, as it often happens in aged individuals. In addition, the study highlights the importance of vascular signals in nerve regeneration, a topic that has gained traction in recent years. Importantly, these results further emphasize the contribution of longneglected SGCs to nerve tissue homeostasis and repair. Although the study does not reach a complete mechanistic understanding, the results are robust and are expected to attract the interest of a broader readership.

We thank the reviewer for the positive comments, especially in regard to the rigor and originality of our study.

Weaknesses:

Despite these positive comments provided above, the following points should be considered:

(1) This study examines the contribution of the ET-1 pathway in the ganglia, and in vitro assays are consistent with the idea that important signaling events take place there. Nevertheless, it remains to be determined whether the accelerated axon regrowth observed in vivo depends also on cellular crosstalk mediated by ET-1 at the lesion site. Are ECs along the nerve secreting ET-1? What cells are present in the nerve stroma that could respond and participate in the repair process? Would these interactions be sensitive to Bosentan? It may be difficult to dissect this contribution, but it should at least be discussed.

We thank the reviewer for this important point and agree that the in vivo effects observed cannot rule out the contribution of ECs or SCs at the lesion site in the nerve. Dissecting the contribution of ETBR expressing cells in the nerve would require cell-specific manipulations that go beyond the scope of this manuscript. We have revised the Discussion section to highlight the potential contribution of ECs, fibroblast and SCs in the nerve.

(2) It is suggested that the permeability of DRG vessels may facilitate the release of "vascular-derived signals" (lines 82-84). Is it possible that the ET-1/ETBR pathway modulates vascular permeability, and that this, in turn, contributes to the observed effects on regeneration?

We thank the reviewer for raising this interesting point. ET-1 can have an impact on vascular permeability. It was indeed shown that in high glucose conditions, increased trans-endothelial permeability is associated with increased Edn1, Ednra and Ednrb expression and augmented ET1 immunoreactivity (PMID: 10950122). It is thus possible that part of the effects observed results from altered vascular permeability. We have included this point in the Discussion section. Future experiments will be required to test how injury and age affects vascular permeability in the DRG.

(3) Is the affinity of ET-3 for ETBR similar to that of ET-1? Can it be excluded that ET-3 expressed by fibroblasts is relevant for controlling SGC responses upon injury/aging?

We thank the reviewer for raising this point. ET-1 binds to ETAR and ETBR with the same affinity, but ET3 shows a higher affinity to ETBR than to ETAR (Davenport et al. Pharmacol. Rev 2016 PMID: 26956245). We attempted to examine ET-3 level in adult and aged DRG by western blot, but in our hands the antibody did not work well enough, and we could not obtain clear

results. We thus cannot exclude the possibility that ET-3 released by fibroblasts contribute to the effects we observe on axon regeneration. Indeed, in cultured cortical astrocytes, application of either ET-1 or ET-3 leads to inhibition of Cx43 expression. We have revised the text in the Discussion section to highlight the possibility that both ET-1 and ET-3 could participate on the ETBR-dependent effect on axon regeneration.

(4) ETBR inhibition in dissociated (mixed) cultures uncovers the restraining activity of endothelin signaling on axon growth (Figure 2C). Since neurons do not express ET-1 receptors, based on scRNA-seq analysis, these results are interpreted as an indication that basal ETBR signaling in SGC curbs the axon growth potential of sensory neurons. For this to occur in dissociated cultures, however, one should assume that SGC-neuron association is present, similar to in vivo, or to whole DRG cultures (Figure 2C). Has this been tested?

We thank the reviewer for this point. In dissociated DRG culture, neurons, SGCs and other nonneuronal cells are present, but SGCs do not retain the surrounding morphology as they do in vivo. Within 24 hours in culture, SGCs lose their adhesive contacts with neuronal soma and adhere to the coverslip (PMID: 22032231, PMID: 27606776). We have included new data in Figure 2B to show that in our culture conditions, SGCs are present, but do not wrap neurons soma as they do in vivo. We also know from prior studies that the density of the culture affects axon growth, an effect that was attributed to trophic factors released from non-neuronal cells (Smith and Skene 1997). Therefore, although SGCs do not surround neurons, the signaling pathway downstream of ETBR may be present in culture and contribute to the release of trophic factors that influence axon growth. We have revised the Results section to better explain our in vitro results and their interpretation.

