

Endothelin B receptor inhibition rescues aging-dependent neuronal regenerative decline

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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 a single cell RNAseq approach, we reveal that in dorsal root ganglia (DRG), satellite glial cells (SGCs), which completely envelop the sensory neuron soma, 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. Bosentan treatment also enhances the expression of connexin 43 in SGCs after injury in adult and aged mice. 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.

eLife assessment

The study presents a **valuable** finding that the Endothelin B receptor (ETBR) expressed by the satellite glial cells (SGCs) in the dorsal root ganglions (DRG) inhibited sensory axon regeneration in both adult and aged mice. The evidence supporting most of the conclusions was **solid**, and the work will be of interest to neuroscientists working on axon regeneration and the involvement of non-neuronal cell types in regulating axon regeneration. Although the proposed mechanism is intriguing and the methodology is robust, the molecular mechanisms by which ETBR regulates axon regeneration are not fully elucidated.

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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 have not been investigated.

Connexin 43 (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). Studies in astrocytes, which share many functional and molecular features with SGCs (Avraham et al., 2022; Avraham et al., 2020; Hanani and Verkhratsky, 2021) have shown that endothelin 1 (ET-1) signaling via the endothelin receptor B (ETBR) inhibits the expression of Cx43 and reduces gap junction mediated coupling (Blomstrand et al., 2004; Rozyczka et al., 2005). In cultured SGCs, ET-1 also reduces gap junction coupling (Feldman-Goriachnik and Hanani, 2017). ET-1 is a potent vasoconstrictor secreted by endothelial cells and is the predominant isoform of endothelin in the human cardiovascular system (Lüscher and Barton, 2000; Yanagisawa et al., 1988). ET-1 production increases with age and is linked to many age-associated diseases (Barton, 2014; Jankowich and Choudhary, 2020; Stauffer et al., 2008). ET-1 signaling was also suggested to play a role in pain (Khodorova et al., 2009; Pomonis et al., 2001). Whether endothelin signaling contributes to axon regeneration and age-dependent axon regenerative decline has not been examined.

To determine the role of endothelin signaling in axon regeneration during aging, we performed unbiased single-cell RNA-sequencing of the DRG cells from mice 3 months of age (adult), 12 months of age (middle-aged) and 21 months of age (aged). Examination of endothelins and their receptors revealed enrichment of *Edn1* in endothelial cells and *Ednrb* 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. Bosentan treatment also increased expression of Cx43 in SGCs after injury in adult and aged mice. These results suggest that ETBR signaling inhibits 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). Additionally, immunofluorescent 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 pathways in the DRG microenvironment during aging, we performed single cell RNA sequencing (scRNA-seq) on lumbar DRGs from mice 3 months of age (adult), 12 months of age (middle-aged) and 21 months of age (aged) 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 18,098 cells (16,923 adult, 602 mid-aged, 573 old) from two biological replicates per age with an average of 3,411 genes and 12,071 transcripts detected per cell (see methods for filtering

