MODULATING NEUROTROPHIN RECPTOR; P75^{NTR} EXERTS VASCULAR PROTECTION IN ISCHEMIC RETINOPATHY

by

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(Under the Direction of AZZA B. EL-REMESSY)

ABSTRACT

Achieving reparative angiogenesis remains an unrealized goal in cardiovascular diseases. We have previously shown that deletion of the neurotrophin death receptor; p75^{NTR} increased activation of the survival receptor; TrkA in bovine retinal endothelial cell cultures. The overall goal of this project is to examine the impact and elucidate the molecular mechanisms by which deletion of p75^{NTR} enhances vascular repair in ischemic tissues. Using oxygen-induced retinopathy mouse model, we demonstrated that expression of p75^{NTR} was upregulated during vaso-obliteration phase, which was accompanied by marked central vascular cell death and pathological neovascularization in WT pups. Deletion of p75^{NTR} receptor attenuated both manifestations, a vascular protective effect that was accompanied by increased activation and expression of TrkA receptor, preservation of mature neurotrophin levels (NGF and BDNF) as well as enhanced VEGF signal. Vascular protection was reversed by intravitreal injection of the staurosporine kinase inhibitor; K-252a. Next, we assessed the impact of deleting p75^{NTR} receptor on increasing vascular homing of mesenchymal stem cells (MSCs), using retinal ischemia-reperfusion (IR) mouse model. Trypsin-digested retinas showed that, deletion of p75^{NTR} protected against ischemia manifested by decreased number of acellular capillaries 10 days after

IR insult. Vascular protection was enhanced by intra-vitreal injection of MSCs 48 hours after IR induction. Knocking down $p75^{NTR}$ receptor on the surface of MSCs increased their vascular homing to ischemic vasculature 7 days after intravitreal injection and increased SDF-1α/CXCR-4& -7 signaling axis. In summary, our results showed deletion of $p75^{NTR}$ receptor is protective in different retinal ischemic models. The underlying mechanism involves, at least in part, activation of the survival receptor; TrkA and increased vascular homing of MSCs. Our findings identified $p75^{NTR}$ receptor as novel therapeutic target for ischemic ocular diseases.

INDEX WORDS: $p75^{NTR}$ receptor, TrkA receptor, Ischemic retinopathy diseases, mesenchymal stem cells (MSCs), angiogenesis, neurotrophins, vascular endothelial growth factor (VEGF)

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DEDICATION

To my father, *Lotfy Elshaer*; the man whom I owe everything I am, everything I have and no matter what, I cannot pay him back. Without his faith in me, guidance and unconditional love, I wont have been able to continue moving on.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Ischemic ocular diseases

Retinal ischemia is a common pathogenic stage in multiple ocular diseases; i.e. retinopathy of prematurity (ROP), proliferative diabetic retinopathy (PDR) as well as diabetic macular edema (DME). ROP can be viewed as an arrest of normal retinal neuronal and vascular development in the preterm infant, with ultimately pathological compensatory mechanisms that result in aberrant vascularization of the retina [1]. Optimum oxygenation to balance risk of retinopathy of prematurity against improved survival is still unknown [2,3]. Oxygen administration is better controlled nowadays than in the past in developed countries, but ROP persists, partly because of the increased survival of infants with extremely low gestational ages and birth weights who are at high risk for the disease. In some developing countries, unmonitored treatment with 100% oxygen is still used, which can even cause more mature babies to develop severe retinopathy of prematurity [1].

Another ocular disease characterized by retinal ischemia is diabetic retinopathy (DR), which is a leading cause of blindness in working-age, affecting both genders equally [4]. DR is estimated to affect 101 million people worldwide [5]. Patients with type 1 diabetes may show evidence of retinopathy as early as 5 years after the onset of diabetes, and almost all patients show varying degrees of retinopathy 20 years later. Background retinopathy may even be present at the time of diagnosis of type 2 diabetic patients, consistent with the long duration of subclinical hyperglycemia in such patients and more than 60% of type 2 diabetic patients will experience retinopathy after 20 years of diabetes onset [6]. Although DR was previously

perceived as a sole microvascular complication, it is now widely accepted that diabetes affects multiple cell types in the retina resulting in neurodegeneration, inflammation and alteration of microvasculature [7]. DR begins with microaneurysms and progress into exudative changes such as leakage of lipoproteins and blood that lead to macular edema. Then ischemic changes, collateralization and dilatation of venules, followed by proliferative changes; abnormal vessels on the optic disk and retina, proliferation of fibroblasts, and vitreous hemorrhage (Reviewed in [8]).

Current treatments for ischemic ocular diseases include laser photocoagulation and anti-VEGF injections [9]. Laser seals leaking blood vessels directly by photocoagulation. It eliminates abnormal blood vessels that form neovascularization, intentionally destroying tissue in retinal periphery [10]. On the other hand, anti-VEGF therapy was proven to be effective for managing DME based on the results of "Rise and Ride" clinical trials [9] and DR as illustrated in another report by Ip et al. 2012 {Ip, 2012 #11}. Manifestations of anti-VEGF therapy include hypertension as well as renal side effects including proteinuria and glomerular thrombotic microangiopathy [11] with pre-existing hypertension, age and body mass index as significant predisposing risk factors [12]. Both laser and anti-VEGF treatments are focused on end stage disease and carry significant sight-threatening side effects [13]. Importantly, they do not address the early and potentially reversible failure of retinal perfusion [14].

Nerve growth factor (NGF)

The long-term goal of my project is to develop novel therapeutic approach to prevent retinal ischemia and induce vascular repair thus, preventing ocular diseases from progressing into neovascular stage, through neurotrophin system, currently studied in our lab. Nerve growth factor; NGF which is the first discovered neurotrophin, plays an important role not only in

neurons but also in vasculature supporting angiogenesis [15-18]. NGF is traditionally released by glia as proform; proNGF that is cleaved to mature form [19]. Mature NGF binds to TrkA, associated with $p75^{NTR}$, initiating survival pathway [20]. Meanwhile, proform binds preferentially to $p75^{NTR}$ bound to sortilin receptor to activate inflammatory and apoptotic pathway [21].

p75 neurotrophin ($p75^{NTR}$) receptor

$p75^{NTR}$, also known as nerve growth factor receptor (NGF-R), is present on the cell surface of sympathetic neurons and neural crest-derived sensory neurons [22,23]. $P75^{NTR}$ is a member of the tumor necrosis factor (TNF) receptor superfamily that can bind all neurotrophins including nerve growth factor (NGF), brain derived nerve factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin4/5 (NT4/5) with approximately equal affinity in most cells [24]. $P75^{NTR}$ is a single trans-membrane protein with an N-terminal extracellular domain; containing four repeated modules of six cysteines [25,26] and a C-terminal intracellular domain (ICD). Because $p75^{NTR}$ lacks intrinsic catalytic activity, it signals by undergoing conformational rearrangements through ligand binding and co-receptor interactions. In addition, a series of proteolytic cleavage and recruitment of diverse intracellular proteins direct its signaling function [27]. Regulated intra-membrane proteolysis of $p75^{NTR}$ consists of ectodomain shedding of $p75^{NTR}$ by α-secretase that results in formation of a membrane-bound C-terminal fragment (CTF). CTF is subsequently cleaved by γ-secretase complex, liberating the intracellular domain (ICD) [28]. The ICD of $p75^{NTR}$ can be palmitoylated at cysteine 279 [29] or phosphorylated on serine and threonine residues [30]. These post-translational modifications confer roles in protein-protein interaction, proper intracellular folding of the receptor or in directing its cellular localization [31].

Signaling and biological functions of $p75^{NTR}$.

Since $p75^{NTR}$ receptor lacks intrinsic catalytic activity, it signals by interaction with several intracellular proteins. The most prominent intracellular feature of $p75^{NTR}$ ICD is the death domain (DD), a ~80 amino acid association module, initially identified in related pro-apoptotic TNFR superfamily members though not showing their self-aggregation property [32]. $P75^{NTR}$ is an unusual member of the TNF receptor family owing to its tendency to dimerize rather than trimerize and its ability to act as a co-receptor with tropomyosin receptor kinases (Trks) [31]. When $p75^{NTR}$ co-receptors with one of the Trks, it refines their affinity and specificity for neurotrophins. For instance, $p75^{NTR}$ increases the affinity of TrkA to NGF [33], while restricting its binding of NT3, and it also relaxes the specificity of TrkC [34].

Survival can be promoted by $p75^{NTR}$ receptor in response to NGF in an NF-*K*B-dependent manner [35]. $P75^{NTR}$ can also co-receptor with Nogo receptor [36] to regulate axonal growth through interaction with the small GTPase; RhoA. In absence of neurotrophins, a constitutive interaction between RhoA and $p75^{NTR}$ receptor maintains RhoA activation and inhibition of axonal growth, whereas, neurotrophin binding to $p75^{NTR}$ dissociates RhoA, blocking its activity and promoting axonal growth [37].

The main action of $p75^{NTR}$ receptor is to mediate apoptosis via forming a co-receptor with sortilin; a member of Vps10p-domain receptor family [38]. Several intracellular proteins were shown to be involved in $p75^{NTR}$-mediated apoptotic action. Interaction of TNF receptor-associated factor-6 (TRAF6) with $p75^{NTR}$ was shown to be required for JNK activation and $p75^{NTR}$-mediated apoptosis [39,40]. Neurotrophin receptor-interacting MAGE homolog (NRAGE) was identified to interact with $p75^{NTR}$ cytosolic region and mediate cell death in JNK-dependent mitochondrial pathway. NRAGE was shown to induce cytosolic accumulation of

cytochrome c, activation of Caspases-3, -9 and -7, and caspase-dependent cell death [41]. The neurotrophin receptor interacting factor (NRIF) was also reported to be essential for $p75^{NTR}$-dependent JNK activation and apoptosis through a mechanism that requires p53 and NRIF nuclear translocation [42,43]. Finally, $p75^{NTR}$-associated cell death executor (NADE) was also shown to be involved in $p75^{NTR}$-induced cell death upon NGF binding [44].

The apoptotic effect of $p75^{NTR}$ receptor is extensively demonstrated in the literature. The first report for apoptotic effect of $p75^{NTR}$ receptor came from Bredesen's group where over-expression of $p75^{NTR}$ receptor increased the rate of cell death [45]. In a subsequent study by Barrett and Bartlett in 1994, down-regulation of $p75^{NTR}$ by antisense oligonucleotides increased the survival of sensory neurons [46]. Several in-vivo reports confirmed the apoptotic action as well. Cheema et al., 1996 reported that $p75^{NTR}$ receptor was responsible of death of axotomized sensory neurons in the dorsal root ganglia of newborn rats [47]. Over-expression of $p75^{NTR}$ ICD resulted in marked increase in neuronal death during development in sensory and sympathetic ganglia [48]. In $p75^{NTR}$ knockout mice, apoptosis was reduced where the number of sympathetic neurons at two weeks of age was much higher than normal [49]. Genetic deletion of $p75^{NTR}$ also reduced apoptosis of spinal cord neurons at E11.5 and of retinal neurons at E15.5 [50]. Apoptosis and cell cycle arrest mediated by $p75^{NTR}$ in neuronal cells was reported in many other studies [51-53]. Given its well-documented role in neurodegeneration, $p75^{NTR}$ was studied in retinal neurodegenerative diseases. However, whether $p75^{NTR}$ can contribute to ischemic microvascular diseases remain poorly understood.