In both in vitro experimental settings (dissociated and whole DRG cultures) how is ETBR stimulated over up to 7 days of culture? In other words, where does endothelin come from in these cultures (which are unlikely to support EC/blood vessel growth)? Is it possible that the relevant ligand here derives from fibroblasts (see point #6)? Or does it suggest that ETBR can be constitutively active (i.e., endothelin-independent signaling)? Is there any chance that endothelin is present in the culture media or Matrigel?

We thank the reviewer for raising this point. Our single-cell data indicate that ET-1 is expressed by endothelial cells and ET-3 by fibroblasts. In dissociated DRG culture at 24h time point, all DRGs cells are present, including endothelial cells and fibroblasts, and could represent the source of ET-1 or ET-3. In the explant setting, it is also possible that both ET-1 and ET-3 are released by endothelial cells and fibroblasts during the 7 days in culture. According to information for the suppliers, endothelin is not present neither in the culture media nor in the Matrigel. While mutations can facilitate the constitutive activity of the ETBR receptor, we are not aware of data showing that endogenous ETBR can be constitutively active. Because the molecular mechanisms governing ETBR-mediated signaling remain incompletely understood (see for example PMID: 39043181, PMID: 39414992) future studies will be required to elucidate the detailed mechanisms activating ETBR in SGCs and its downstream signaling mechanisms. We have now expanded the Results and discussion sections to clarify these points.

(5) The discovery that ET-1/ETBR signaling in SGC curtails the growth capacity of axons at baseline raises questions about the physiological role of this pathway. What happens when ETBR signaling is prevented over a longer period of time? This could be addressed with pharmacological inhibitors, or better, with cell-specific knock-out mice. The experiments would certainly be of general interest, although not within the scope of this story. Nevertheless, it could be worth discussing the possibilities.

We agree that this is an interesting point. As mentioned above in response to point #1 of reviewer 1, the physiological role of this pathway could be to limit plasticity and prevent maladaptive neural rewiring that can happen after injury (Costigan et al 2009, PMID: 19400724), but can also hinder beneficial recovery after injury. Other mechanisms that limit axon regeneration capacity have been described and involve local mRNA translation and Rho signaling. We have revised the Discussion section to include these points. We agree that understanding the consequence of blocking ETBR over longer time periods is beyond the scope of the current study, but we now discuss the possibility that blocking ETBR with a cell specific KO approach could unravel its physiological function on target innervation and behavior.

(6) Assessing Cx43 levels by measuring the immunofluorescence signal (Figure 5E-F) is acceptable, particularly when the aim is to restrict the analysis to SGCs. The modulation of Cx43 expression by ET-1/ETBR plays an important part in the proposed model. Therefore, a complementary analysis of Cx43 expression by quantitative RT-PCR on sorted SGCs would be a valuable addition to the immunofluorescence data. Is this attainable?

We agree and have attempted to perform these types of experiments but encountered technical difficulties. We attempted to sorting SGCs from transgenic mice in which SGCs are fluorescently labeled. However, the cells did not survive the sorting process and died in culture. We think that increasing the viability of cells after sorting would require capillary-free fluorescent sorting approaches. However, we do not currently have access to such technology. We attempted this experiment with cultured SGCs, following a previously published protocol (Tonello et al. 2023 PMID: 38156033). In these experiments, SGCs are cultured for 8 days to obtain purity. We did not observe any difference in Cx43 protein or mRNA level upon treatment with ET-1 with or without BQ788. However, in these SGCs cultures, Cx43 displayed a diffuse localization, rather than puncta as observed in vivo. Therefore, despite our multiple attempts, quantifying Cx43 on sorted or purified SGCs was not attainable.

(7) The conclusions "We thus hypothesize that ETBR inhibition in SGCs contributes to axonal regeneration by increasing Cx43 levels, gap junction coupling or hemichannels and facilitating SGC-neuron communication" (lines 303-305) are consistent with the findings but seem in contrast with the effect of aging on gap junction coupling reported by others and cited in line 210: "the number of gap junctions and the dye coupling between these cells increases (Huang et al., 2006)". I am confused by what distinguishes a potential, and supposedly beneficial, increase in coupling after ETBR inhibition, from what is observed in aging.

