Endothelin-B Receptor Activation in Astrocytes Regulates the Rate of Oligodendrocyte Regeneration during Remyelination.

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Highlights
- EDNRA and EDNRB are upregulated after demyelination in reactive astrocytes
- Pharmacological inhibition of EDNRB, but not EDNRA, accelerates remyelination
- EDNRB loss in astrocytes, but not in OPCs, accelerates remyelination
- Endothelin indirectly inhibits OPC differentiation through astrocytes

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In Brief
Astrocyte-derived endothelin-1 (ET-1) inhibits remyelination through unknown mechanisms. Using pharmacological and genetic approaches, Hammond et al. demonstrate that ET-1 signals through endothelin receptor-B in reactive astrocytes, indirectly inhibiting oligodendrocyte progenitor cell (OPC) differentiation and remyelination. Inhibiting this pathway could provide an exciting therapeutic strategy to promote remyelination in MS.

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Endothelin-B Receptor Activation in Astrocytes Regulates the Rate of Oligodendrocyte Regeneration during Remyelination

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SUMMARY

Reactive astroglia is an essential and ubiquitous response to CNS injury, but in some cases, aberrant activation of astrocytes and their release of inhibitory signaling molecules can impair endogenous neural repair processes. Our lab previously identified a secreted intercellular signaling molecule, called endothelin-1 (ET-1), which is expressed at high levels by reactive astrocytes in multiple sclerosis (MS) lesions and limits repair by delaying oligodendrocyte progenitor cell (OPC) maturation. However, as ET receptors are widely expressed on neural cells, the cell- and receptor-specific mechanisms of OPC inhibition by ET-1 action remain undefined. Using pharmacological approaches and cell-specific endothelin receptor (EDNR) ablation, we show that ET-1 acts selectively through EDNRB on astrocytes—and not OPCs—to indirectly inhibit remyelination. These results demonstrate that targeting specific pathways in reactive astrocytes represents a promising therapeutic target in diseases with extensive reactive astroglia, including MS.

INTRODUCTION

Reactive astroglia is the cellular and biochemical transformation of astrocytes in response to brain injury, and it significantly impacts—both positively and negatively—neural regeneration (Sofroniew and Vinters, 2010; Williams et al., 2007). Reactive astroglia was once thought to be an all-or-nothing transformation, but emerging evidence suggests that reactive astrocytes (RAs) are highly dynamic and tailor their transcriptional response to the type of injury and the region in which it occurs (Zamanian et al., 2012). This response includes production of growth factors, cytokines, and other intercellular signaling molecules that influence the ability of progenitor cell populations to repair damaged tissue. Therefore, it is essential to understand how specific signals produced by RAs impact neural regeneration so that we can develop targeted approaches to enhance the beneficial aspects of the astrocyte response while preventing the deleterious ones.

Endothelin-1 (ET-1) is upregulated by astrocytes in a number of brain pathologies, including stroke, traumatic brain injury, Alzheimer’s disease, cancer, and multiple sclerosis (MS) (D’haeseleer et al., 2013; Hammond et al., 2014; Palmer et al., 2012; Petrov et al., 2002; Schinelli, 2006; Stiles et al., 1997; Torbidoni et al., 2005). While ET-1 has been well characterized for its role as a secreted signaling peptide in the cardiovascular system, its role in the normal and pathological brain is not well defined (Rubanyi and Botelho, 1991). Both neurons and glia, including astrocytes, express endothelin receptors (EDNRs), and ET-1 has been shown to promote reactive astroglia in vitro and in vivo (Gadea et al., 2008; Schinelli, 2006). Interestingly, EDNR inhibition improves recovery in several animal models of brain injury (Guo et al., 2014a; Hammond et al., 2014; Moldes et al., 2012), suggesting that ET-1 plays a deleterious role in the pathological lesion environment. However, given the widespread expression of EDNRs, the mechanisms by which ET-1 signaling impacts the regenerative response, including its effect on specific cellular targets, are not well understood.