Role of $p75^{NTR}$ in ischemic diseases of the eye.

$P75^{NTR}$ receptor was genetically proven to be involved in cell death in the developing mouse retina [50] that was facilitated through NRAGE (neurotrophin receptor interacting MAGE

homolog) [54]. In the adult retina, localization of $p75^{NTR}$ was first shown to be in both RGCs [55] and Muller cells [56,57] at the level of light microscope. Later in 1998, in SD rat retinas, it was clear that $p75^{NTR}$ is localized in the processes of Muller cell that wrap around RGCs and not in RGCs themselves [58].

proNGF/$P75^{NTR}$ axis and diabetic retinopathy (DR).

One of the early and land mark studies that established the link between diabetes and $p75^{NTR}$ receptor, was reported by Hammes et al., in 1995. They investigated whether NGF plays a role in diabetes-induced degeneration of neurons and the development of occluded (acellular) retinal capillaries, a surrogate marker for ischemia. They reported that diabetes induced upregulation of $p75^{NTR}$ receptor mainly within Muller cells, that was associated with programmed cell death in RGCs and Muller cells. Diabetes-induced programmed cell death in RGCs and Muller cells could progress into pericyte loss and formation of acellular occluded capillaries [59]. These effects were ameliorated by exogenous NGF treatment for 3 months, suggesting that diabetes downregulates NGF levels or function in the retina [59].

Later, in 2008, our group has reported significant $p75^{NTR}$ expression in diabetic retinas of human and rat samples. Paradoxically, diabetes stimulated retinal NGF at the mRNA level and triggered significant neurodegeneration in diabetic rat retinas [60]. In the same study, we have seen that, diabetes-induced peroxynitrite formation impaired NGF survival signal by selective nitration and inhibition of TrkA receptor at tyrosine 490 [60]. In a subsequent study conducted in 2011, treatment of diabetic animals with epicatechin, a selective nitration inhibitor resulted in restoration of TrkA phosphorylation at Y490 and reduction of $p75^{NTR}$ expression [61]. Blocking nitration or silencing $p75^{NTR}$ resulted in protecting retinal neurons from cell death in vitro [61].

These studies established a possible link and cross talk between the activation of the survival receptor; TrkA and the expression of $p75^{NTR}$ in diabetic retina.

In 2011, we made the discovery that diabetes impairs the processing of the NGF precursor (proNGF) resulting in accumulation of proNGF at the expense of NGF [62]. While NGF binds the survival receptor TrkA, proNGF binds with high affinity to $p75^{NTR}$ to mediate cell death. Imbalance of proNGF to NGF was associated with retinal neurodegeneration and breakdown of the blood retina barrier [62]. The contribution of $p75^{NTR}$ to retinal inflammation and vascular dysfunction was demonstrated under diabetic condition. The genetic deletion of $p75^{NTR}$ blunted diabetes-induced proNGF/NGF imbalance, increase in NF-kB and TNF-α, ganglion cell loss and vascular permeability in vivo and in vitro [15]. The apoptotic effect of $p75^{NTR}$ was not limited only to neurodegeneration observed in early diabetes. Apoptotic effect of proNGF/$p75^{NTR}$ signaling pathway was also demonstrated in brain endothelial cells [63] and endothelial lineage cells [64]. Our recent work showed that stable overexpression of the cleavage-resistant form of proNGF in the rodent retina triggered $p75^{NTR}$ expression [65,66] that was associated with development of occluded (acellular) capillaries. Overexpression of the cleavage-resistant proNGF induced endothelial cell apoptosis that was inhibited by silencing $p75^{NTR}$ receptor in retinal endothelial cells in vivo and in vitro [66]. All of these findings underscore the importance of $p75^{NTR}$ receptor as a potential therapeutic target in diabetic retinopathy.

Clinically, Humpert et al. studied the alteration of the three distinct isoforms of $p75^{NTR}$ receptor; full length, extracellular domain (ECD) and iNTRacellular domain (ICD) in the serum of type 2 diabetic patients. Levels of the ECD of $p75^{NTR}$ (~24 KD) were reduced and levels of the ICD (~51 KD) were increased, whereas the immune-reactivity of full length $p75^{NTR}$ was not

altered. Nevertheless, none of the three forms seemed to be a marker of peripheral or autonomic neuronal function in patients with type 2 diabetes [67]. Our group has reported that, the alteration of proNGF and NGF levels observed in retina and vitreous is mirrored in serum of diabetic patients. In addition, C-terminal fragment (CTF, 27 KD) and ICD (22 KD) fragments of $p75^{NTR}$ receptor were significantly increased in vitreous and serum samples of diabetic patients and thus, both imbalance in the proNGF to NGF ratio and $p75^{NTR}$ shedding pattern could serve as biomarkers for diabetic retinopathy [68].

So far, the contribution of $p75^{NTR}$ is well-characterized in neuro- and microvascular degeneration in DR, however its role in the angiogenic phase (PDR) remain unclear. Our group reported positive correlation between proNGF and progression of DR, with proNGF level getting higher in the proliferative stage of the disease [62]. Diabetes triggers early $p75^{NTR}$ expression [60] and the level remain steady in PDR. Previous literature identified clear angiogenic effects of NGF via activation of TrkA in models of angiogenesis [18]. Our group was the first to show that proNGF can be a potential player in angiogenic behavior in PDR [69]. Interestingly, proNGF was shown to mediate angiogenic signal at least in part through activation of TrkA receptor. Furthermore, inhibition of $p75^{NTR}$ did not significantly alter angiogenic response of retinal endothelial cells to exogenous proNGF. Yet, its inhibition was associated with enhanced TrkA activation [69]. These findings highlight the cross talk between the two main receptors for neurotrophins; Trk and $p75^{NTR}$.

$P75^{NTR}$ and Retinopathy of prematurity (ROP).

ROP, an ischemic ocular disorder is the dominant cause of severe visual impairment in childhood in North America and Europe [70]. ROP proceeds following an initial phase of degeneration of the retinal microvasculature (vaso-obliteration) that is associated with cessation

of progression of vascular growth toward the retinal periphery resulting in ischemic retina [71]. In the subsequent phase of the disease, the ensuing retinal ischemia predisposes to abnormal compensatory neovascularization [72]. Various risk factors that have been linked to the development of ROP include low birth weight, low gestational age, supplemental and oxygen therapy [73]. ROP is associated with significant sequelae, the most serious being retinal detachment, which results in blindness. However, even milder forms of ROP increase the incidence of pathologies that negatively impact visual acuity [74].

Although neurotrophins are heavily involved in the growth, survival, proliferation and migration of neurons in the developing brain and retina, there are limited data whether premature birth and development of ROP is associated with alteration in neurotrophins. Clinically, serum levels of BDNF were found to be lower in preterm infants compared to full-term [75], [76]. In humans, levels of NGF, BDNF and NT-3 were significantly decreased in amniotic fluids in mothers that went through sever infections and later were associated with cerebral abnormalities [77]. The role of BDNF in ROP was further supported by a recent study that examined single nucleotide polymorphism (SNP) in a large cohort of premature infants with levels of ROP severity. The results showed that two intronic SNPs in the gene BDNF (rs7934165 and rs2049046) were associated with severe ROP in a large candidate gene study of infants with threshold ROP [78]. Nevertheless, no data exist on the levels or role of $p75^{NTR}$ in ROP from clinical studies.

Experimentally, the role of neurotrophins and $p75^{NTR}$ is beginning to unfold. The study by Liu X et al., 2010 examined the contribution of NGF and its receptor to retinal pathological neovascularization using oxygen-induced retinopathy (OIR) mouse model. The results showed increased expression of NGF mRNA that was associated with pathological retinal

neovascularization by postnatal day (p17). Inhibiting TrkA receptor using compound K252a prevented retinal neovascularization [79]. Yet, the role of $p75^{NTR}$ was not examined in this study. More recent work identified a critical role of $p75^{NTR}$ in retinal angiogenesis. Genetic deletion of $p75^{NTR}$ receptor protected against vaso-obliteration and retinal neovascularization in OIR model [80]. Deletion of $p75^{NTR}$ was linked to decreased stabilization of HIF-1α and subsequent decreasing induction of HIF-1α target genes including VEGF in hypoxia [80]. These results lend further support to ongoing studies showing that pharmacological inhibition of $p75^{NTR}$ receptor prevented glial activation, inflammation and protected against retinal neovascularization [81]. Moreover, we have observed that genetic deletion of $p75^{NTR}$ prevented vascular cell death, enhanced reparative central capillary growth and prevented pathological retinal neovascularization. The mechanisms involve restoring levels of NGF and BDNF and improving TrkA-mediated survival and angiogenic signal [82,83]. Thus, inhibition of $p75^{NTR}$ can provide potential therapeutic target for ischemic retinopathy.

$p75^{NTR}$ receptor and mesenchymal stem cells (MSCs)

Mesenchymal stem cells (MSCs) are multipotent non-hematopoetic, fibroblast-like cells that can be isolated from various tissues, including bone marrow (BM), adipose tissue, placenta, and umbilical cord blood [84]. MSCs have the potential to differentiate into lineages of mesenchymal tissues, including bone, cartilage, fat, tendon, muscle and marrow stroma [85]. In addition to their differentiation capabilities, MSCs exert vascular therapeutic effects via secretion of paracrine factors that may have anti-inflammatory and immunomodulatory effects [86,87]. Of note, immune-modulation is a unique feature for MSCs, making their combination with other stem cells subtypes very appealing to enhance allogeneic injection for inducing repair [88-90].

Among increasing prospective stem cells marker, $p75^{NTR}$ receptor, also known as CD271 enriches several progenitor/stem cells subtypes [91]. $p75^{NTR}$ was first identified as a genuine neural crest stem cell marker [92]. Since then, it has been widely used to isolate putative stem cells from neural crest derived tissues. Its involvement in MSCs differentiation has been also exploited. Nonetheless, $p75^{NTR}$ expression and function in vivo as well as its underlying mechanisms in MSCs biology, have not been sufficiently addressed. MSCs were stained positive for $p75^{NTR}$ as early as 1988 in a study done by Thomson et al [93]. $p75^{NTR}$ was shown to directly inhibit differentiation of MSCS into multiple cell types through inhibition of transcription factors, including Runx2 and OSX, which are essential for osteoblast differentiation and for expression of chondrogenesis marker Sox9 and the myogenic marker Myf5 as reported by Mikami et al [94].