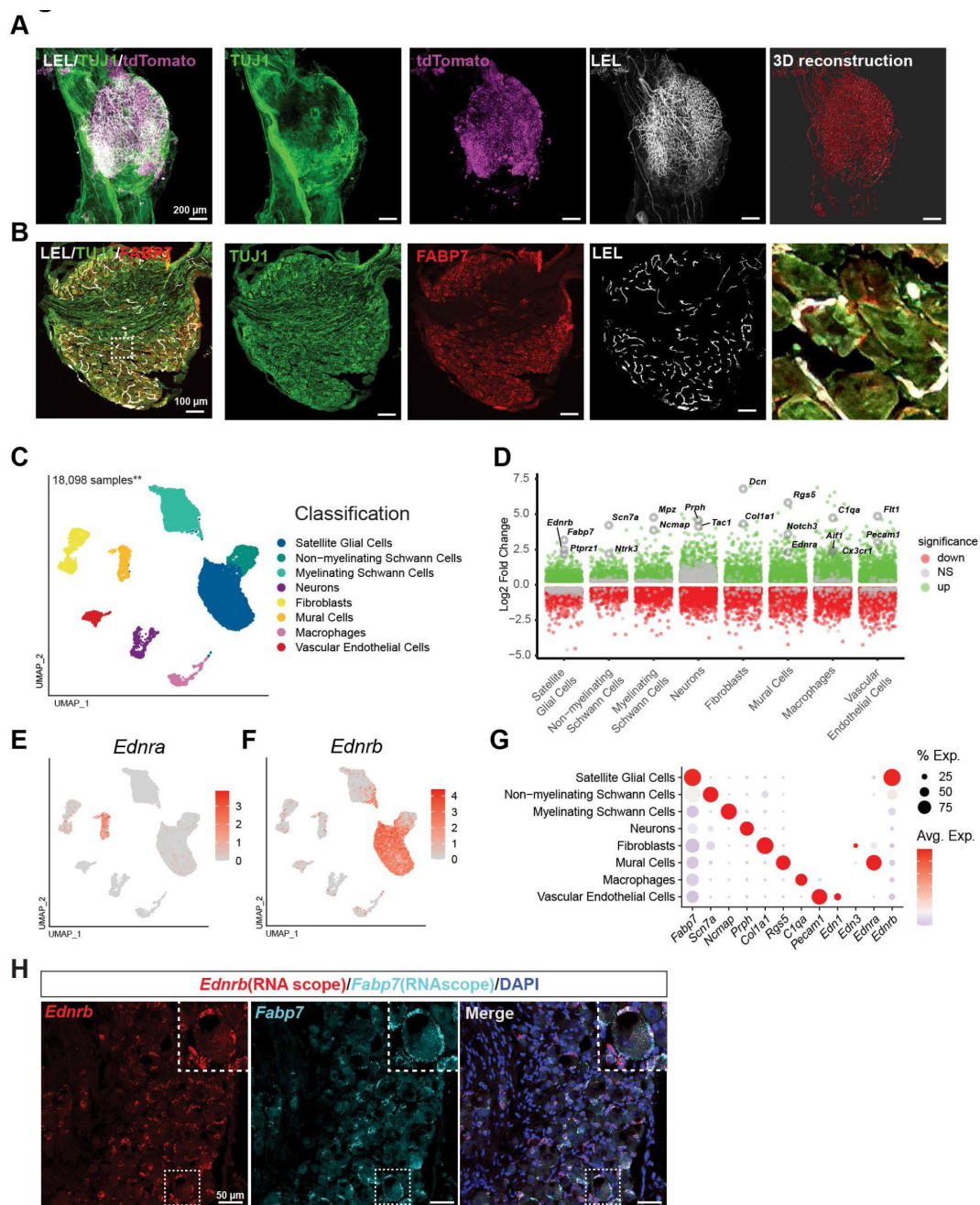


Fig. 1

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).

B. Representative z-stack 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 identified 7 cell clusters based on known marker genes.

D. Differential gene expression analysis revealed up- and down-regulated genes across cell clusters. Red or green indicates adjusted $p < 0.01$, while gray indicates $p \geq 0.01$.

E-F. UMAP overlay for expression of *Ednra* (E) and *Ednrb* (F).

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

H. 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).

criteria). Following condition integration and unsupervised clustering, we identified 8 major clusters containing cells from all age groups (**Fig 1C**). Examination of cluster-specific marker genes revealed major cellular subtypes including endothelial cells (*Flt1*, *Pecam1*), SGCs (*Fabp7*, *Ptprz1*), fibroblasts (*Col1a1*, *Dcn*), non-myelinating Schwann Cells (*Scn7a*, *Ntrk3*), myelinating Schwann cells (*Mpz*, *Ncmnp*), neurons (*Prph*, *Tac1*), mural cells (*Rgs5*, *Notch3*), and macrophages (*C1qa*, *Aif1*) (**Fig.1D**, Supplementary Fig.1A, Supplementary File 1 related to **Fig.1**). *Ednrb* was highly enriched in SGCs, with lower levels of expression in non-myelinating and myelinating Schwann cells (**Fig.1F**), whereas *Ednra* was expressed mainly in Mural cells (**Fig.1E**). *Ednrb* enrichment in SGCs is consistent with prior studies (Mapps et al., 2022; Pomonis et al., 2001; Tasdemir-Yilmaz et al., 2021). Endothelin-1 (*Edn1*) and endothelin-3 (*Edn3*) were expressed in endothelial cells and fibroblasts, respectively, whereas *Edn2* was not detected (**Fig 1G**, Supplementary Fig.1A). *Ednrb* levels decreased with age, whereas *Edn1* increased with age (Supplementary Fig.1B). *Ednrb* mRNA in SGCs was validated by RNA *in situ* hybridization, which showed *Ednrb* co-localized with the SGCs marker *Fabp7* (Avraham et al., 2020) (**Fig.1H**). Using our previously generated scRNAseq data sets (Avraham et al., 2022), we also found that *Ednrb* is highly expressed in SGCs in rat, and exclusively expressed in SGCs in humans (Supplementary Fig.1C,D).

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. Adult DRG mixed cultures, containing neuronal and non neuronal cells, were plated and treated with an ETBR antagonist (BQ788), ETBR agonist (IRL1620), endothelin A receptor (ETAR) antagonist (BQ123), or vehicle for 24 hours. 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.2A**-C). 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.2A**-C). These results indicated that ETBR signaling inhibits axonal growth *in vitro*.

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. Radial axon growth was significantly increased in explants treated with BQ788 compared to vehicle treatment (**Fig.2D**-F). To confirm that the *in vivo* morphology of SGCs enveloping neuronal somas was intact, DRG explants were immunostained with TUBJ1 for neurons and FABP7 for SGCs at DIV 7 (**Fig 2G**). These results further confirmed that ETBR signaling inhibits axon growth 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* and *ex-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., 2002). Bosentan is an antagonist of both ETAR and ETBR (Clozel et al., 1994), while Ambrisentan is an antagonist selective for ETAR (Humbert et al., 2004; Newman et al., 2007). Adult mice were treated orally with vehicle, Bosentan or Ambrisentan 2 hours