MS is a disease characterized by oligodendrocyte (OL) death, focal demyelinated CNS lesions, and extensive RA scar formation (Compston and Coles, 2008; Williams et al., 2007). In response to demyelination, OL progenitor cells (OPCs) can replace lost OLs by maturing into new myelin-producing cells in a process called remyelination (Franklin and Ffrench-Constant, 2008). However, stalled OPC differentiation is frequently found in patients with progressive MS (Chang et al., 2002; Wolswijk, 1998), possibly due to the aberrant expression of signals within the demyelinated lesions (Franklin and Ffrench-Constant, 2008). These signals could derive, at least in part, from permanent astrocytic scars that are common in MS brain tissue.

ET-1 is highly upregulated in RAs in human chronic active MS lesions and in experimentally induced demyelinated lesions in mice (D’haeseleer et al., 2013; Hammond et al., 2014). We recently showed that ET-1 contributes to stalled OPC differentiation by promoting inhibitory astrocyte-OPC signaling (Hammond et al., 2014). This led to delayed remyelination, an effect that was reversed using EDNR antagonists during the repair phase. Consistent with these findings, others demonstrated that overexpression of ET-1 by astrocytes exacerbates experimental autoimmune encephalomyelitis (EAE), a mouse model...
of MS (Guo et al., 2014b). Despite these findings, it is still unclear whether astrocyte-derived ET-1 solely acts by modulating the astrocyte response to injury, including overactivation of inhibitory astrocyte-OPC signaling pathways (as previously described; Hammond et al., 2014), or whether astrocyte-derived ET-1 also acts directly on OPCs, which also express EDNRs.

In our study, we used pharmacological approaches and cell-specific genetic manipulation to understand the contribution of indirect signaling through RAs to the mechanism of ET-1 action following demyelinating injury in mice. We found that selective inhibition of EDNRB signaling, but not EDNRA, accelerated OL regeneration and remyelination in demyelinated lesions. Conditional deletion of Ednrb in RAs accelerated OPC differentiation, OL regeneration, and increased myelin production, whereas deletion of Ednrb in OPCs had no effect. Our results demonstrate that RAs are a key intermediary that modulate OPC differentiation in response to demyelinating injury, and that the response of RAs, but not OPCs, to ET-1 can drastically inhibit the rate of remyelination.

**RESULTS**

Our previous study demonstrated that the EDNRA/B pan-antagonist PD142,893 accelerated OPC differentiation, myelin sheath production, and remyelination in mouse demyelinated lesions (Hammond et al., 2014). Conditional ablation of ET-1 in RAs also produced the same phenotype (Hammond et al., 2014), indicating that activation of EDNRA and/or EDNRB by astrocyte-derived ET-1 limits OPC differentiation. EDNRA and EDNRB are G protein-coupled receptors that trigger distinct downstream signal transduction cascades through Gq/Gs and Gq/Gi, respectively (Bigaud and Pelton, 1992; de Nucci et al., 1988; Schinelli, 2006). While multiple ligands (ET-1, ET-2, and ET-3) can bind to the EDNRA and EDNRB receptors, we found in our previous study that ET-1 was the only isoform expressed in the subcortical white matter (WM) (Hammond et al., 2014).

To date, the expression patterns and cellular responses induced by ET-1 on EDNRA and EDNRB have not been defined in the context of demyelinating injury. Here we used a mouse model of lysolecithin (LPC)-induced demyelination to address these questions in vivo.

LPC was used to induce focal demyelinated lesions in one hemisphere of the subcortical WM in mice. As a control, saline (NaCl) was injected into the contralateral hemisphere. We first examined the protein expression patterns of EDNRA and EDNRB in adult WT mice following LPC-induced demyelination at 7 dpl.

**Figure 1. EDNR Expression Is Upregulated in RAs following Demyelination**

EDNRA and EDNRB expression was examined in adult WT mice following LPC-induced demyelination of subcortical WM at 7 dpl. Images of brain sections stained with anti-EDNRA and anti-EDNRB antibodies. Increases in EDNRA (A) and EDNRB (B) expression were found in LPC lesions, as compared to saline (NaCl)-injected contralateral control hemispheres. (C) Quantification of EDNRA and EDNRB protein expression by mean fluorescent intensity within the outlined regions in LPC and NaCl tissue is shown (n = 4; *p < 0.05, unpaired t test; mean ± SEM).