We agree that the aging impact of Cx43 level and gap junction number appears contradictory. Procacci et al 2008 reported that Cx43 expression in SGCs decreases in the aged mice. Huang et al 2006 report that both the number of gap junctions and the dye coupling between these cells were found to increase with aging. Procacci et al suggested as a possible explanation for this apparent discrepancy that additional connexin types other than Cx43 may contribute to the gap junctions between SGCs in aged mice. Our snRNAseq data did not allow us to verify this hypothesis, because there were less SGCs in aged mice compared to adult, and connexin genes were detected in only 20% or less of SGCs. Furthermore, our quantification did not look specifically at gap junctions, but just at Cx43 puncta. Cx43 can also form hemichannels in addition to gap junctions, and can also perform non-channel functions, such as protein interaction, cell adhesion, and intracellular signaling. Thus, more research examining the role of Cx43 in SGCs is necessary to address this discrepancy in the literature. We have expanded the Discussion section to include these points.

(8) I find it difficult to reconcile the results in Figure 5F with the proposed model since (1) injury increases Cx43 levels in both adult and aged mice, (2) the injured aged/vehicle group has a similar level to the uninjured adult group, (3) upon injury, aged+Bosentan is much lower than adult+Bosentan (significance not tested). It seems hard to explain the effect of Bosentan only through the modulation of Cx43 levels. Whether the increase in Cx43 levels following ETBR inhibition actually results in higher SGC-neuron coupling has not been assessed experimentally.

We thank the reviewer for this point and agree that the effect of Bosentan is likely not exclusively through the modulation of Cx43 levels in SGCs, and that Cx43 levels may simply correlate with axon regenerative capacity. We have revised the manuscript to clarify this point. We have also added the missing significance test in Figure 5F.

Cell specific KO of Cx43 and ETBR would allow to test this hypothesis directly but is beyond the scope of the current study. We have not tested SGCs-neuron coupling, as these experiments are currently beyond our area of expertise. Cx43 has also other functions beyond gap junction coupling, such as protein interaction, cell adhesion, and intracellular signaling. Investigating the precise function of Cx43 would require in depth biochemical and cell specific experiments that are beyond the scope of this study. Furthermore, as we now mentioned in response to reviewer #2 point 5, ETBR signaling may also have other downstream effects in SGCs, such as glutamate transporters expression, or affect other cells in the nerve during the regeneration process. We have revised the Discussion section to include these alternative mechanisms.

Reviewer #3(Public Review):

Summary:

This manuscript suggests that inhibiting ETBR via the FDA-approved compound Bosentan can disrupt ET-1-ETBR signalling that they found detrimental to nerve regeneration, thus promoting repair after nerve injury in adult and aged mice.

Strengths:

- (1) The clinical need to identify molecular and cellular mechanisms that can be targeted to improve repair after nerve injury.*
- (2) The proposed mechanism is interesting.*
- (3) The methodology is sound.*

We thank the reviewer for highlighting the strengths of our study

Weaknesses:

- (1) The data appear preliminary and the story appears incomplete.*

We appreciate the reviewer's point. We would like to emphasize that our results provide compelling evidence that ETBR signaling is a default brake on axon growth, and inhibiting this pathway promotes axon regeneration after nerve injury and counters the decline in regenerative capacity that occurs during aging. We also provide evidence that ETBR signaling regulates the levels of Cx43 in SGCs. Furthermore, our results document the use of an FDA approved compound to increase axon regeneration may be of interest to the broader readership, as there is currently no therapies to improve or accelerate nerve repair after injury. We agree that the detailed mechanisms operating downstream of ETBR will need to be elucidated. Answering these questions would first require cell specific KO of ETBR and Cx43

to confirm that this pathway is operating in SGCs to control axon regeneration. We would also need to identify how SGCs communicate with neurons to regulate axon regeneration, which is a large area of ongoing research that remains poorly understood. This extensive and highly complex set of experiments is beyond the scope of the current study. As we discussed in our response to reviewer #1 and #2 we attempted to perform numerous additional experiments to better define the role of ETBR signaling in SGCs in aging and have included additional results in Fig. 2B, Fig 3G-H, Fig 5A-E, and Figure 4- Figure Supplement 1 and Figure 5- Figure Supplement 1. We have expanded the

Discussion to acknowledge the limitation of our study and to discuss possible mechanisms.

(2) Lack of causality and clear cellular and molecular mechanism. There are also some loose ends such as the role of connexin 43 in SGCs: how is it related to ET-1- ETBR signalling?