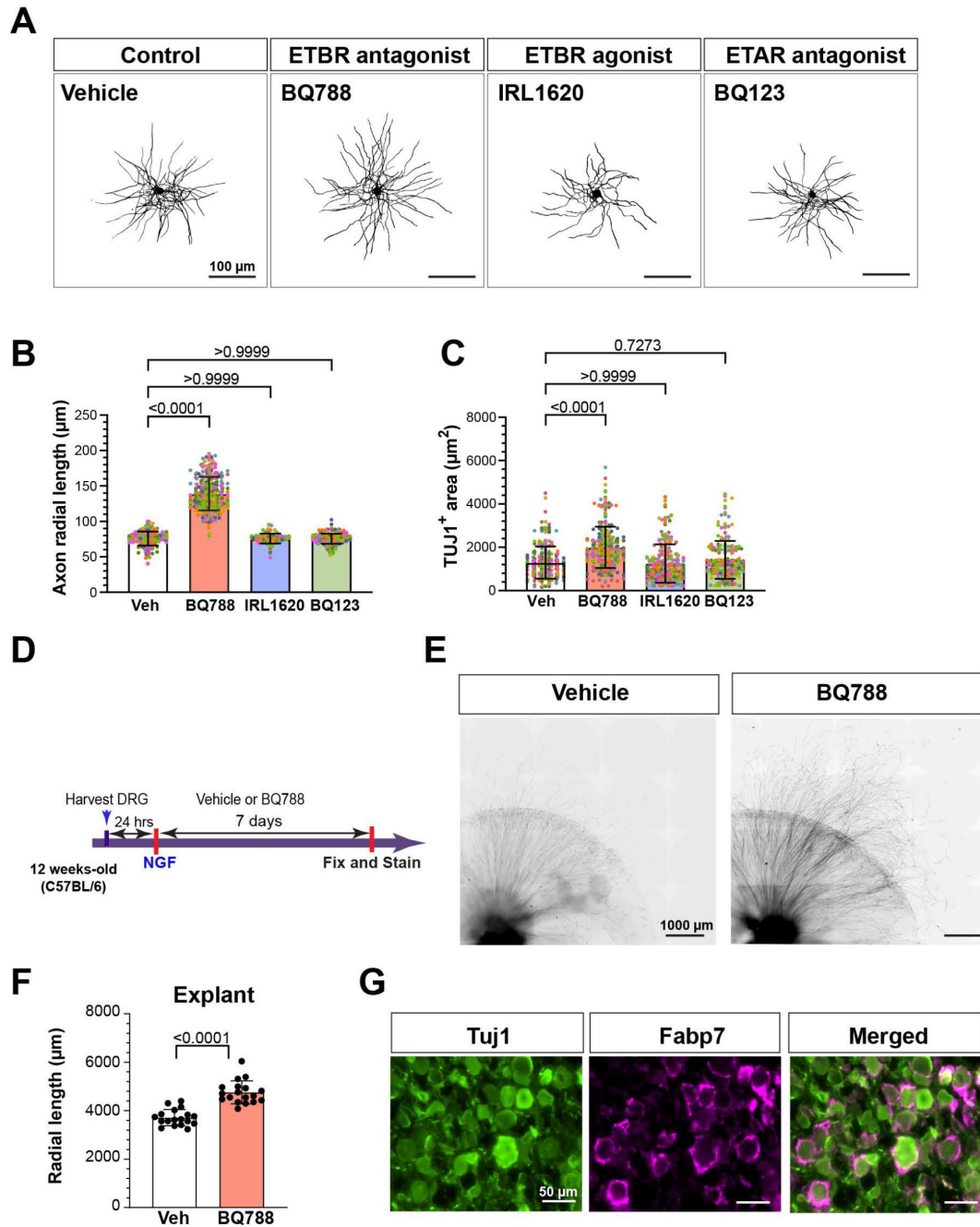


Fig. 2

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-C. 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.

D. Scheme of drug treatment and DRG explant model.

E. Representative images of DRG explants 7 days after drug treatment, immunostained for TUJ1 (black) (scale bars, 1000 μ m).

F. Quantification of radial length of the 35 longest axons from DRG explants from indicated groups N=36 (BQ788; 18 replicates, Veh; 18 replicates).

G. Representative images of DRG explants immunostained for TUJ1 (green), FABP7 (magenta), and merged (scale bars, 50 μ m).

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) (Fig.3A-E). 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; Cho et al., 2013; Feng et al., 2023; Shin et al., 2014) and axon elongation was quantified by measurement of the 10 longest axons, as previously described (Carlin et al., 2019) (Fig.3B). 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; Feng et al., 2023) (Fig.3C). 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). 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). 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 (Supplementary Fig.2A-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 (Supplementary Fig.2F-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 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). 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.3G-I). In uninjured condition, neurons extend short neurites, whereas a prior nerve injury leads to neurons growing longer neurites (Fig 3G,H), as expected (Smith and Skene, 1997). 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.3G,H). Since the selective ETAR antagonists, BQ123 and Ambrisentan, had no significant effects on axon growth regeneration after nerve injury (Fig 2A-C, 3A-E), our results indicate that ETBR limits axon regeneration 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 (Supplementary Fig.3A). Following DRC injury, axon growth occurs at about half the rate of peripheral axons (Oblinger and Lasek, 1984; Wujek and Lasek, 1983) and our previous work 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 (Supplementary Fig.3B-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