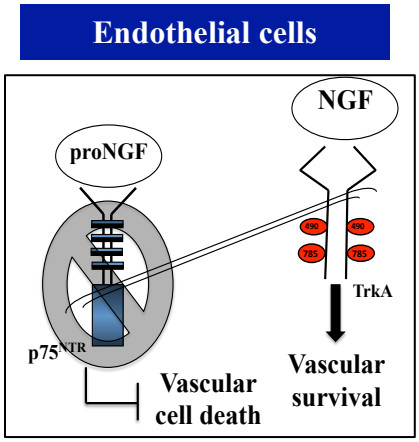
Gap in knowledge

Our group was the first to show that diabetes-induced oxidative milieu impairs maturation of proNGF, increasing its expression and decreasing NGF [62]. This imbalance was associated with upregulation of $p75^{NTR}$ and reduction in TrkA-Y490 activation [15,60,61]. Recently, we have reported the neuro- and vascular protective effects of inhibiting $p75^{NTR}$ in diabetic retina [15,69]. While prior work demonstrated that NGF stimulates angiogenesis via TrkA activation [38], there is a gap in knowledge about the extent and how proNGF/$p75^{NTR}$ axis modulates retinal ischemia and angiogenic response in models of ischemic retinopathy.

The **central hypothesis** is that modulating proNGF/$p75^{NTR}$ axis prevents retinal ischemia and enhances vascular repair by restoring endogenous NGF/TrkA angiogenic signal in endothelial cells and improving recruitment of mesenchymal stem cells (MSCs) to perivascular tissues. The central hypothesis will be examined by three specific aims:

<u>Specific Aim 1.</u> To elucidate role of proNGF in ischemic retinopathy.

Rationale: Our group was the first to demonstrate increase in proNGF expression and decrease in NGF level in clinical and experimental diabetes [62], which coincided with up-regulation of death receptor; $p75^{NTR}$ and decreased activation of survival receptor; TrkA [60]. proNGF was shown to be associated with microvascular damage of myocardium [95] as well as neuronal damage in alzheimer disease [96].



Working hypothesis for specific aims 1&2

Yet, its role in ischemic retinopathy diseases remains to be elucidated. In this aim, we will evaluate the specific contribution of proNGF to ischemic retinopathy diseases.

Working hypothesis: we hypothesize that proNGF contributes to pathological angiogenesis seen in PDR patients. We are going to test role of proNGF in aqueous humor samples collected from PDR patients using invitro angiogenic assays in human retinal endothelial cells (HREs). HREs will be treated with aqueous humor samples (10 µL/mL) from PDR patients in presence or absence of anti proNGF (1 µg/mL). Cells will be subjected to cell migration, proliferation and tube formation assays as previously described in our lab [97]. Aqueous humor samples from PDR patients is expected to enhance angiogenic response in HREs, an effect which would be blunted by anti-proNGF. To further elucidate underlying mechanism of proNGF-mediated angiogenic response, HREs cultures will be treated with proNGF (50 ng/mL) in presence of TrkA inhibitor; K252 (0.1 µM, Calbiochem) or $p75^{NTR}$ inhibitor (20 µM, McGill University). HREs cultures will be subjected to in-vitro angigenic

assays as previously described in our lab [97]. proNGF is expected to enhance angiogenic response in HREs in TrkA-dependent but not $p75^{NTR}$-dependent manner. This aim is completed and manuscript is published in Journal of Diabetes Research (chapter 2).

Aim 2. To elucidate role of $p75^{NTR}$ receptor in ischemic retinopathy.

Rationale: We have seen increased $p75^{NTR}$ expression in human diabetic retinas [60] as well as OIR retinal lysates by p12. Enhanced $p75^{NTR}$ expression can induce neuronal and endothelial cell apoptosis [60,64] that involve activation of pro-apoptotic JNK pathway [98,99]. Its deletion was associated with restored balance between NGF and its pro-form in diabetic mice [15]. NGF is well known to play an important role in reparative angiogenesis [16-18] through activation of TrkA [79]. TrkA activation can enhance Akt and MAPK ERK 1,2 angiogenic signal [100,101]. In this aim, we will evaluate the vaso-protective effect of deleting $p75^{NTR}$ receptor in ischemic retinopathy and the specific contribution of NGF-TrkA signaling in it.

Sub aim 2.1. Deletion of $p75^{NTR}$ is vaso-protective in ischemic retinopathy.

Working hypothesis: We hypothesize that deletion of $p75^{NTR}$ protects against retinal ischemia. To test our hypothesis, WT and $p75^{NTR-/-}$ mice pups will be exposed to oxygen-induced retinopathy (OIR) mouse model which is known to be one of the most frequently used models for ischemic retinopathy [102]. Briefly, on postnatal day 7 (p7), pups along with their dams will be subjected to 70% oxygen for 5 days. Pups will then be returned to room air (21% oxygen, relative hypoxia) for 5 days (p12-p17). In OIR, exposing the developing neonatal mouse retina to high oxygen level (70% O_2, p7-p12) induces significant vascular cell death and capillary dropout (CDO) at the center of the retina by p12. Upon returning to room air (21% O_2, p12-p17), the retina will experience relative hypoxia and release of angiogenic factors that drives both reparative angiogenesis in the central retina and pathological at mid-periphery by p17 [103].

Retinas will be flat-mounted and stained with Isolectin to localize vasculature as previously described by our lab [97].

Hyperoxia (p12 time point) will induce significant central vascular cell death in WT mice that is expected to reduce in $p75^{NTR-/-}$ pups. Moreover, deletion of $p75^{NTR}$ is expected to enhance central vascular repair and ameliorate pathological angiogenesis during hypoxia (p17 time point). The results are complied and presented in chapter (3). The manuscript is submitted to Journal of Angiogenesis.

Sub aim 2.2. To investigate underlying vaso-protective mechanism of deleting $p75^{NTR}$ in ischemic retinopathy

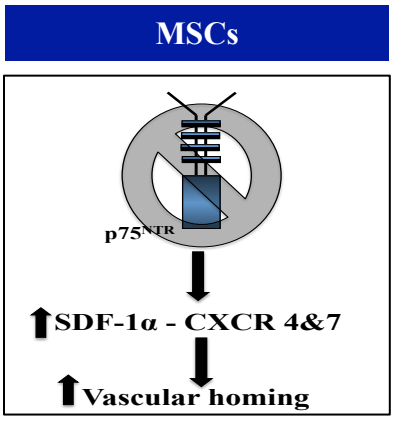
Working hypothesis: vascular protection observed in $p75^{NTR-/-}$ pups is assumed to occur through enhanced mature neurotrophin/Trk(s) survival and angiogenic signal. To test our hypothesis, retinal lysates of WT and $p75^{NTR-/-}$ pups subjected to OIR will be tested for expression of NGF, proNGF, BDNF and proBDNF as well as activation of TrkA and TrkB. Survival markers; Akt and VEGF as well as apoptotic markers; JNK, c-caspase and c-PARP will be assessed as well, as previously described in our lab [60,62].

During hyperoxia (p12 time point), WT pups will experience increased proNGF and proBDNF expression on the expense of NGF and BDNF, an imbalance that is expected to reduce in $p75^{NTR-/-}$ pups. Enhanced expression of apoptotic markers; cleaved caspase-3 and cleaved PARP is expected in WT mice pups by p12 but not $p75^{NTR-/-}$. Deletion of $p75^{NTR}$ is expected to enhance angiogenic signal manifested by, increased TrkA and TrkB activation and maintained Akt and VEGF signaling. The results are complied and presented in chapter (3). The manuscript is submitted to Journal of Angiogenesis.

Sub aim 2.3. Deletion of $p75^{NTR}$-induced vascular protection is regulated through Trk(s) receptors.

Working hypothesis: We hypothesize that vascular protection observed in $p75^{NTR-/-}$ pups is regulated through enhanced survival/angiogenic signal of Trk(s) receptors, activated by mature neurotrophins; NGF and BDNF. To test our hypothesis, WT and $p75^{NTR-/-}$ mice pups will be subjected to OIR (70%O2, p7-p12) then returned to room air till p17. Mice will be treated with vehicle (DMSO) or general Trk(s) inhibitor (K252a, 0.5 µg/eye) intravitreally at p12. Changes in vasculature will be assessed by p17 in isolectin-stained and FITC-perfused retinal flat mounts. K252a compound is expected to abolish vascular protection seen in $p75^{NTR-/-}$ mice. The results are complied and presented in chapter (3). The manuscript is submitted to Journal of Angiogenesis.

Specific Aim 3. Modulation of $p75^{NTR}$ enhances vascular repair via interplay with MSCs.



Working hypothesis for specific aim 3

Rationale: We have reported that deletion of $p75^{NTR}$ is associated with restored NGF expression in diabetic mice [15]. Prior evidence showed that NGF promotes angiogenesis through activation of endothelial progenitor cells (20), yet NGF effect on mesenchymal stem cells (MSCs) remains unknown. MSCs are fibroblast-like multipotent stem cells, isolated from various tissues, with the most intensely studied, those derived from bone marrow (BM). BM-MSCs could exert neuro-protective effects in ischemia-reperfusion (28) and neuro-degeneration Royal College Surgeons rat models (29). NGF was shown to stabilize HIF-1α which induces transcription of the angiogenic cytokine, SDF-1 and its receptor; CXCR-4

(30). SDF-1 can increase MSCs migration and homing in-vivo to vasculature (31) where they can exert supportive effect, likely through differentiation into pericytes (32). Our preliminary data using unilateral hind limb ischemia model showed that deletion of $p75^{NTR}$ preserved expression of SDF-1β and its receptors CXCR-4&7 under diabetic conditions. In this aim, we will further investigate how modulation of $p75^{NTR}$ exerts vascular protection through interplay with MSCs.

Sub aim 3.1. Deletion of $p75^{NTR}$ enhances MSCs recruitment to vasculature.

We will examine vascular protection exerted by deletion of $p75^{NTR}$ interplaying with MSCs using ischemia-reperfusion (IR) model in the eye, previously described by Zheng et al., 2007 [104]. Briefly, IOP will be elevated to 120 mmHg for 45-60 minutes by perfusing saline into anterior chamber of the eye, resulting in retinal ischemia. I/R injury and choroidal non-perfusion will be evident by blanching of the posterior segment via fundus examination. After ischemia, rapid reperfusion of retinal vasculature will be allowed. GFP-labeled MSCs will be injected intravitreally 48 hours post I/R to evaluate their reparative effect on ischemic vasculature interplaying with $p75^{NTR}$ receptor. I/R insult results in degenerated capillaries, which seem comparable to acellular capillaries found in DR patients. Degenerated, acellular capillaries are not perfused, and are believed to represent a discrete event that progressively contributes to the development of retinal ischemia, and ultimately, to neovascularization [105], [106].