We thank the reviewer for this point and agree that the molecular mechanisms downstream of ETBR remain to be elucidated. However, we believe that our manuscript reports an interesting potential of an FDA-approved compound in promoting nerve repair. We focused on Cx43 downstream of ETBR signaling because decreased Cx43 expression in SGCs in ageing was previously established, but the mechanisms were not elucidated. Furthermore, it was reported that ET1 signaling in cultured astrocytes, which share functional similarities with SGCs, leads to the closure of gap junctions and reduction in Cx43 expression. Our study thus provides a mechanism by which ETBR signaling in SGCs regulates Cx43 expression. Whether Cx43 directly impact axon regeneration remains to be tested. Cell specific KO of Cx43 and ETBR would be required to answer this question. We have revised the Introduction and Discussion section extensively to provide a link between ETBR and Cx43 and to acknowledge the lack of causality in Cx43 in SGCs, as well as to provide additional potential mechanisms by which ETBR inhibition may promote nerve repair.

Reviewer #2 (Recommendations For The Authors):

In addition to the points listed in the Public Review section, please consider the following comments:

(1) ETAR, which is high in mural cells, does not seem to be implicated in the reported proregenerative effects. Even so, can vasoconstriction be ruled out as an underlying cause of the age-dependent decline in axon regrowth potential and, more generally, in the effects of ET-1 inhibition on regeneration? This could be discussed.

We agree that we can't exclude a role in vasoconstriction or effect on vascular permeability in the age-dependent decline in axon regrowth potential. However, our in vitro and ex vivo experiments, in which vascular related mechanisms are unlikely, suggest that vasoconstriction may not be a major contributor to the effects we observed.

(2) The manuscript (e.g. line 287-288) would benefit from a discussion of the role that blood vessels play in the peripheral nervous system, and possibly CNS, repair. Vessels were shown to accompany regenerating fibers and instruct the reorganization of the nerve tissue to favor repair potentially through the release of pro-regenerative signals acting on stromal cells, glia, and other cellular components. Highlighting these processes will help put the current findings into perspective.

We agree and have revised the Discussion section to better explain the role of blood vessels in orientating Schwann cells migration and guiding axon regeneration.

(3) The vast majority of the cells that are sequenced and shown in the UMAP in Figure 1C are from adult (3-month-old) mice [16,923 out of 18,098]. It would be useful to include the UMAP split (or color-coded) by timepoint to appreciate changes in cell clustering that may occur with aging.

We apologize for this misunderstanding, Figure 1C had all cells from all ages. However, the number of cells we obtained from the age group was insufficient to perform in depth analysis of each cell type. We have thus revised this section and Figure 1, now only presenting the data from adult mice.

It is not discussed why fewer cells were sequenced at later stages. Additionally, I do not know how to interpret the double asterisks next to the labeling "18,098 samples" in Figure 1C.

Since our original sequencing of adult and aged mice using 10x yielded so few cells from the aged DRG, we tested and optimized a new technology for single cell preparation of DRG using Illumina Single Cell 3' RNA Prep. This preparation creates templated emulsions using a vortex mixer to capture and barcode single-cell mRNA instead of a microfluidics system. This method yielded much better results for nuclei recovery from aged DRG, with more nuclei and better quality of nuclei. Thus, we now present in Figure 5 and Figure 5- Figure Supplement 1 the results from snRNA-sequencing of aged and adult DRG using the Illumina single cell kit. The results of the snRNA-sequencing show a decreased abundance of SGCs in aged mice, consistent with the results from our morphology analysis with EM. We were also able to perform SGCs-specific pathway analysis because of the increased number of nuclei captured in the aged SGCs, which we included in the manuscript.

(4) The in vivo studies are designed to examine the effects of ETBR inhibition during the first phase of axon regrowth after nerve injury (1-3 days post-injury, dpi). Is there a reason why later stages have not been studied? It would be interesting to understand whether ETBR inhibition improves long-term recovery or is only effective at boosting the initial growth of axons through the lesion. It is possible that early inhibition will be enough for long-term recovery. If so, these experiments would define a sensitivity window with therapeutic value.