(D–F) Images show LPC lesions co-immunolabeled with anti-EDNRA and anti-GFAP (RAs) (D), anti-IBA1 (microglia) (E), and anti-Olig2 (OPCs/OLs) (F).

(G–I) Images show LPC lesions co-immunolabeled with anti-EDNRB and anti-GFAP (G), anti-IBA1 (H), and anti-Olig2 (I). Scale bars, 200 μm (A and B) and 50 μm (D–F and G–I).
lesions (Figures 2G–2J). No changes were found in total Olig2+ cell numbers (Figure S1A), indicating that changes in the BQ788-infused mice were due to precocious OPC differentiation and not changes in the total number of OPCs/OLs.

We have shown previously that EDNRA/B antagonist PD142,893 accelerates OPC differentiation by inhibiting Notch activation in the transgenic Notch reporter (TNR) mouse following demyelination (Hammond et al., 2014). Since BQ788 also increased rates of OPC differentiation (Figure 2F), we infused BQ788 into the demyelinated lesions of TNR mice to determine if EDNRB inhibition also led to a reduction in Notch signaling. At 7 dpl, we observed a significant reduction in EGFP+Olig2+ cells within the lesion (Figures 2K–2M), demonstrating that inhibition of EDNRB activation is sufficient to reduce Notch activation during remyelination.

Altogether, our results demonstrate that, despite elevated expression of both receptors in demyelinated lesions (mostly in RAs), only inhibition of the EDNRB accelerated OPC differentiation. This could be due, in part, to reduced activation of Notch signaling in OPCs.

Our previous studies showed that RAs regulate the rate of remyelination through activation of EDNRs on RAs themselves through an indirect pathway (Hammond et al., 2014). However, several reports have demonstrated that OPCs also express EDNRAs and EDNRBs and respond to endothelins directly (Gadea et al., 2009; Jung et al., 2011; Yuen et al., 2013). Therefore, we sought to assess the relative contribution of ET-1 signaling to OPCs and RAs individually by generating cell-specific conditional knockouts of EDNRs. Based on pharmacological results from our selective antagonist infusions, we genetically ablated EDNRB on each cell type using conditional knockout mice. Floxed Ednrb mice were bred to Pdgfrα creERT2 and hGfap creERT2 mice to ablate EDNRB expression in OPCs and RAs, respectively. Tamoxifen (Tam) was injected once a day for 3 days prior to LPC-induced demyelination and brains were analyzed at 14 dpl (Figure 3A).

By 14 dpl, EDNRB expression was noticeably reduced in OPCs in Ednrbfl/fl;PdgfrαCREERT2 (Figure 2A) and Ednrbfl/fl;hGfapCREERT2 (Figure 2B) mice treated with Tam, as compared to Tam-treated Ednrb+/−CRE+ controls. At 14 dpl, we found no change in the total number of mature OLs in Ednrbfl/fl;PdgfrαCREERT2 and hGfapCREERT2 (Figure 2B) mice treated with Tam, as compared to Cre-negative and vehicle-injected control littermates (Figures 3B–3D and 3L). There was also no change in the total number of NG2+ OPCs (Figures S3A–S3D), the CC1+ to NG2+ cell ratio (Figure S3E), or the number of Olig2+ OPC/OLs (Figure S1B). The last finding shows that the loss of EDNRB in OPCs had no effect on their ability to populate the demyelinated lesion. In addition, loss of...
EDNRB in OPCs had no effect on remyelination since there were no changes in MBP immunostaining (compared to control, Figures S4A and S4B) or the number of myelinated axons and myelin thickness (g ratio) as assayed by transmission electron microscopy (EM) (Figures 3E, 3F, 3N, and 3O). To ensure that EDNRB ablation in OPCs had no effect on the rate of OPC differentiation, we characterized the terminal fate of Rosa-YFP+ OPCs by co-staining with CC1 (Figures S3K–S3N). We found no differences in the total number of Rosa-YFP+CC1+ cells (Figure S3M) or the percentage of Rosa-YFP+CC1+ cells (Figure S3N) between Ednrbfl;PdgfrcreER2 and Ednrbfl;PdgfrcreER2 controls at 14 dpl.