Working hypothesis: We hypothesize that, genetic deletion of $p75^{NTR}$ receptor will protect against ischemic insult after IR, manifested by decreased number of acellular capillaries. Genetic deletion of $p75^{NTR}$ is also hypothesized to increase vascular homing of MSCs. MSCs are expected to exert vascular protection complementary to genetic deletion of $p75^{NTR}$. Acellular capillaries will be counted on trypsin-digested retina and retinal lysates will be assessed for

hypothesized angiogenic signaling pathway (SDF-1/CXCR-4&7). The results are complied and presented in chapter (4). Manuscript will be submitted to jornal of Stem Cells.

Sub aim 3.2. Inhibition of MSCs-p75^{NTR} improves reparative function of vasculature.

Working hypothesis: Among increasing prospective stem cells marker, p75^{NTR} receptor, also known as CD271 enriches several progenitor/stem cells subtypes including MSCs [91]. We hypothesize that inhibition of p75^{NTR} on the surface of MSCs will potentiate their paracrine effect on endothelial cells enhancing their angiogenic capabilities. MSCs will be treated with pharmacological inhibitor against p75^{NTR} receptor (LM11A-31, 200 nM, kind gift from Dr. Frank Longo, Stanford University) and conditional media will be used as treatment for HREs cultures, which will be subjected to in vitro angiogenic assays. Inhibition of p75^{NTR} on MSCs surface is expected to increase SDF-1/CXCR-4&7 signaling axis and enhance angiogenic behavior in HREs. In vivo approach will be carried out in parallel to in vitro studies. GFP-labeled MSCs will be transfected with Si-RNA against p75^{NTR} receptor. Transfected cells (100,000 cells/ 2 μL) will be injected in WT mice 48 hours after IR. Deleting p75^{NTR} from MSCs is expected to potentiate their vascular protective effect after I/R by increasing their incorporation into vasculature, decreasing number of acellular capillaries and increasing expression of SDF-1, CXCR-4&7. The results are complied and presented in chapter (4). Manuscript will be submitted to Journal of Stem Cells.

In summary, our studies demonstrate the potential role of modulating proNGF/p75^{NTR} axis as a therapeutic alternative for ischemic ocular diseases. Currently available treatments are anti-VEGF therapy and laser photocoagulation, which are known for their devastating side effects as well as late intervention during mal-adaptive neovascularization phase. Our results

support the notion that, modulating proNGF/ $p75^{NTR}$ axis can enhance mature neurotrophin/Trk(s) signaling and increase vascular homing of MSCs, thus, combating ischemic ocular diseases in the early ischemic stage.

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CHAPTER 2

PRO-NERVE GROWTH FACTOR INDUCES ANGIOGENESIS VIA ACTIVATION OF TRKA: POSSIBLE ROLE IN PROLIFERATIVE DIABETIC RETINOPATHY [1]

[1] Sally L. Elshaer*, Mohammed A. Abdelsaid*, Ahmad Al-Azayzih*, Parag Kumar, Suraporn Matragoon, Julian J Nussbaum and Azza B. El-Remessy (*, equal contribution)

Abstract

Proliferative diabetic retinopathy (PDR) is the leading cause of blindness in working-age Americans. Our previous analyses showed that diabetes disturbs the homeostasis of nerve growth factor (NGF) resulting in accumulation of its precursor proNGF. Increases in proNGF were positively correlated with progression of diabetic retinopathy, having the highest level in ocular fluids from PDR patients compared to non-diabetic patients. However whether proNGF can contribute to angiogenesis and PDR remain unknown. Here, we attempted to evaluate the contribution and the possible mechanism of proNGF to PDR. The angiogenic response of aqueous humor samples from PDR patients was examined in human retinal endothelial (HRE) cells in the presence or absence of anti-proNGF antibody. Additional cultures were treated with mutant-proNGF in the presence of specific pharmacological inhibitors of TrkA and $p75^{NTR}$ receptors. Results showed that PDR-aqueous humor samples exerted significant angiogenic response including cell proliferation, migration and alignment into tube-like structures in HRE cells. These effects were reduced by anti-proNGF antibody, but not with anti-IgG control antibody. Treatment of retinal endothelial cells with mutant-proNGF activated phosphorylation of TrkA and p38 MAPK, however did not alter $p75^{NTR}$ expression. Inhibition of TrkA but not $p75^{NTR}$ significantly reduced mutant-proNGF-induced cell proliferation, cell migration and tube formation. Together, these results provide preliminary evidence that proNGF can contribute to PDR at least in part via activation of TrkA.

Introduction

Diabetic retinopathy (DR) is the leading cause of blindness among working aged adults in the US. It affects 80% of individuals with a 10 years history of diabetes, adding 63,000 new cases of DR each year [1]. DR is characterized by neuro- and vascular degeneration that eventually lead to ischemia and subsequent release of angiogenic growth factors including vascular endothelial growth factor (VEGF) into the vitreous cavity resulting in retinal neovascularization and proliferative diabetic retinopathy (PDR) [2, 3]. PDR is characterized by vitreous hemorrhage, neovascular glaucoma, and tractional retinal detachment, which can result in visual loss [4]. Current treatment options for PDR include laser photocoagulation and anti-VEGF ocular injection, which are invasive and limited by side effects. Repeated injections of anti-VEGF can deprive the retina from the survival actions of VEGF on neurons and vasculature (reviewed in [2, 5]). Therefore, there is a great need to identify contributing factors in PDR other than VEGF; in the hope of devising treatments that will preserve both retina vasculature and neuronal function.

Diabetes-induced oxidative stress disturbs retinal homeostasis by activating glial cells, reducing neurotrophic support and increasing proinflammatory cytokines including VEGF, IL-1β and TNF-α [6, 7]. In addition to these known growth factors, recent findings using ocular fluids from diabetic patients and experimental models of diabetes suggest that neurotrophins including nerve growth factor (NGF) are emerging as critical mediators of DR [5, 8-11]. NGF is produced by neurons and many non-neuronal cell types such as immune cells, inflammatory cells and smooth muscle cells [12]. It was originally characterized by its ability to stimulate growth, differentiation and survival of neurons; however NGF appears as a pleiotropic modulator of wound-healing and reparative angiogenesis [13-15]. NGF activates two different receptors including the high affinity tropomyosin-related receptor A (TrkA), which is a tyrosine kinase and

the low affinity $p75^{NTR}$ neurotrophin receptors ($p75^{NTR}$) [16]. Previous studies demonstrated that the angiogenic response of NGF was mediated via activation of TrkA [15, 17, 18].

NGF is synthesized and secreted by glial cells as the precursor proNGF which is cleaved, by furin intracellularly and by the matrix metalloproteinase-7 (MMP-7) extracellulary, to generate mature NGF [19]. Our studies showed that diabetes-induced peroxynitrite formation impairs maturation of NGF, leading to accumulation of its precursor proNGF both in experimental models and in clinical diabetes [10, 11]. In these studies we used specific antibodies to detect NGF (13 kDa) and proNGF (32 kDa) rather than ELISA assays that detect both NGF and proNGF. Our results showed that increases in proNGF positively correlated with progression of the disease where ocular fluids from PDR patients showed the higher level of proNGF (5-fold) and lower level of NGF (65% less) compared to non-diabetic samples [10]. Interestingly, earlier studies utilizing ELISA showed higher NGF levels in PDR patients than in controls and non-proliferative diabetic retinopathy (NPDR) patients [9]. Because many NGF antibodies can detect both NGF and proNGF, these increases may reflect the combined presence of both NGF and proNGF. Based on these observations, it appears that proNGF may contribute to development and progression of proliferative diabetic retinopathy clinically. Here, we attempted to evaluate the specific contribution of proNGF to angiogenic response of ocular fluids from PDR patients within retinal endothelial cells and to elucidate the possible role of TrkA and $p75^{NTR}$ in mediating the angiogenic signal.

Materials and Methods

2.1. Human aqueous humor samples.

Human specimens were obtained with Institutional Review Board approval from the human assurance committee at Georgia Regents University. Aqueous humor samples were

collected from Eye Clinic at Georgia Regents University from patients undergoing intravitreal injections and were identified as being from patients with PDR. Table (1) shows the clinical characteristics of participants providing aqueous humor samples.

2.2 Cell culture

Human retinal endothelial (HRE) cells and cell culture medium were purchased from Cell Systems Corporations (Kirkland, WA) and VEC Technologies (Rensselaer, NY), respectively. Experiments were performed using cells between passages (4-6) at 37 °C in a humidified atmosphere of 5% CO2. Cells were switched to serum free medium overnight prior to stimulation with aqueous humor samples (10µl/ml) from various patients in the presence or absence of either anti-proNGF anti-body or isotope control rabbit IgG (1µg/ml). Mutant (cleavage-resistant) proNGF protein and anti-proNGF antibody were purchased from Alomone Labs (Jerusalem, Israel) and IgG was purchased from Cell Signaling Technology (Danvers, MA). For proNGF studies, bovine retinal endothelial (BRE) cells were cultured as described previously [20]. Cells from passages 4 to 8 were used in all experiments. Cells were maintained in M199 supplemented with 10% fetal bovine serum, 10% CS-C complete medium, 2 mM glutamine, 100U/ml penicillin, and 100µg/ml streptomycin at 37°C in a humidified CO_2 incubator. Cells were stimulated with proNGF (50ng/ml) in the presence or absence of either TrkA antagonist, K252a (0.1µM) from Calbiochem/Millipore (Temecula, CA) or $p75^{NTR}$ selective p75 antagonist A (C30-35, 20µM), a kind gift from Uri Saragovi, McGill University, Canada [21].

2.3 Endothelial cell migration

HRECs and BRECs were grown to confluence then were wounded with a single sterile cell scraper of constant diameter as described previously [22]. Images of wounded areas were

taken immediately after adding the treatment and after 18 h and % cell migration was calculated. Each condition was verified in triplicate and was repeated using independent cultures.

2.4. Tube formation

Tube formation assay was performed using growth factor-reduced Matrigel (BD Biosciences) as described previously [23, 24]. HRE cells and BRE cells were counted and plated at 2×10^4 cells/ml with Matrigel in a 96 well-plate. Eighteen hours later, images of the tube-like structures were captured and analyzed using Zeiss Axiovert microscope software. Each condition was verified in triplicate and was repeated using independent cultures.

2.5. Endothelial cell proliferation

Cells were seeded at a density of $0.5x10^5$/ml, switched to medium containing 0.5% FBS, and incubated overnight. Cells were incubated with and without various treatments in medium containing 0.2% FBS for 24 h. After trypsinization, the cell number was determined using a hemocytometer [23, 24]. Each condition was verified in triplicate and was repeated using independent cultures.