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

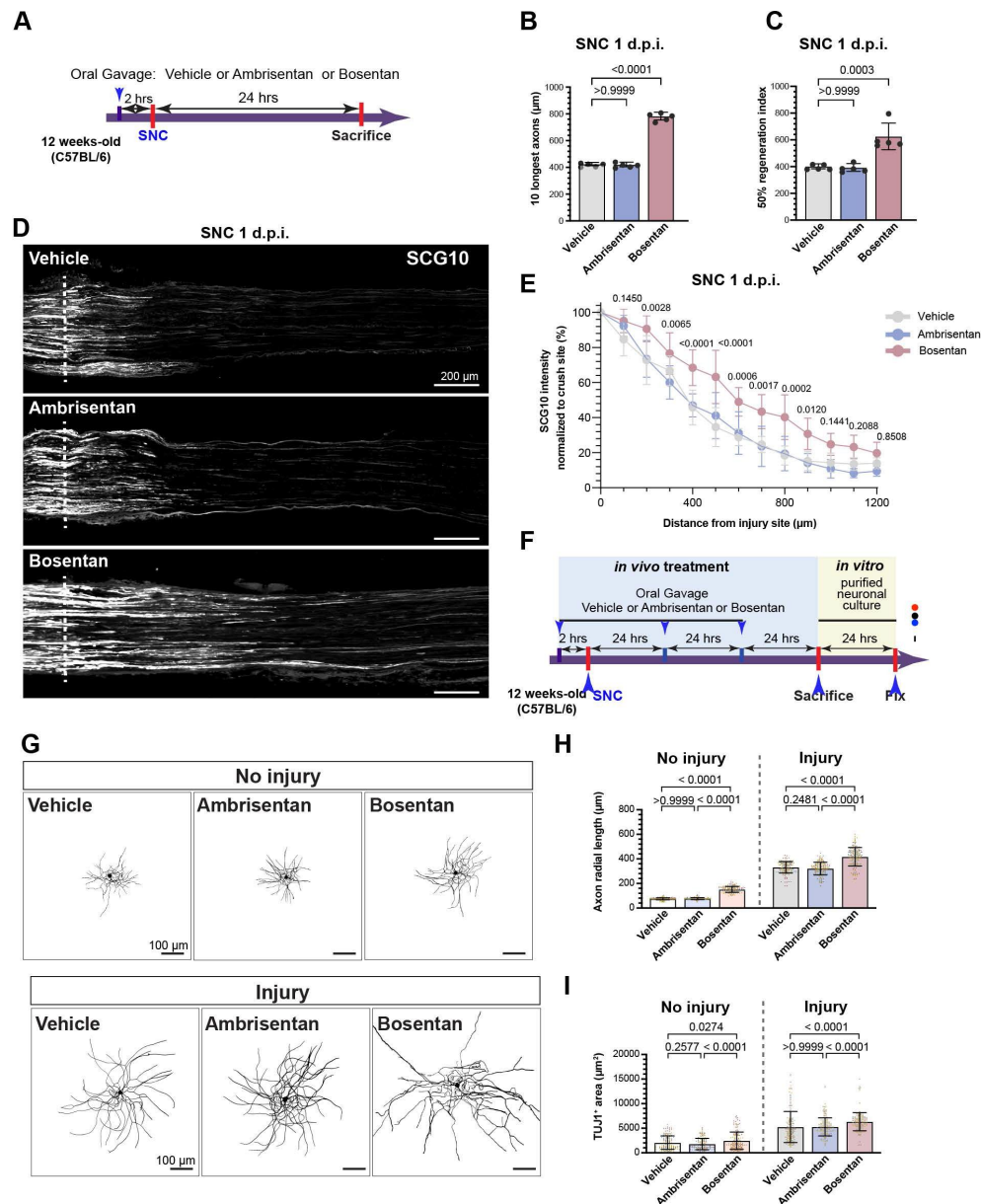


Fig. 3

Bosentan treatment improves axonal 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 SCG10 intensity is 50% of the SCG10 intensity at crush site.

D. Representative longitudinal sections of sciatic nerves 24h after SNC, immunostained for SCG10, from mice with the indicated treatment. Dotted line indicates the crush site, determined as the maximal SCG10 intensity (scale bars, 200 μ m).

E. Quantification of SCG10 intensity at the indicated distance normalized to the intensity at the crush site for each condition. N = 5 mice/condition.

F. Scheme of adult DRG neuronal culture and treatments.

G. Representative images showing TUJ1 (black) immunostaining of neurons in DRG cultures (scale bars, 100 μ m).

H-I. 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.

fact, aged explants treated with BQ788 had significantly more radial axon growth than to that of adult (3-month old) vehicle explants (**Fig 4C**). Axon regeneration after peripheral nerve injury in aged mice was also 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.

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 sensory neurons (Hanani and Spray, 2020; Retamal et al., 2017). Past studies in mice have also shown that Cx43 expression in SGCs decreases during aging (Procacci et al., 2008), while both the number of gap junctions and the dye coupling between these cells increases (Huang et al., 2006). Our scRNA-sequencing data revealed that *Gja1*, the gene for Cx43, is enriched in SGCs in both mice and human (**Fig.5A,B**) (Avraham et al., 2022) and that *Gja1* in SGCs decreased with age in mice (**Fig.5C**). In contrast, *Gjc1*, the gene for Cx45 displayed a trend of increased expression with age (**Fig.5C**), suggesting that Cx45 may contribute to the previously observed increase in gap junction (Huang et al., 2006). At the protein level, Cx43 is highly expressed in SGCs in mice (**Fig.5G** and Movie S1). The level of Cx43 expression, measured by both the Cx43 expression area and the number of Cx43 puncta in Fabp7 positive SGCs surrounding neuron, was significantly lower in aged mice compared to adult mice (**Fig.5E-G**), consistent with prior studies (Ohara et al., 2008; Procacci et al., 2008).

In cultured SGCs and astrocytes, endothelin signaling reduces the expression Cx43 (Blomstrand et al., 2004; Feldman-Goriachnik and Hanani, 2017; Rozyczka et al., 2005), while nerve injury increases SGCs gap junction coupling (Hanani et al., 2002; Kim et al., 2016; Pannese et al., 2003). To elucidate a potential mechanism by which ETBR antagonism increases axonal regeneration, the effect of Bosentan treatment on expression of Cx43 in SGCs was assessed. Cx43 expression level on SGCs was increased 3 days after SNC in both adult and aged vehicle-treated mice (**Fig.5E-G**). Bosentan treatment led to an increase in Cx43 expression in both adult and aged mice (**Fig.5E-G**). 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 (Supplementary Fig.4A-C). These results demonstrate that Bosentan treatment increases Cx43 expression in SGCs, providing a possible mechanism by which ETBR inhibition promotes axon regeneration after injury.