Since ablation of the EDNRB in OPCs had no effect on the rate of OPC differentiation, we sought to determine if loss of the EDNRB in RAs was sufficient to accelerate repair. Interestingly, we found large increases in the number of CC1+Olig2+ OLs in the lesions of Ednrbfl;hGfaprcre + Tam mice (Figures 3I and 3M), as compared to Cre-negative and vehicle-injected controls (Figures 3G, 3H, and 3M). These increases were similar in magnitude to those seen following BQ788 infusion (Figure 2F). Increases in mature OLs were accompanied by a significant decrease in the number of NG2+ OPCs (Figures S3F–S3I) and an increase in the CC1+ to NG2+ ratio (Figure S3J).

Insignificant decrease was observed in the total number of Olig2+ cells in the Ednrbfl;hGfaprcre + Tam mice (Figure S1C). Consistent with the increase in CC1+Olig2+ population, Ednrbfl;hGfaprcre + Tam mice displayed increased remyelination. There was a significant increase in the number of myelinated axons and myelin thickness (decreased g ratio) in Ednrbfl;hGfaprcre + Tam mice compared to Ednrbfl;hGfaprcre + vehicle controls (Figures 3J, 3K, 3P, and 3Q), as well as increased MBP expression in the lesions (Figures S4A and S4B). There was no change in the average axon diameter for any condition (Figure S4C).

BQ788 is a potent accelerator of OPC differentiation when infused into demyelinated lesions (Figure 2F). Therefore, we wanted to test whether infusion of BQ788 into lesions of Ednrbfl;PdgfrcreER2 mice could stimulate precocious OPC differentiation by blocking astrocytic EDNRBs, or whether it would provide any additional benefit in Ednrbfl;PdgfrcreER2 mice. BQ788 was infused from 6 to 14 dpl following Tam administration before LPC demyelination (Figure 4A). In Ednrbfl;PdgfrcreER2 mice, we found a significant increase in the number of CC1+Olig2+ mature OLs following BQ788 infusion compared to saline-infused controls (Figures 4B–4D), indicating that inhibition of non-OPC EDNRBs in the lesion was sufficient to accelerate OPC differentiation. On the other hand, infusion of

**Figure 3. Selective EDNRB Ablation in Astrocytes, but Not OPCs, Accelerates Remyelination**

(A) Ednrbfl;PdgfrcreER2 and Ednrbfl;hGfaprcreER2 mice were generated, and, prior to LPC demyelination, Tam was injected once a day from −3 to −1 dpl (3 to 1 days prior to LPC injection). (B–D) Images of Ednrbfl;PdgfrcreER2 + Tam (B), Ednrbfl;PdgfrcreER2 + Vehicle (C), and Ednrbfl;PdgfrcreER2 + Tam (D) lesions at 14 dpl co-immunolabeled with anti-CC1 and anti-Olig2 antibodies. White arrowheads indicate CC1+Olig2+ cells.

(E and F) EM images show LPC-injected lesions of Ednrbfl;PdgfrcreER2 + Vehicle (E) and Ednrbfl;PdgfrcreER2 + Tam (F) at 14 dpl.

(G–I) Images show Ednrbfl;hGfaprcreER2 + Tam (G), Ednrbfl;hGfaprcreER2 + Vehicle (h), and Ednrbfl;hGfaprcreER2 + Tam (i) lesions at 14 dpl co-immunolabeled with anti-CC1 and anti-Olig2 antibodies.

(J and K) EM images show LPC-injected lesions of Ednrbfl;hGfaprcreER2 + Vehicle (J) and Ednrbfl;hGfaprcreER2 + Tam (K) at 14 dpl.

(L) No changes were found in the total number of CC1+Olig2+ cells between groups (n = 4–5; N.S., not significant, ANOVA Bonferroni post hoc; mean ± SEM).

(M) Significant increases in the number of CC1+Olig2+ cells were found in Ednrbfl;hGfaprcreER2 + Tam lesions as compared to Ednrbfl;hGfaprcreER2 + vehicle and Control (n = 4–5; *p < 0.05, ANOVA Bonferroni post hoc; mean ± SEM).