Western blot analysis

For analysis of protein, bovine retinal endothelial cells were homogenized in a modified RIPA buffer [20 mM Tris-HCl (pH 7.4), 2.5 mM ethylenediamine tetraacetic acid, 50 mM NaF, 10 mM $Na_4P_2O_7$, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 1% sodium deoxycholate, 1 mM phenylmethyl sulfonyl fluoride]. Total protein concentrations were measured using Bio-Rad protein assay. Protein samples (20μg) were separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, probed with the following antibodies: TrkA (Chemicon/Millipore, Temecula, CA), phospho-TrkA (Santa Cruz Biotechnology, Santa Cruz, CA), p38MAPK and phospho-p38 MAPK (Cell Signaling

Technology, Danvers, MA), rabbit anti-$p75^{NTR}$ provided by Dr. Bruce Carter, Vanderbilt University, School of Medicine, Nashville, TN) and tubulin (abcam, Cambridge, MA, USA) followed by secondary horseradish peroxidase-conjugated sheep anti-rabbit antibody and enhanced chemiluminescence (GE Health Care). The films were subsequently scanned and band intensity was quantified using densitometry software (fluorchem FC2) and expressed as relative optical density to controls.

2.7 Data analysis

The results are expressed as mean ± SEM. Differences between experimental groups were evaluated by ANOVA and the significance of differences between groups was assessed by the post-hoc test (Fisher's protected least significant difference) when indicated. Significance was defined as $p < 0.05$.

Results

3.1. PDR-aqueous humor stimulates cell migration in a proNGF-dependent manner.

Our previous studies have shown that diabetes-induced oxidative stress disturbs the homeostasis of nerve growth factor (NGF) resulting in accumulation of its precursor proNGF at the expense of the mature NGF in diabetic rat [11] and ocular fluids from diabetic patients [10]. Interestingly, the accumulation of proNGF was positively correlated with severity of diabetic retinopathy, where patients identified with proliferative diabetic retinopathy (PDR) showed higher levels (5-fold) of proNGF compared to non-diabetic samples [10]. Here, we examine the angiogenic response of aqueous humor samples from PDR patients using human retinal endothelial (HRE) cells in the presence or absence of anti-proNGF antibody (1μg/ml). Each aqueous humor sample (total of 100μl) was tested at least in duplicates on HRE cell culture (n=7). As shown in Fig.1, treatment of HRE cells with PDR-aqueous humor significantly

stimulated cell migration by 1.7-fold compared to the control group. Prior treatment of aqueous humor samples with anti-proNGF antibody significantly reduced the stimulatory effect of untreated-aqueous humor on cell migration to 1.2-fold of the control level. Whereas prior treatment with the isotope IgG maintained stimulatory effect (1.6-fold) of aqueous humor on cell migration. Treatment of control HRE cells with anti-proNGF antibody did not significantly impact cell migration compared to untreated control group.

3.2 PDR-aqueous humor stimulates tube-like structures in a proNGF-dependent manner.

We next examined the effects of PDR-aqueous humor on alignment of endothelial cells to tube-like structures. As shown in Fig.2, aqueous humor from PDR patients increased the relative mean tube length by 1.75-fold compared to the control group (n=7). Prior treatment of aqueous humor samples with anti-proNGF antibody (1μg/ml) blunted the stimulatory effect of aqueous humor on inducing tube formation whereas IgG did not significantly affect tube formation. Meanwhile, treatment of control cells with anti-proNGF antibody did not markedly reduce tube formation compared to untreated control group.

3.3. PDR-aqueous humor stimulates cell proliferation in a proNGF-dependent manner.

We next examined the effect of aqueous humor on HRE cell proliferation. As shown in Fig. 3, PDR-aqueous humor stimulated cell proliferation by 1.8 compared to the control group (n=7). Prior treatment of aqueous humor samples with anti-proNGF antibody (1μg/ml) blunted the stimulatory effect of aqueous humor on cell proliferation whereas; prior treatment with IgG did not markedly reduce cell proliferation. Treatment of control cells with anti-proNGF antibody did not affect relative number of proliferating cells compared to untreated control.

3.4. ProNGF activates TrkA/p38 MAPK in retinal endothelial cells

Previous studies showed that Trk receptors play a key role of mediating the mitogenic and angiogenic response of neurotrophins including NGF and BDNF [17, 25, 26]. Our previous work demonstrated significant upregulation of $p75^{NTR}$ receptor expression in clinical and experimental diabetes ([11, 27]). Therefore, we examined the impact of proNGF on activating TrkA and $p75^{NTR}$ receptors in endothelial cells. As shown in Fig. 4A, there was no significant difference in $p75^{NTR}$ expression among various groups. As shown in Fig.4B, treatment of BRE cells with proNGF (50ng/ml) stimulated phosphorylation of TrkA. Prior treatment of BRE cells with the TrkA antagonist K252a (0.1μM) blocked proNGF-mediated TrkA activation confirming the possibility that proNGF can activate TrkA. Interestingly, inhibition of $p75^{NTR}$ using a selective antagonist modestly increased TrkA activation in both control and proNGF-stimulated cells suggesting mutual regulation of the two receptors TrkA and $p75^{NTR}$. We next examined expression pattern of $p75^{NTR}$ in retinal endothelial cells. We next examined activation of p38 MAPK, and the results showed that proNGF activated p38 MAPK and this effect was abolished with TrkA antagonist (Fig. 4C). Prior treatment of BRE cells with $p75^{NTR}$ antagonist (20μM) significantly reduced proNGF-mediated activation of p38 MAPK (Fig. 4**C**). These results suggest that proNGF can activate the mitogenic p38 MAPK signal in retinal endothelial cells.

3.5. Inhibiting TrkA prevents proNGF-mediated retinal endothelial cell proliferation.

We next attempted to examine the effects of inhibiting TrkA on the mitogenic and angiogenic function of proNGF. As shown in Fig.5, treatment with the mutant proNGF (50ng/ml) induced cell proliferation (1.6-fold) compared to untreated control. This effect was blocked by the specific TrkA receptor antagonist K252a (0.1μM) meanwhile, it was not reduced

by $p75^{NTR}$ inhibitor (20μM). Inhibition of TrkA in control group did not significantly inhibit cell proliferation compared to untreated controls.

3.6. Inhibiting TrkA prevents proNGF-mediated retinal endothelial cell migration.

As shown in Fig.6, proNGF (50ng/ml) increased the relative percentage of BRE cell migration by 1.8-fold compared to the control group. These effects were blocked with the specific TrkA receptor antagonist K252a (0.1μM), but not with $p75^{NTR}$ inhibitor (20μM). Inhibition of TrkA in control group did not significantly inhibit cell migration compared to untreated controls.

3.7. Inhibiting TrkA prevents proNGF-mediated retinal endothelial tube formation.

As shown in Fig. 7, proNGF (50ng/ml) stimulated alignment of BRE cells into tube-like structures and tube length by 1.5-fold compared to the control group. This effect was blocked by the specific TrkA receptor antagonist K252a (0.1μM), but not with $p75^{NTR}$ inhibitor (20μM). Inhibition of TrkA in control group did not significantly inhibit tube formation compared to untreated controls.

Discussion

Although increases in cytokines and growth factors including VEGF, TNF-α, IL-1β, IL-6 have been well-documented in vitreous from diabetic patients [28-30], little is known about the role of proNGF in PDR. The current study was conducted to evaluate the contribution of proNGF to the angiogenic response and to identify the possible underlying mechanisms. The main findings of the current study are that aqueous humor samples from PDR patients stimulate the angiogenic response in HRE cells in a proNGF-dependent manner and that exogenous proNGF mediates pro-angiogenic action via activation of TrkA/p38 MAPK pathway in retinal endothelial cells. We believe that this study is the first one to demonstrate evidence that proNGF

can contribute to PDR and to provide insight on the possible mechanism. Future studies are warranted to further elucidate the complex role of proNGF in angiogenesis.

Angiogenesis/neovascularization can be detrimental in pathological diseases, including diabetic retinopathy, arthritis, and tumor growth as well as beneficial during wound healing and post-ischemic repair (reviewed in [31, 32]). Under diabetic conditions, pro-oxidative stress and pro-inflammatory milieu stimulate apoptosis of retinal vascular endothelial cells and capillary drop out leading to ischemia ([6]). Normally to counteract the ischemic condition and salvage injured ischemic tissue, growth of collateral arteries from preexisting arterioles (reparative angiogenesis) is initiated [33]. This reparative mechanism appears to be impaired in the diabetic retina; however in an effort to meet the metabolic demand of the retina, sprouting of capillaries and pathological neovascularization is triggered eventually leading to PDR. In response to ischemic stress, several growth factors including NGF are secreted to induce reparative angiogenesis via activation of TrkA receptor, promoting endothelial cell survival and angiogenesis [34]. Prior studies detected NGF at mRNA level or utilized ELISA assays, both of which cannot distinguish NGF from its precursor**,** showed a positive correlation of NGF with progression of PDR in human [9, 35] or experimental retinal neovascularization models [25]. NGF is secreted as precursor form (proNGF) that gets cleaved to the mature NGF. Our previous analyses showed that diabetes-induced oxidative stress disturbs the homeostasis of NGF by hampering the cleavage of proNGF resulting in accumulation of proNGF and reducing NGF levels in experimental [11] and ocular fluids from PDR patients [10]. Therefore it is conceivable that the previously reported increases in NGF are mixed proNGF/NGF rather than NGF alone. So far, researchers have focused on studying angiogenic response of NGF in retinal endothelial cells [18, 25, 36-38], however, until now no studies have evaluated the possible angiogenic

action of proNGF. Therefore, we tested the hypothesis that accumulated proNGF can contribute to angiogenic response elicited by ocular fluids from PDR patients. Treatment of HRE cells with aqueous humor samples from diabetic patients stimulated endothelial cell migration, cell proliferation and tube formation. All of which, were inhibited by prior treatment with anti-proNGF antibody but not with rabbit IgG, confirming that proNGF directly contributes to angiogenesis. These results are consistent with the concept that diabetes deprives the retina from the neurotrophic support of NGF and favors accumulation of pro-inflammatory proNGF that can contribute to pathological neovascularization and PDR.

Neurotrophins including NGF, BDNF and neurotrophin-3 (NT-3) have been extensively studied for their actions on the nervous system. However, recent studies demonstrated the effects of neurotrophins as pleiotropic modulators of wound-healing and angiogenesis [13-15, 39, 40]. The angiogenic response was either mediated through direct activation of the corresponding tropomyosin kinase receptor such as TrkA and TrkB in endothelial cells or indirectly via paracrine effects from the release of angiogenic factors from other cells [14, 34, 40]. Our results clearly show that proNGF can induce early activation (within 4 hours) of TrkA in retinal endothelial cells without significant effect on $p75^{NTR}$ expression (Fig.4). Our results also show that inhibiting TrkA activation blocked proNGF-induced angiogenic response in retinal endothelial cells (Fig. 5-7). Our results lend further support to a recent study showing that the angiogenic effect of proNGF in cancer cells is exerted mainly via TrkA rather than $p75^{NTR}$ receptor [41]. The inhibitory effect of K252a, staurosporine-related compound [42], on angiogenic response have been demonstrated in several studies [14, 15, 18, 25, 36-38], nevertheless we believe that our results are the first to demonstrate involvement of TrkA activation in response to proNGF in retinal endothelial cells.