Aging alters SGCs morphology and metabolism

Changes in morphology of SGCs have been reported with age, with a decrease in SGCs number and SGCs retracting and leaving the neuronal soma exposed to the extra cellular DRG environment in aged rabbit (Pannese et al., 1997; Pannese et al., 1996). In the DRG of aged mice, neurons are usually enveloped by their own sheath of SGCs (Huang et al., 2006). We thus examined the impact of age on SGCs morphology by transmission electron microscopy (TEM). In adult mice, each sensory neuron was enveloped by its own SGCs sheath as described previously (Pannese, 1981, 2010). In aged mice, SGCs appeared thinner compared to adult mice (**Fig 6A**). Quantification of the average width of SGCs per neuron demonstrated a significant decrease during aging (**Fig.6B**). Quantification of the total number of SGCs nuclei per neuron soma in TEM images revealed that 21-month-old mice had a higher frequency of neurons with 0 or 1 SGC nuclei per

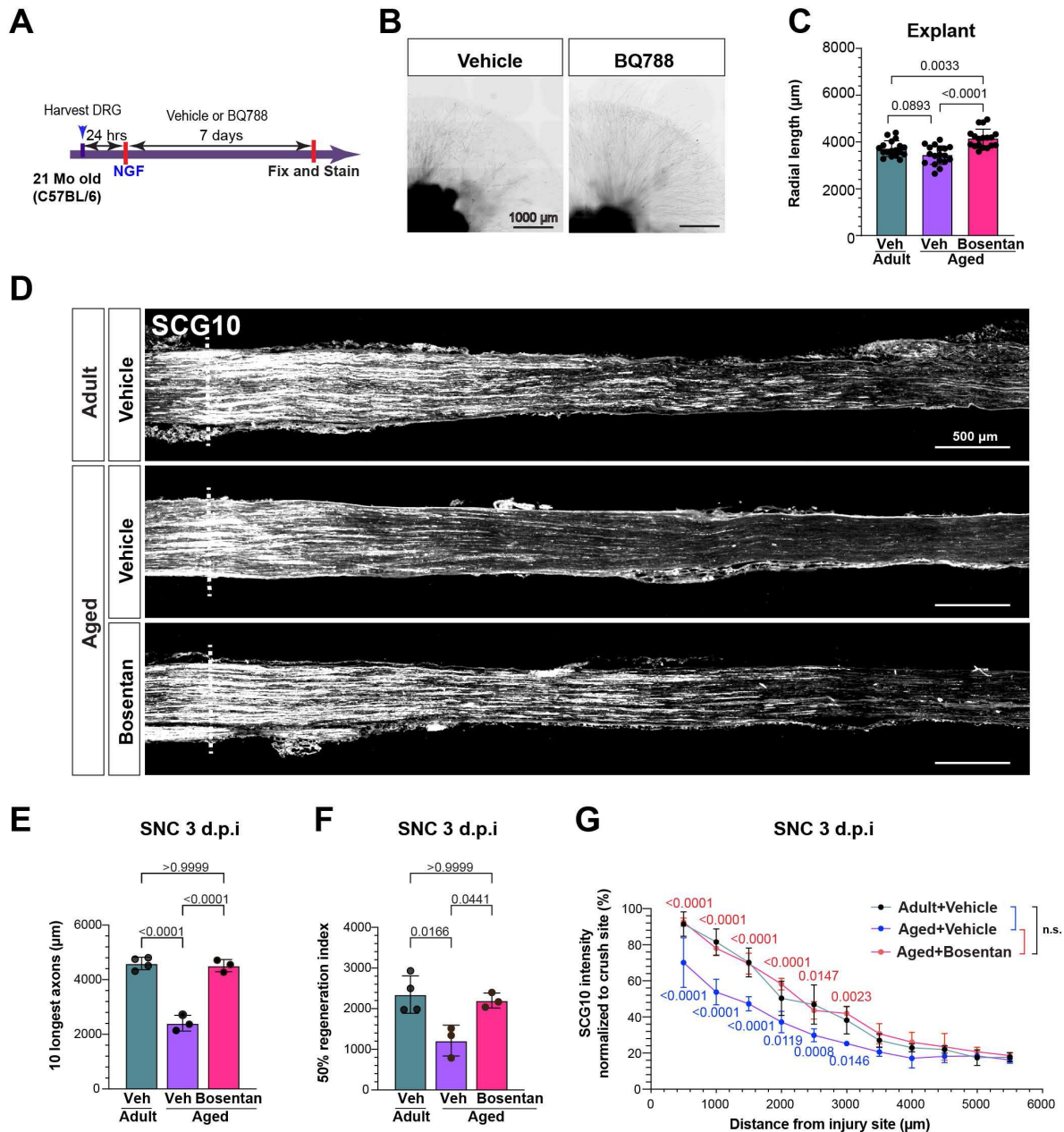


Fig. 4

Bosentan treatment rescues aging-dependent neuronal regenerative decline.

A. Scheme of drug treatment and DRG explant model.

B. Representative images of DRG explants 7 days after drug treatment (scale bars, 1000 μm).

C. Quantification of radial length of the 50 longest axons from DRG explants. N=36 (BQ788; 18 replicates, Veh; 18 replicates).

D. Representative longitudinal sections of sciatic nerves 3 d after SNC immunostained for SCG10 from mice with the indicated treatment. Dotted line indicates the crush site, determined as the maximal SCG10 intensity (scale bars, 200 μm).

E-F. Quantification of the 10 longest axons in indicated groups (**E**). Quantification of 50% regenerative index, calculated as the distance along the nerve where the SCG10 intensity is 50% of the SCG10 intensity at crush site (**F**).

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.