(N and O) No changes in the number of the myelinated axons (N) or the g ratio (O) were found for either condition (n = 3–4; N.S., not significant, unpaired t test; mean ± SEM).

(P and Q) Significant increases in the number of the myelinated axons (P) and decreased g ratio (Q) were found in Ednrbfl;hGfaprcreER2 + Tam versus controls (n = 3–4; *p < 0.05, unpaired t test; mean ± SEM).

Scale bars, 40 μm (B–D and G–I) and 2 μm (red scale bar; E, F, J, and K). Also see Figures S1–S4.
BQ788 into Ednrb<sup>fl/fl</sup> hGfap<sup>cre+</sup> + Tam mice produced no additional increase in the number of CC1<sup>+</sup>Olig2<sup>+</sup> cells compared to saline-infused littermates (Figures 4E–4G).

Altogether, these results demonstrate that blocking ET signaling through EDNRB in astrocytes alone is responsible for modulating downstream effects on OPC differentiation and remyelination through our previously proposed indirect ET-1-signaling pathway (Hammond et al., 2014), rather than through direct activation of EDNRB on OPCs.

DISCUSSION

Prior to our study, the cell- and receptor-specific action(s) of ET-1 following demyelinating injury were largely unexplored. Here we found that ET-1 acts almost exclusively through EDNRB, and not EDNRA, on astrocytes to inhibit remyelination. RA-specific EDNRB ablation accelerated OPC differentiation and remyelination, while activation of EDNRB signaling in OPCs played little or no role in the differentiation or remyelination process. Since it has been shown that EDNRB levels are elevated in active MS lesions (Yuen et al., 2013), our results provide strong evidence that selective pharmacological inhibitors of the EDNRB could be used to promote remyelination and prevent OPC differentiation failure in patients with MS.

Transcriptome analysis of the postnatal mouse forebrain has revealed that the Ednrb is enriched 7.6-fold in astrocytes versus neurons, OPCs, and OLs (Cahoy et al., 2008). Consistent with these findings, our data show that the enrichment is conserved in demyelinated lesions, where EDNRB protein expression was predominantly confined to RAs (Figure 1G). Higher levels of expression in RAs could explain why ablating the receptor in astrocytes, but not OPCs, had the greatest effect on OPC differentiation. Despite these findings, OPC EDNRBs do exist at low levels and in vitro studies have shown that direct ET-1 exposure can impact OPC differentiation. However, these studies in cultured OPCs have produced conflicting results; our lab found that ET-1 exposure maintained OPCs in a premyelinating state (Gadea et al., 2009), but another group found that ET-1 promoted OPC maturation and myelin production (Jung et al., 2011). Interestingly, ET-2 exposure also was found to promote differentiation of cultured OPCs (Yuen et al., 2013). It is possible that these discrepant results are due to low and variable levels of EDNRBs in cultured OPCs. In the absence of other important cell types like RAs, microglia, and the vasculature, it is hard to interpret how these culture assays relate to the demyelinated lesion environment, as in vivo we found no significant effects of OPC EDNRB signaling on OPC differentiation.

Figure 4. Astrocyte EDNRB Activation Predominantly Regulates OPC Differentiation

(A) Ednrb<sup>fl/fl</sup>Pdgf<sup>creER<sup>T2</sup></sup> and Ednrb<sup>fl/fl</sup>hGfapER<sup>T2</sup> mice were injected with Tam from −3 to −1 dpl. Saline or BQ788 was infused into demyelinated lesions from 6 to 14 dpl.

(B and C) Images of Ednrb<sup>fl/fl</sup>Pdgf<sup>cre</sup> saline- (B) or BQ788-infused (C) lesions co-immunolabeled with anti-Olig2 (OPC/OL) and anti-CC1 (OL) antibodies at 14 dpl. White arrowheads indicate CC1<sup>+</sup>Olig2<sup>+</sup> cells.

(D) Significant increases in the number of CC1<sup>+</sup>Olig2<sup>+</sup> mature OLs were found in BQ788-infused Ednrb<sup>fl/fl</sup>Pdgf<sup>cre</sup> lesions, as compared to saline-infused animals (n = 4–5; *p < 0.05, unpaired t test; mean ± SEM).