Activation of TrkA leads to its phosphorylation at Tyr^{490}, which recruits the adaptor proteins GRB2-associated binding protein-1 and SH2-containing protein, activating MAPK/ERK kinase and promotes neurite and endothelial growth [43]. Our results showing that proNGF activates TrkA/p38 MAPK and that inhibition of TrkA significantly inhibited proNGF-mediated cell proliferation, migration and tube formation lend further support to other studies of the role of TrkA/p38 MAPK promoting cell growth, migration and invasion of cancer cells [44, 45]. A study in smooth muscle cells showed also that activation of p38 MAPK and ERK was necessary for TrkA-mediated cell proliferation [45].

The $p75^{NTR}$ receptor, a member of the tumor necrosis factor (TNF) receptor superfamily [46], has multiple and cell-specific functions dependent upon availability of ligands and co-receptors (reviewed in [47-49]). In the retina, $p75^{NTR}$ is expressed predominately by Müller cells, however stress can induce expression of $p75^{NTR}$ in other retina cells types including retinal ganglion cells [50] and endothelial cells [26, 51]. ProNGF has great affinity to bind and activate $p75^{NTR}$ along with the sortilin receptor to mediate cell death [52]. We and others have shown that upregulation of proNGF induces $p75^{NTR}$-mediated retinal neurodegeneration [10, 11, 53, 54]; inflammation [54, 55] as well as endothelial cell death [26, 51, 56]. Interestingly, in the present study, results showed that inhibition of $p75^{NTR}$ modestly activated TrkA (Fig.4A) and did not significantly alter proNGF-induced angiogenic response in retinal endothelial cells (Fig.5-7). These results lend further support to previous work demonstrating that inhibition of $p75^{NTR}$ contributes to endothelial cell survival and inhibition of apoptosis rather than angiogenic function [26, 56]. Recent studies showed that $p75^{NTR}$ played critical role in guiding migration of neuronal precursor cells and repair of vasculature in ischemic stroke model [57, 58]. Another study showed that $p75^{NTR}$ is required for nitric oxide production in pulmonary endothelial cells

[59]. As such, the proNGF/p75^{NTR} pathway is more likely involved in paracrine effects of other retina cell types in the diabetic retina rather than direct angiogenic process within endothelial cells. Further studies warrant characterization of the complex signaling pathway of proNGF/p75^{NTR} using in vivo models of retinal angiogenesis.

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Author Disclosure Statement

We do not have any commercial associations that might create a conflict of interest in connection with our manuscripts.

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Figure 1.1.

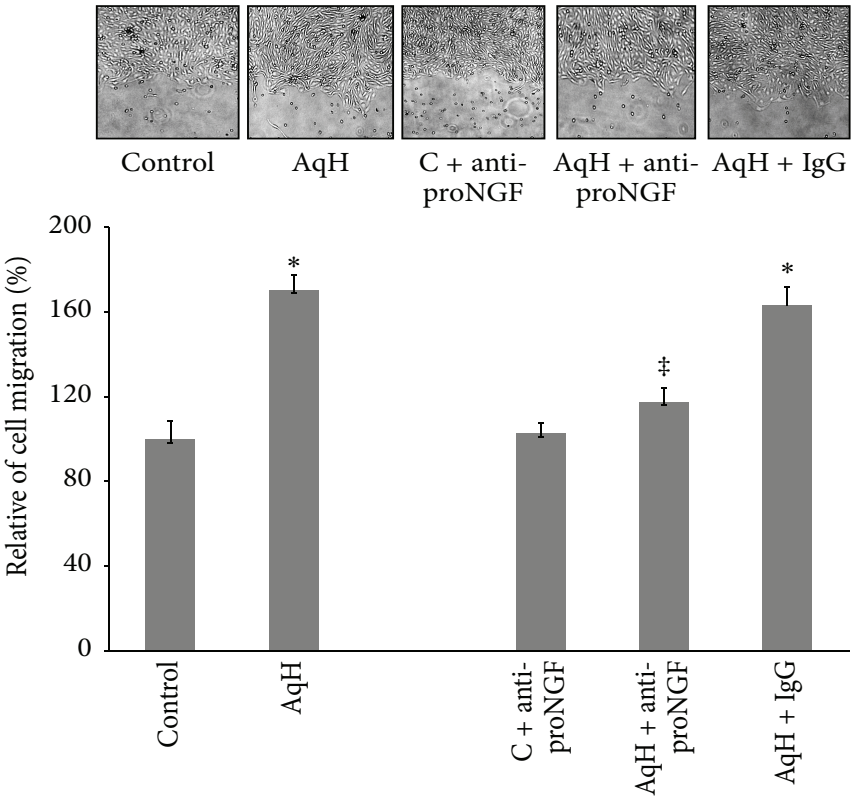

Control
AqH
C + anti-proNGF
AqH + anti-proNGF
AqH + IgG
Relative of cell migration (%)
200
160
120
80
40
0
*
*
‡
Control
AqH
C + anti-proNGF
AqH + anti-proNGF
AqH + IgG

Figure 1.1. PDR-aqueous humor stimulates cell migration in a proNGF-dependent manner.

HRE cells were grown to confluence then scratched using a standard cell scrapper. Cells were switched to serum free medium and treated with aqueous humor samples (10μl/ml) in the presence or absence of either anti-proNGF antibody or rabbit IgG (1μg/ml). Representative micrographs for wounded HRE cells are shown after 18 hours of various treatments. Statistical analysis showed that aqueous humor increased mean cell migration by 1.8-fold compared to the control group. Addition of Anti-proNGF antibody to aqueous humor samples significantly reduced the mean percent of cell migration to the level of the control group whereas IgG did not significantly impact stimulatory effect of aqueous humor samples. Addition of anti-ProNGF antibody to control HRE cells did not significantly affect percent cell migration compared to control group. (*, ‡ statistically significant compared to control and aqueous humor groups respectively ($P<0.05$), n of aqueous humor samples = 7, n of cell cultures =14-16).

Figure 1.2.

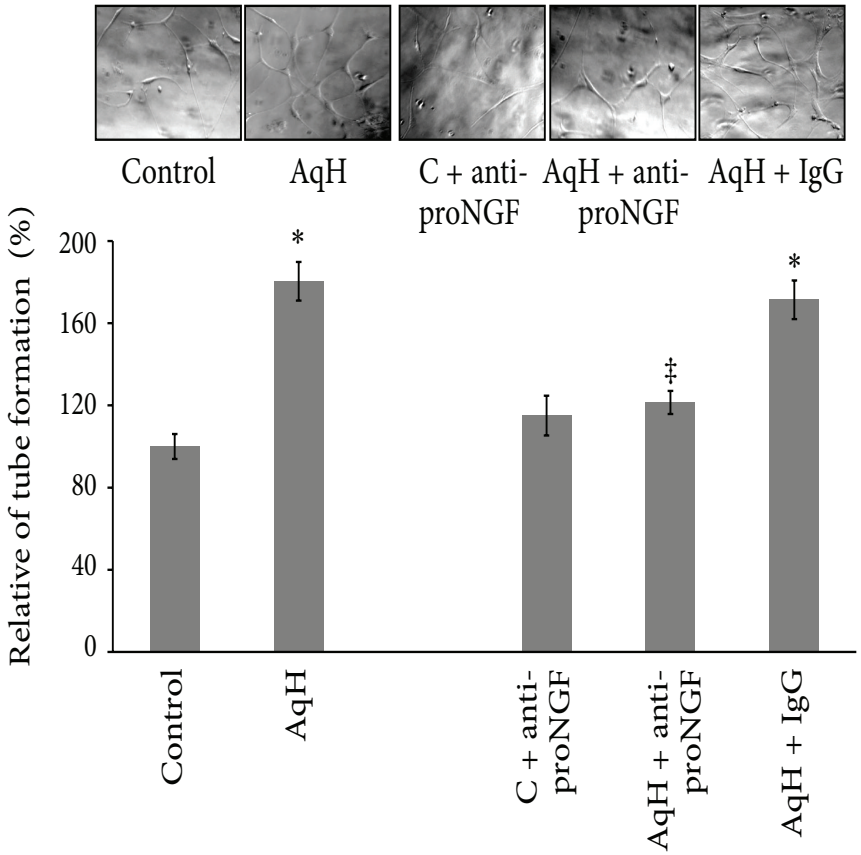

Control
AqH
C + anti-proNGF
AqH + anti-proNGF
AqH + IgG
Relative of tube formation (%)
200
160
120
80
40
0
*
*
‡‡
Control
AqH
C + anti-proNGF
AqH + anti-proNGF
AqH + IgG

Figure 1.2. PDR-aqueous humor stimulates tube-like structures in a proNGF-dependent manner.

HRE cells were grown into confluence then trypsinized and mixed with reduced-growth factor Matrigel and treated with aqueous humor samples (10μl/ml) in the presence or absence of either anti-proNGF antibody or rabbit IgG (1μg/ml). Representative micrographs for alignment of HRE into tube-like structures are shown after 18 hrs of incubation. Statistical analysis of tube length showed that aqueous humor increased mean tube formation 1.7-fold compared to the control group. Addition of anti-proNGF antibodies to aqueous humor samples significantly reduced the relative mean tube length but did not affect control group. Prior treatment of humor samples with rabbit IgG did not significantly reduce relative mean length when compared to the untreated aq. humor group. (*, ‡ statistically significant compared to control and aqueous humor groups respectively ($P<0.05$), n of aqueous humor samples =7, n of cell cultures =14-16).

Figure 1.3.