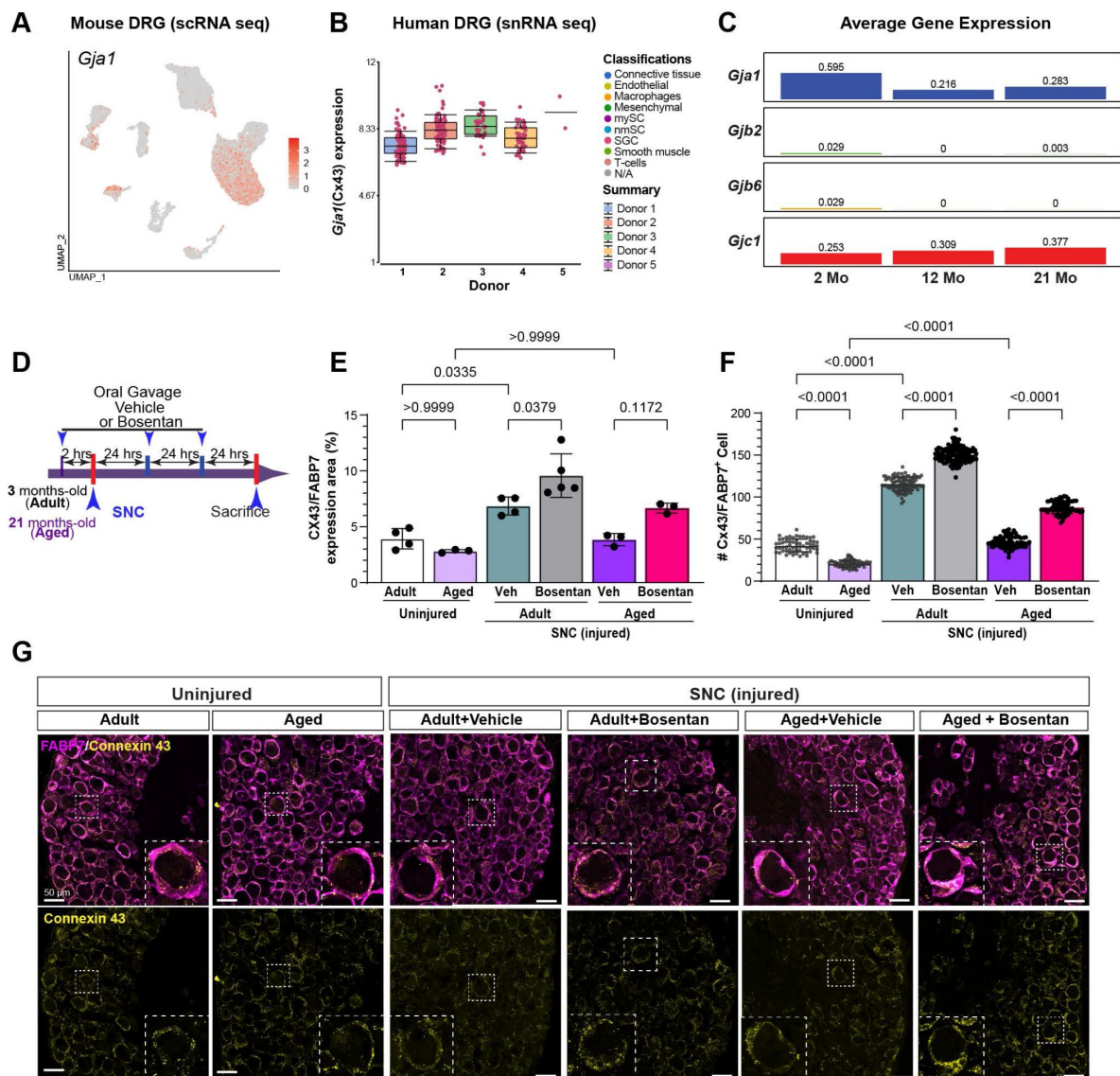


Fig. 5

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

A. UMAP overlay for expression of *Gja1* in mouse DRG.

B. Box and whisker plot of *Gja1* gene expression in 5 donors, each dot represents a single cell.

C. Plot showing the average gene expression of *Gja1*, *Gjb2*, *Gjb6* and *Gjc1* genes in SGCs in adult (2M), mid-aged (12M), and aged (21M) mice.

D. Scheme of drug treatment and peripheral nerve injury model.

E. Quantification of the percentage of the Cx43/FABP7 expression area.

F. 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.

G. Representative immunostaining images showing Connexin 43 (Cx43), FABP7 and TUJ1 in L4 DRGs from the indicated condition (scale bars, 50 μ m).

neuron than 2-month-old mice (**Fig. 6C**), suggesting a decrease in SGCs number around neuron soma with age. These results suggest that SGCs may undergo atrophy or senescence during aging, consistent with the hypothesis that a prominent decrease in Cx43 is a marker of senescence (Procacci et al., 2008).

To further examine the gene expression changes during aging, we performed differential gene expression analysis to identify common patterns of aging gene expression in mid-aged and aged samples across all cell types. Due to fewer cells recovered from mid-aged and old DRG samples, all cell types were pooled for DEG analysis. A considerable number of genes were differentially expressed when comparing mid-aged to adult (1977 upregulated, 3968 downregulated), and old to adult (1768 upregulated, 4053 downregulated) samples, with substantial overlap between the two aged groups (**Fig. 6D**-F, Supplementary File 2 related to **Fig. 6**). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and Gene Ontology (GO) analysis for the DEGs overlapping in mid-aged or aged groups compared to adults revealed upregulation of a limited number of pathways including Rap1 signaling and ribosome function (**Fig. 6G**, Supplementary Fig. 5B-C). Conversely, the pathways and terms identified from the set of downregulated genes were numerous (**Fig. 6H**, Supplementary File 3 related to **Fig. 6**). Interestingly, the strongest downregulated pathways in aged samples were related to fatty acid and cholesterol metabolism, as well as PPAR α (**Fig. 6H**). Our previous studies have shown that fatty acid metabolism upstream of PPAR α in SGCs promotes axon regeneration (Avraham et al., 2020; Avraham et al., 2021), suggesting that in addition to a reduction in Cx43, a decrease in fatty acid metabolism and PPAR α signaling in SGCs may contribute to age-dependent regenerative decline.