(E and F) Images of Ednrb<sup>fl/fl</sup>hGfap<sup>cre</sup> saline- (E) or BQ788-infused (F) lesions co-immunolabeled with anti-Olig2 and anti-CC1 antibodies at 14 dpl. White arrowheads indicate CC1<sup>+</sup>Olig2<sup>+</sup> cells.

(G) No difference in the number of CC1<sup>+</sup>Olig2<sup>+</sup> mature OLs were found in BQ788-infused Ednrb<sup>fl/fl</sup>hGfap<sup>cre</sup> lesions, as compared to saline-infused animals (n = 4–5; N.S., not significant, unpaired t test; mean ± SEM).

Scale bar, 40 μm for all images.
Our findings beg the question of how, if at all, ET-1 directly impacts OPCs following injury. One possibility is that ET-1 stimulates a pro-migratory phenotype in OPCs, a result we previously demonstrated in cultured OPCs and subventricular zone (SVZ) explants (Gadea et al., 2009). However, here we found no major differences in total OPC/OL cell numbers following drug infusion or in the EdnrbCreERT2,PdgfrαCreERT2 mice. It is still possible that ET-1 signaling could affect the contribution of SVZ versus parenchymal OPCs to the repair process or the timing of OPC recruitment. Since significant OPC/OL EDNRA expression was found in LPC lesions, this raises the question of whether EDNRA signaling impacts OPC recruitment. Further studies will be needed to address this and uncover the role of the EDNRA in the injured brain.

In the brain, ET-1 promotes reactive astrogliosis, including increased astrocyte proliferation and expression of the RA marker GFAP (Gadea et al., 2008; Koyama et al., 1999). In cultured astrocytes, BQ788 blocked ET-1-induced increases in astrocyte proliferation, but EDNRA inhibition had little or no effect (Gadea et al., 2008). These findings, together with our results in this study, suggest that activation of astrocytes through the EDNRB and subsequent changes in the astrocyte activation state have the greatest impact on regeneration following injury. Although RAs are essential for normal recovery following brain injury, it appears that targeting specific aspects of their signaling response to injury could be beneficial. This is especially true for disorders where excessive or prolonged astrocyte activation, including MS, could lead to altered activation of signaling pathways that limit normal recovery.

All endothelin isoforms bind EDNRB with equally high affinity, and EDNRB stimulation results in activation of JNK, p38MAPK, ERK, and c-Jun pathways in RAs (Gadea et al., 2008; Schinelli et al., 2001). Activation of these pathways can be blocked using BQ788, but not by the EDNRB antagonist BQ123. ET-1 also induces Jagged1 expression in astrocytes, which is inhibitory to OPC differentiation (Hammond et al., 2014). Interestingly, BQ788 also effectively blocked Notch activation in LPC lesions (Figure 2M), suggesting that BQ788 also might reduce Jagged1 expression in RAs. While it is still unclear how EDNRA activation leads to changes in Jagged1 transcription, it has been shown that the activator protein-1 (AP-1) transcription factor binds the promoter sequence of the Jagged1 gene (Johnston et al., 2009). Furthermore, c-Jun, which is directly upregulated by EDNRB activation, is a component of the heterotrimeric AP-1 complex (Horinouchi et al., 2013). This pathway will need to be explored in greater detail, and it could have great importance in other disorders where Notch activation contributes to pathology.

Independent of Jagged-Notch signaling, it is likely that analysis of ET-1-treated astrocytes also would reveal other signals that indirectly influence OPC development and remyelination. Our analysis has shown that ET-1-treated astrocytes undergo profound changes in vitro, including transcriptional changes in hundreds of genes (T.R.H. and V.G., unpublished data). Because RAs play such an important and influential role in the signaling microenvironment in demyelinated tissue (Brosnan and Raine, 2013; Williams et al., 2007), it is essential to understand how these cells respond to signals like ET-1, which are released at very high levels after several types of CNS injury both in rodents and humans (Armstead and Kreipke, 2011; D’haeseleer et al., 2013; Hammond et al., 2014; Palmer et al., 2012; Petrov et al., 2002; Stiles et al., 1997; Toribondi et al., 2005).