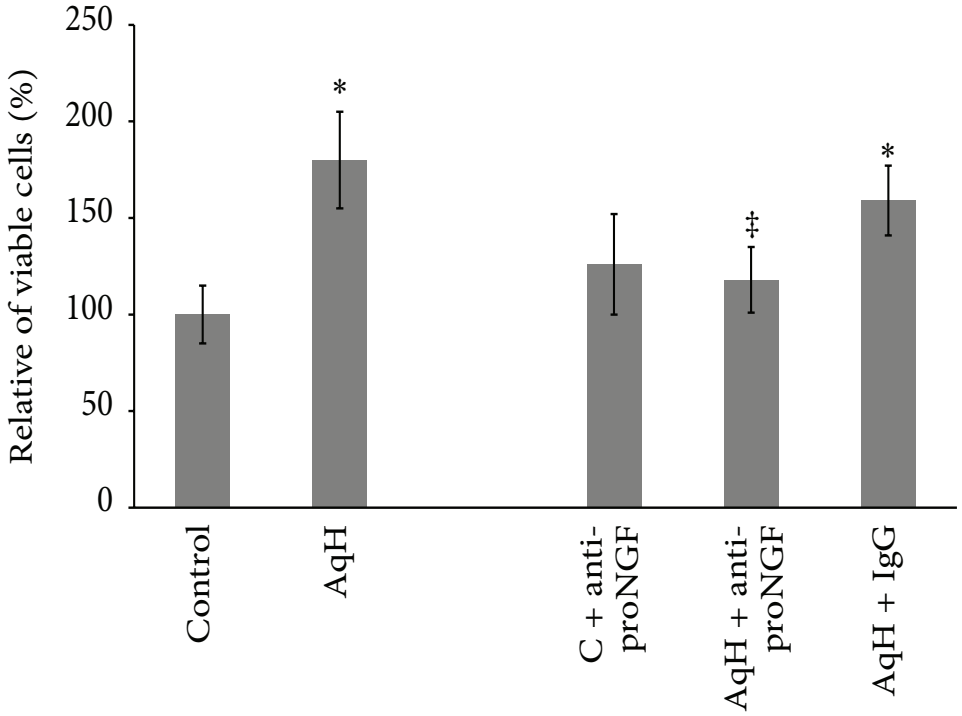
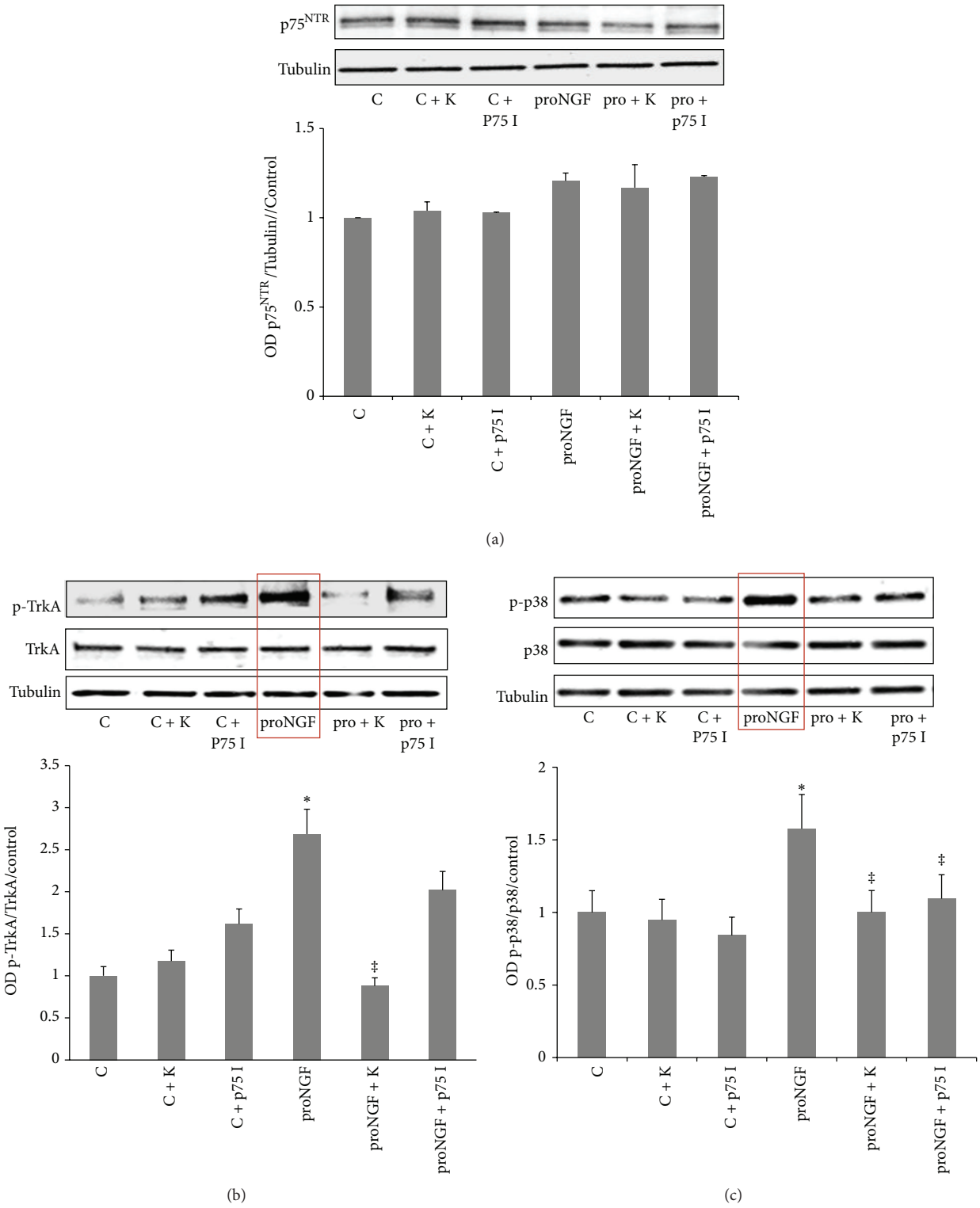

Relative of viable cells (%)
250
200
150
100
50
0
*
*
‡‡
Control
AqH
C + anti-proNGF
AqH + anti-proNGF
AqH + IgG

Figure 1.3. ProNGF and aqueous humor from PDR patients stimulated cell proliferation.

HRE cells were grown into confluence then trypsinized and plated as described in methods section. Cells were switched to serum free medium and treated with aqueous humor samples (10 μl/ml) in the presence or absence of either anti-proNGF antibody or rabbit IgG (1 μg/ml) for 24 hours then cells were trypsinized and counted. Statistical analysis showed that aqueous humor from PDR patients stimulated cell proliferation by 1.8-fold compared to the control group. Adding anti-proNGF antibody to aqueous humor samples significantly reduced the relative number of proliferating cells while IgG did not. Addition of anti-proNGF antibody to HRE cells did not affect number of proliferating cells. (*, ‡ statistically significant compared to control and aqueous humor groups respectively ($P<0.05$), n of aqueous humor samples = 6, n of cell cultures =12-14)

Figure 1.4.


p75NTR
Tubulin
C
C + K
C + P75 I
proNGF
pro + K
pro + p75 I
OD p75NTR /Tubulin//Control
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1
0.5
0
C
C + K
C + p75 I
proNGF
proNGF + K
proNGF + p75 I
(a)
p-TrkA
TrkA
Tubulin
C
C + K
C + P75 I
proNGF
pro + K
pro + p75 I
OD p-TrkA/TrkA/control
3.5
3
2.5
2
1.5
1
0.5
0
*
‡
C
C + K
C + p75 I
proNGF
proNGF + K
proNGF + p75 I
(b)
p-p38
p38
Tubulin
C
C + K
C + P75 I
proNGF
pro + K
pro + p75 I
OD p-p38/p38/control
2
1.5
1
0.5
0
*
‡
‡
C
C + K
C + p75 I
proNGF
proNGF + K
proNGF + p75 I
(c)

Figure 1.4. ProNGF activates TrkA/p38 MAPK in retinal endothelial cells.

BRE cells were grown to sub-confluence then switched to serum free medium and treated with mutant proNGF (50ng/ml). Cells were harvested after 4 hours and subjected to Western Blot. **A.** Representative image of $p75^{NTR}$ and tubulin showing no significant change in $p75^{NTR}$ expression among various groups. **B.** Representative image and statistical analysis showed that proNGF is capable of activating TrkA in BRE cells compared to control cells. Treatment of BRE cells with the TrkA antagonist K252a abolished the ability of proNGF to activate TrkA while pharmacological inhibition of $p75^{NTR}$ modestly increased TrkA activation. **C.** Representative image and statistical analysis showed that proNGF activates p38 MAPK compared to controls. Inhibiting TrkA or $p75^{NTR}$ abolished the ability of proNGF to activate p38 MAPK. (*, ‡ statistically significant compared to control and proNGF groups respectively ($P<0.05$), n=3-5)

Figure 1.5.

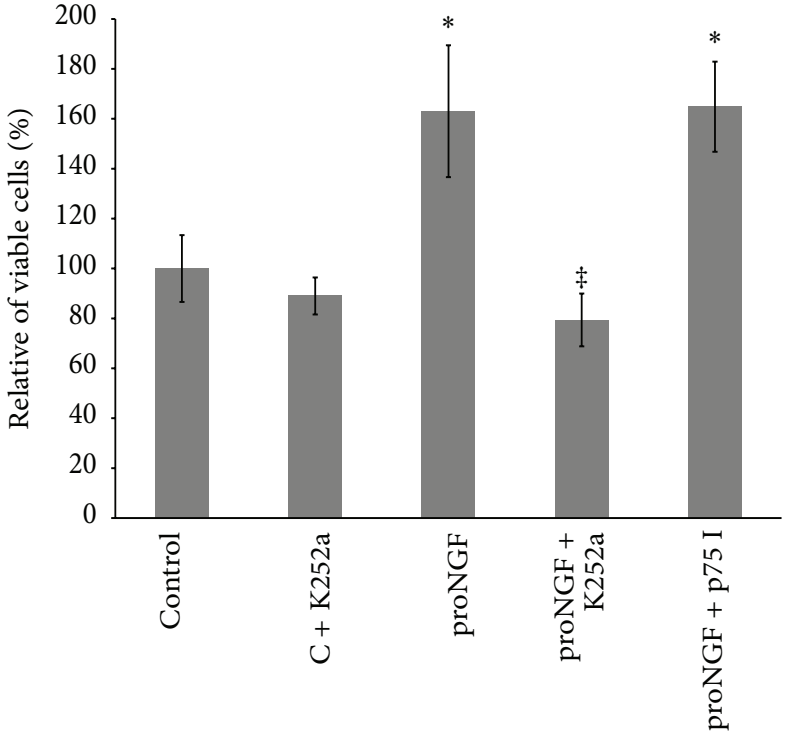
Relative of viable cells (%)
200
180
160
140
120
100
80
60
40
20
0
*
*
‡‡
Control
C + K252a
proNGF
proNGF + K252a
proNGF + p75 I

Figure 1.5. Inhibiting TrkA prevents proNGF-mediated retinal endothelial cell proliferation.

BRE cells were grown into confluence then trypsinized and plated as described in method section. Cells were switched to serum free medium and treated with mutant proNGF (50ng/ml) in the presence or absence of K252a, TrkA inhibitor (0.1μM) or $p75^{NTR}$ inhibitor (20μM) for 24 hours then cells were trypsinized and counted. Statistical analysis showed that proNGF increased the percentage of proliferated cells by 1.6-fold compared to the control group. This effect was blocked by the specific TrkA receptor antagonist (K252a) but not with p75 inhibitor. (*, ‡ statistically significant compared to control and proNGF groups respectively ($P<0.05$), n=5-7)

Figure 1.6.