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 increase the risk of long-term disability (Pestronk et al., 1980; Vaughan, 1992; Verdu et al., 2000; Yun, 2015). Our study showed that ETBR functions in part to limit axon regenerative capacity and that Bosentan, an FDA-approved ETBR/ETAR antagonist, increased axonal regeneration after peripheral and central axon nerve injury. Furthermore, Bosentan rescued 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 explants increases axon outgrowth, suggesting that Bosentan treatment increases axon growth after nerve injury via inhibition of ETBR on SGCs. It is important to note that, in the nerve, ETBR is also expressed on Schwann cells and has been shown to play a role in Schwann cell generation (Brennan et al., 2000). Thus, we cannot rule out that Bosentan treatment *in vivo* may also promote axon regeneration through ETBR on Schwann cells. However, as 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 significantly contributes to axon outgrowth. As our sequencing data showed that *Edn1*/ET-1, a ligand for ETBR, is enriched in endothelial cells, and there is a dense and permeable vascularization in the DRG, we hypothesize that ET-1 arises from endothelial cells lining blood vessels. However, other studies have suggested that ET-1 is also expressed by small diameter neurons (Giaid et al., 1989), thus it is possible that ET-1 from neurons is also acting on ETBR in SGCs. The observation that elevated tissue or plasma concentrations of ET-1 occurs with age (Jankowich and Choudhary, 2020) supports our results that ETBR functions to limit axon regenerative capacity in aged mice.

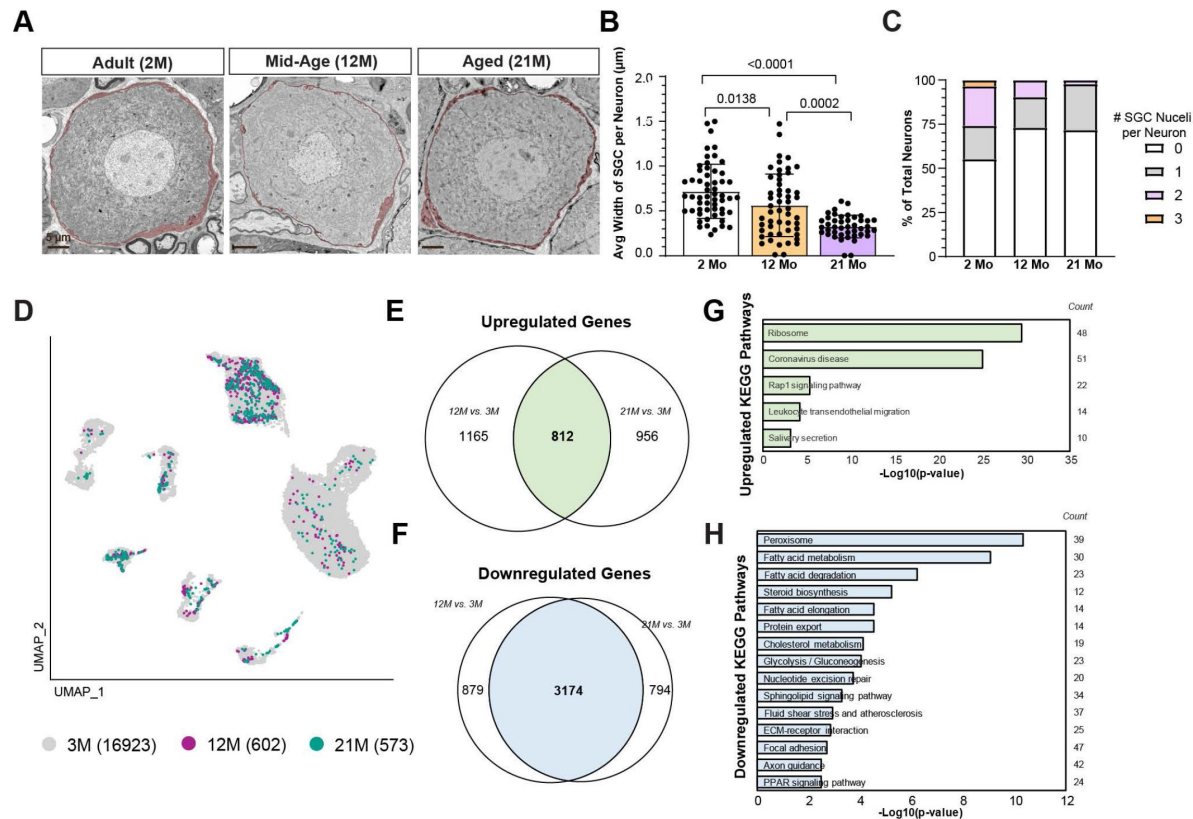


Fig. 6

Aging alters SGCs morphology and metabolism.

A. 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).

B. Quantification of the average width of SGCs per neuron in μ m.

C. Frequency of neurons with 0, 1, 2, or 3 SGC nuclei per neuron in TEM images for 2M, 12M, and 21M old mice.

D. UMAP analysis of 6430 SGCs from mice of different age.

E-F. Venn diagrams were constructed to compare the sets of differentially expressed genes (DEGs) that were upregulated (**E**) and downregulated (**F**) in the mid-aged vs. adult comparison and in aged vs. adult. (FDR ≤ 0.05 , fold-change ≥ 2).

G-H. KEGG analysis was performed on the common upregulated DEGs (**G**) and common downregulated DEGs (**H**). The dots in the diagram represent the count of genes associated with each pathway.