EXPERIMENTAL PROCEDURES

Animals

TNR (005854), floxed Ednrb (011080), and PdgfrαCreERT2 (018280) mice were purchased from the Jackson Laboratory. C57bl/6n mice were purchased from Charles River Laboratories. The hGfapCreERT2 mice were obtained from Dr. Flora Vaccarino at Yale University and were generated as previously described (Ganat et al., 2006). Mice used for all experiments were 8–14 weeks old unless otherwise specified. All mouse colonies were maintained in the animal facility of Children’s National Medical Center, and all animal procedures complied with the guidelines of the NIH and with the Children’s Research Institute Institutional Animal Care and Use Committee (IACUC) guidelines.

Tam Injections

Tam (Sigma, 90 mg/ml) was dissolved in 100% ethanol and then diluted in autoclaved sunflower oil (Sigma) to a final concentration of 10 mg/ml. EdnrbCreERT2, hGfapCreERT2 and EdnrbCreERT2,PdgfrαCreERT2 mice were injected with 75 mg/kg Tam once per day from ~3 to ~1 dpl. A vehicle solution of 10% ethanol in sunflower oil was injected into control animals.

LPC Injection

Mice were deeply anesthetized using 100 mg/kg ketamine and 10 mg/kg xylazine. LPC (1% Lyso, 2 mg/ml) was injected unilaterally into the external capsule of 8- to 14-week-old mice using a Hamilton syringe. On the contralateral side, 2 µl 0.9% NaCl was injected for control purposes. Injections were made using a stereotaxic apparatus at the following coordinates: 1.0 mm anterior to bregma, 1.5 mm lateral, and 3.0 mm deep. The date of injection was denoted as 0 dpl. Mice were then left for a period of 7, 8, or 14 dpl and subsequently perfused for immunohistochemical analysis.

Mini-Osmotic Pump Installation

Mice were deeply anesthetized using 100 mg/kg ketamine and 10 mg/kg xylazine and unilateral LPC injections were performed in 8- to 14-week-old mice. Mini-osmotic pumps (Durect) were assembled using a brain infusion kit (Durect). Brains were then used for immunohistochemical analysis.

Immunohistochemistry

Mice were anesthetized by isoflurane inhalation and perfused intracardially with 0.9% saline, BQ123, or BQ788. The approximate BQ123 and BQ788 delivery rates were 300 pmol/day. Mice were re-anesthetized and the pumps were installed into a subcutaneous pocket at the base of the neck. The catheter tubing and cannula were led to the initial injection site, and the cannula was inserted into the same skull perforation used for LPC injection, which was still visible. The cannula was attached to the skull using cyanoacrylate adhesive (5008670, Durect). Brains were then used for immunohistochemical analysis.
biotin also were performed using the Vector Avidin Biotin Blocking Kit (SP-2001) according to the manufacturer’s instructions. Image acquisition and analysis details and antibodies used are described in the Supplemental Experimental Procedures.

**EM**

Mice were perfused in Millonig’s buffer containing 4% paraformaldehyde and 2.5% glutaraldehyde. A brain matrix was used to isolate the regions of interest, which were postfixed in 1% osmium tetroxide, dehydrated, and embedded in PolyBed resin. Thick (1-μm) and thin sections were stained with toluidine blue and a combination of uranyl acetate and lead citrate, respectively. A minimum of ten electron micrographs taken at 5,000× were captured per mouse using a JEOL JEM 1230 transmission electron microscope equipped with a Gatan 4K × 4K Ultrascan digital camera.

**Statistical Analysis**

Specific numbers of animals or cultures are denoted in each figure legend. Significance was calculated using GraphPad Prism software using unpaired t tests for comparisons between two groups. For multi-group comparisons, a one-way ANOVA with a Bonferroni post hoc analysis was used.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.11.002.

**AUTHOR CONTRIBUTIONS**

T.R.H., B.M., and V.G. designed and conceptualized the experiments. T.R.H. and B.M. performed and analyzed all the experiments in this project. J.D. performed EM imaging and analysis. P.D.M. contributed to analysis of the conditional knockout mice and helped to revise the manuscript. M.R. contributed to the EDNR expression analysis. T.R.H., B.M., and V.G. wrote and edited the manuscript.

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