We agree that assessing functional recovery requires proper behavioral tests or morphological evaluations of reinnervation. To determine if Bosentan treatment has long-term effects on recovery, we administered Bosentan or vehicle for 3 weeks (daily for 1 week, and then once a week for the subsequent 2 weeks) after sciatic nerve crush. At 24 days after SNC, we assessed intraepidermal nerve fiber density (IENFD) in the injured paw and saw a trend towards increased fibers/mm in the treated animals (new Figure 3G,H). Future studies will examine how long-term Bosentan treatment affects functional recovery and innervation at later time points. Additionally, behavior assays will be needed to determine if these morphological changes relate to behavioral improvements using IENFD and behavior assays.

(5) I am unsure if the gene expression analysis shown in Figure 6 fits well into this story. It is interesting per se and in line with previous work from this group showing the relevance of fatty acid metabolism in SGCs for axon regeneration. Nevertheless, without a mechanistic link to endothelin signaling and Cx43/gap junction modulation, the observations derived from DEG analysis are not well integrated with the rest and may be more distracting than helpful. One limitation is that there is no cell-type information for the DEGs due to the small number of cells recovered from aged mice. For instance, if ETBR inhibition rescued gene downregulation associated with fatty acid/cholesterol metabolism, then the DGE results would become more relevant for understanding the

cellular basis of the pro-regenerative effect, which at this point remains quite speculative (lines 264-265; lines 318-319).

We agree and have added new snRNA sequencing data to replace these findings (see above response to point #4, new Figure 5 and Figure 5- Figure Supplement 1. The new data shows a decreased abundance of SGCs in aged mice, consistent with our TEM results. Pathway analysis revealed that aging triggers extensive transcriptional reprogramming in SGCs, reflecting heightened demands for structural integrity, cell junction remodeling, and glia–neuron interactions within the aged DRG microenvironment.

(6) It would be interesting to determine whether Bosentan increases SGC coverage of neuronal cell bodies in aged mice (Figures 6A-C).

We agree that this would be very interesting, but will require extensive EM analysis at different time points and is beyond the scope of the current manuscript.

(7) Finally, adding a summary model would help the readers.

We agree and have made a summary model, now presented in Figure 6F.

Reviewer #3 (Recommendations For The Authors):

Longer time points post-injury and assessment of functional recovery after Bosentan would be of great value here.

We agree that assessing functional recovery requires proper behavioral tests or morphological evaluations of reinnervation. To determine if Bosentan treatment has long-term effects on recovery, we administered Bosentan or vehicle for 3 weeks (daily for 1 week, and then once a week for the subsequent 2 weeks) after sciatic nerve crush. At 24 days after SNC, we assessed intraepidermal nerve fiber density in the injured paw and saw a trend towards increased fibers/mm in the treated animals (Fig 3). While the results do not reach significance, we decided to include this new data as it provides evidence that Bosentan treatment may also improve long term recovery. Future studies will be required examine how long-term Bosentan treatment affects functional recovery and innervation at later time points. Additionally, behavior assays will be needed to determine if these morphological changes relate to behavioral improvements.

It would be important to know how ET-1- ETBR signalling axis promotes the regeneration of axons: this remains unaddressed. What are the cells that are specifically involved? Endothelial cells SGC- neurons- SC? There are no experiments addressing the role of any of these?

We agree that the molecular and cellular mechanisms by which ETBR signaling in SGCs promote axon regeneration remains to be elucidated. Answering these questions would first require cell specific KO of ETBR and Cx43 to confirm that this pathway is operating in SGCs to control axon regeneration. We would also need to identify how SGCs communicate with neurons to regulate axon regeneration, which is a large area of ongoing research that remains poorly understood. While these are important experiments, because of numerous technical and temporal constraints, we believe they are beyond the scope of the current manuscript.

How does connexin 43 in SGCs related to ET-1- ETBR signalling?

The relation between connexin 43 and ETBR signaling stems from observations made in astrocytes. ET1 signaling in cultured astrocytes, which share functional similarities with SGCs, was shown to lead to the closure of gap junctions and the reduction in Cx43 expression. Because Cx43 expression, a major connexin expressed in SGCs as in astrocytes, was previously shown to be reduced at the protein level in SGCs from aged mice, we decided to explore if this ETBR-Cx43 mechanism also operates in SGCs. We have revised the Introduction and Discussion section extensively to acknowledge the lack of causality in Cx43 expression SGCs and to provide additional potential mechanisms by which ETBR inhibition may promote nerve repair.

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