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 have an important role in communication between SGCs and sensory neurons (Retamal et al., 2017 [DOI](#)). Published work from our lab and others, as well as the current study, show that Cx43 is the most abundant member of the connexin family in SGCs and its expression increases following nerve injury (Avraham et al., 2021 [DOI](#); Hanani and Spray, 2020 [DOI](#)) and decreases with age (Procacci et al., 2008 [DOI](#)). In the current study, Cx43 in SGCs from both adult and aged injured mice were 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](#))(Blomstrand et al., 2004 [DOI](#)). Mechanistically, 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. In astrocytes, connexins hemichannels also regulate astrocyte volume (Quist et al., 2000 [DOI](#)) and adhesive contacts (Cotrina et al., 2008 [DOI](#)). The decrease in Cx43 expression in aged mice may thus contribute to the decrease SGCs volume we observed, and is consistent with the hypothesis that a prominent decrease in Cx43 is a marker of senescence (Procacci et al., 2008 [DOI](#)). Emerging evidence also suggest that connexins can perform non-channel functions, such as protein interaction, cell adhesion, and intracellular signaling (Mazaud et al., 2021 [DOI](#)). Future studies are aimed at determining the mechanisms by which the ETBR and Cx43 in SGCs regulate axon regeneration.

In our previous studies, we showed that PPAR α signaling downstream of fatty acid synthase in SGCs promotes axon regeneration, in part via regulation of pro-regenerative genes expression in neurons (Avraham et al., 2020 [DOI](#); Avraham et al., 2021 [DOI](#)). In the current study, pathway analysis of transcriptional changes in the DRG during aging revealed downregulation of pathways related to lipid metabolism and fatty acid synthesis. This result suggests that reduction in lipid metabolism in SGCs may contribute to age related decline in axon regeneration in addition to the ETBR-Cx43 axis. Our data also indicate that the number of SGCs surrounding each soma decreases with age, suggesting SGCs loss in addition to SGCs atrophy with age may be linked to the neuronal loss observed with age (Nagashima and Oota, 1974 [DOI](#)). In addition to loss of support, aged SGCs may also transition into a senescent phenotype that limit axon regeneration. 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 [DOI](#)), 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.

Taken together, our results suggest that ETBR signaling inhibits 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 analysis on DRG samples from adult, mid-age, and aged mice, along with comparisons to previous datasets. To achieve these objectives, we conducted single-cell RNA sequencing (scRNA-seq) analysis on samples collected from 2-3-month-old (adult), 12-month-old

(mid-age), and 21-month-old (aged) mice. Two biological duplicates were utilized and compared with our previous dataset (Avraham et al., 2021 [DOI](#)). Rat single-cell and Human single-nucleus RNA sequencing (snRNA-seq) data were obtained from our previous dataset (Avraham et al., 2022 [DOI](#)). Considering that the previous mouse datasets only involved female mice, we exclusively employed female mice for scRNA-seq in the mouse samples. 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), and RNA *in situ* hybridization (RNAscope). For *in vitro* cell assays, data were collected from at least three independent cultures, as indicated in figure legends. *In 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) 1 μ M, 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-wk- old mice were collected into cold dissection medium [HBSS (ThermoFisher, Gibco; Catalog#:14175-079)] with 10% 1M HEPES (ThermoFisher, Gibco; Catalog#15630080) and 18 mM D-Glucose (Sigma; Catalog# G7021). DRG roots were trimmed to 2mm with a sharp scalpel, then seeded on PDL/ laminin-coated cell culture plates. 24 hours after plating, the media was removed and replaced with vehicle (0.1% DMSO) or BQ788 (1 μ M) in complete medium (Neurobasal™-A Medium, ThermoFisher, Gibco; Catalog# 12349015) with B-27™ Plus Supplement (ThermoFisher, Gibco; Catalog# A3582801); GlutaMAX™ Supplement (ThermoFisher, Gibco; Catalog#35050061), and 2.5% Fetal Bovine Serum (FBS) (Gibco, Catalog#A31604). 7 days after plating, explants were fixed and stained for immunofluorescence.

Sciatic nerve and dorsal root injuries

Sciatic nerve crush injuries were performed following established protocols (Avraham et al., 2021 [↗](#)). 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 [↗](#)). 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 [↗](#)) and Bosentan (Sigma, Catalog#PHR2708, 10 mg/kg body weight) (Pinho-Ribeiro et al., 2014 [↗](#)) 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 an 10% Ready Gel® Tris- HCl Precast Gels (BioRad, catalog#1611119) in running Buffer (BioRad, Catlog#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 Rabbit-anti-ETBR (Abcam, Catalog# ab117529, 1:1000) 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 GAPDH 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 blots, the intensity of ETBR was normalized to the intensity of GAPDH. 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 ΔCq 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 µL of Lycopersicon Esculentum Lectin (Vector, Catalog# DL-1178-1) through the tail vein to label the blood vessels (Robertson et al., 2015). 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), 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-technique, 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.

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 20,584 cells with >300 genes were retained for downstream analysis. Sample QC, integration, clustering, DGE, quantification, and statistical analysis were performed using the Seurat package (v4.3.0). Following normalization with SCTransform, anchor-based integration by sample age, and graph-based clustering, three 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, low average genes per cell, and/or low average UMIs per cell. After removal of low-quality samples, the remaining clusters 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. All cell types were pooled for DEG analyses. 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 × 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 ± 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 sequencing data have been deposited at NCBI Gene Expression Omnibus under accession number GSE223910 and will be publicly available upon publication.

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Author contributions

R.F. and V.C. conceptualized the study, and V.C. provided study supervision. R.F., S.J. and I.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. C.G and V.C. provided study supervision. R.F., S.F.R and V.C. wrote the manuscript and all authors edited the manuscript.

Competing interests

Washington University in St Louis has filed a provisional patent application with the US Patent and Trademark Office on this work.

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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.

(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?

(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.

(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 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.

(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.

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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 long-neglected 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.

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.
- (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?
- (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?
- (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? 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?

(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.

(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?

(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.

(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.

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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.

Weaknesses:

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

(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?

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