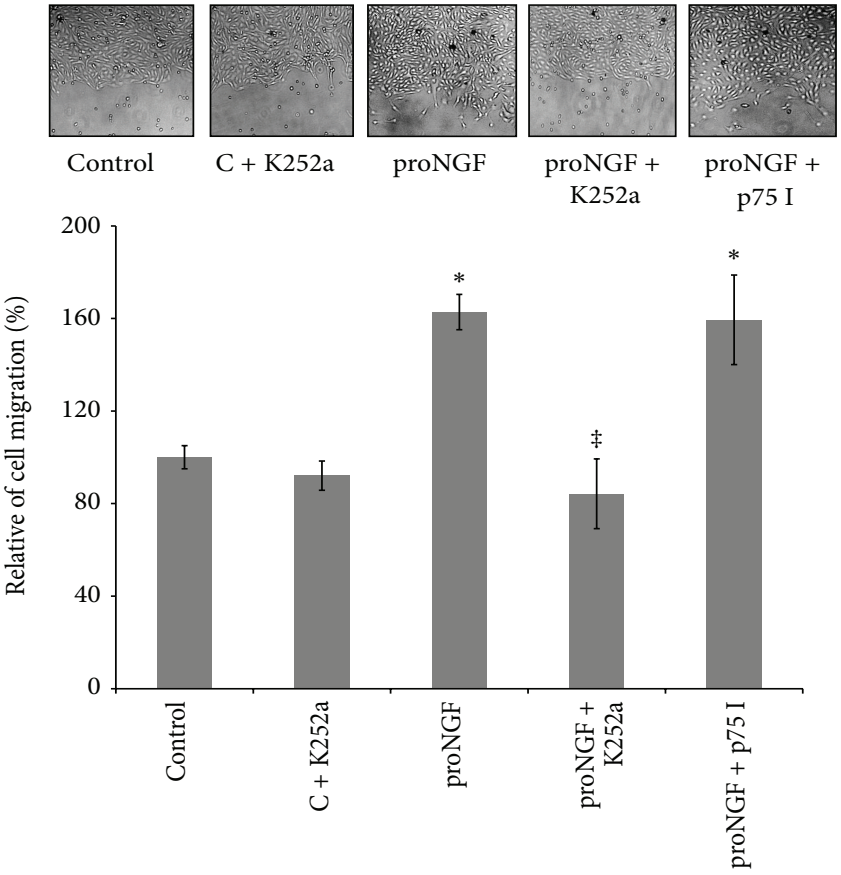
Control
C + K252a
proNGF
proNGF + K252a
proNGF + p75 I
Relative of cell migration (%)
200
160
120
80
40
0
*
‡
*
Control
C + K252a
proNGF
proNGF + K252a
proNGF + p75 I

Figure 1.6. Inhibiting TrkA prevents proNGF-mediated retinal endothelial cell migration.

BRE cells were grown to confluence then scratched using a standard cell scrapper. Cells were switched to serum free medium and treated with mutant proNGF (50ng/ml) in the presence or absence of either TrkA receptor antagonist, K252a (0.1μM) or p75 inhibitor (20μM). Representative micrographs for wounded BRE cells are shown after 18 hours of various treatments. Statistical analysis showed that proNGF increased mean cell migration by 1.98-fold compared to the control group. This effect was blocked by the specific TrkA receptor antagonist (K252a) but not with p75 inhibitor. (*, ‡ statistically significant compared to control and proNGF groups respectively ($P<0.05$), n=6).

Figure 1.7.

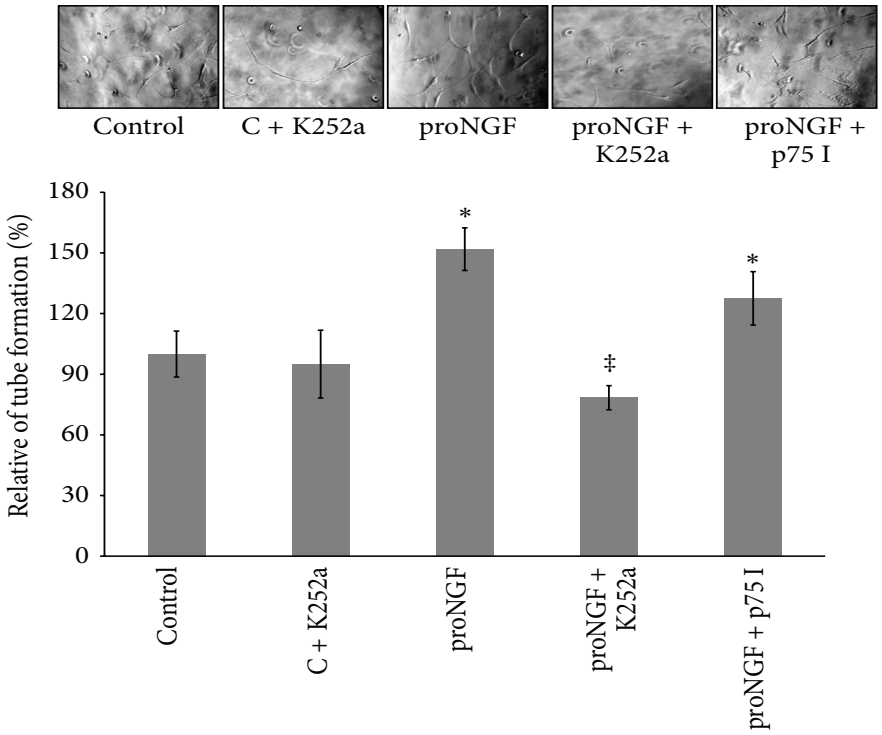
Control
C + K252a
proNGF
proNGF + K252a
proNGF + p75 I
Relative of tube formation (%)
180
150
120
90
60
30
0
*
‡
*
Control
C + K252a
proNGF
proNGF + K252a
proNGF + p75 I

Figure 1.7. Inhibiting TrkA prevents proNGF-mediated retinal endothelial cell tube formation.

BRE cells were grown into confluence then trypsinized and mixed with reduced-growth factor Matrigel and treated with mutant proNGF (50ng/ml) in the presence or absence of either TrkA receptor antagonist, K252a (0.1μM) or $p75^{NTR}$ inhibitor (20μM). Representative micrographs for alignment of BRE into tube-like structures are shown after 18 hrs of incubation. Statistical analysis of tube length showed that proNGF increased mean tube formation 1.5-fold compared to the control group. This effect was blocked by the specific TrkA receptor antagonist (K252a) but not with p75 inhibitor. (*, ‡ statistically significant compared to control and proNGF groups respectively ($P<0.05$), n=6).

Table 1.1. Clinical characteristics of participants providing aqueous humor samples.

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7
Gender	F	F	F	M	M	F	M
Race	Black	Black	White	White	Black	White	Black
Years of DM	20	26	17	18	16	28	19

CHAPTER 3

DELETION OF P75^{NTR} STIMULATES REPARATIVE ANGIOGENESIS AND PREVENTS RETINAL NEOVASCULARIZATION IN ISCHEMIC RETINOPATHY: POSSIBLE CONTRIBUTION OF TRKA RECEPTOR [1]

[1] Sally L. Elshaer and Azza B. El-Remessy.
Submitted to journal of angiogenesis, Nov., 2016

Abstract

Ischemic retinopathy is characterized by an initial microvascular degeneration followed by a maladaptive pathological retinal neovascularization (RNV) resulting in impaired neuro-vascular function and visual impairment. There is urgent need to identify druggable targets to overcome ischemic retinopathy. Given the role of neuron-secreted growth factors in regulating angiogenesis, we examined the vascular protective effects of genetic deletion of the neurotrophin $p75^{NTR}$ receptor in an oxygen-induced retinopathy mouse model. Vascular density, number of tip cells, and perfusions of capillaries were assessed. Western Blot and PCR were used to assess levels of growth factors including vascular endothelial growth factor (VEGF), nerve growth factor (NGF), brain-derived nerve factor (BDNF) and its precursor proNGF, proBDNF as well as expression and activation of the receptors, sortilin, TrkA and VEGFR2. The results showed that deletion of $p75^{NTR}$ prevented hyperoxia-associated central vascular cell death and hypoxia-associated RNV and enhanced reparative angiogenesis compared to WT. These effects were associated with decreased expression of apoptotic markers; c-PARP, total PARP and preserved survival signal Akt and restored the balance of increased NGF and decreased proNGF at the hyperoxic stage. During hypoxia, deletion of $p75^{NTR}$ restored NGF/proNGF and BDNF/proBDNF levels, and maintained VEGF and VEGFR2 activation compared to WT. Deletion of $p75^{NTR}$ resulted in significant increases in expression and activation of TkA, while WT showed decreases in both. Pharmacological inhibition of TrkA using compound K-252a (0.5μg/1μl) resulted in 2-fold increase in RNV and 1.34-fold increase in capillary dropout in $P75^{NTR}$ KO mice, but not in WT compared to vehicle-injected controls. Deletion of $p75^{NTR}$ protected against retinal ischemia, through restoring neurotrophins levels and activating TrkA receptor. Thus, targeting $p75^{NTR}$ offers potential therapeutic strategy for treatment of ischemic retinopathy

Introduction

Ischemic retinopathy is a common characteristic of several ocular diseases that can cause visual impairment and eventually blindness. Examples include diabetic retinopathy, retinopathy of prematurity and retinal vein occlusion [1]. Ischemic retinopathy is characterized by an initial microvascular degeneration followed by a mal-adaptive pathological neovascularization, consequent to hypoxia, in an attempt to reinstate metabolic equilibrium [2]. The disease is initially asymptomatic, yet loss of sight is provoked primarily by blood retinal barrier breakdown, macular edema, vitreous hemorrhages due to growth of leaky capillaries and in advanced cases, tractional retinal detachment [3]. Current therapeutic approaches including intravitreal injection of vascular endothelial growth factor (VEGF) neutralizing therapies are sight saving, yet hindered by serious concerns such as late intervention, invasiveness and cost-prohibition [3]. Therefore, there is pressing need to develop new therapeutics that can preserve both neuronal and vascular function in ischemic retinopathy diseases.

Given that retina is a typical neurovascular unit, neurons and glial cells may interact with blood vessels to contribute to pathologic neovascularization by secreting growth factors and guidance cue [4-6]. Nerve growth factor (NGF) is well known for its roles in regulating survival, growth, and functional maintenance of neuronal cells as well as vasculature. The biological functions of NGF are mediated through two different classes of cell surface receptors, a high-affinity tyrosine kinase receptor (TrkA) and a low-affinity nerve growth factor $p75^{NTR}$ [7]. In response to ischemic insult and hypoxia, NGF will be released with other angiogenic factors to stimulate angiogenic response. Several studies showed that TrkA receptor activation mediates NGF-survival and angiogenic signal via stimulation of the VEGF expression in various models [8-10].

NGF is synthesized and released as precursor, proNGF that normally get cleaved to its mature form. We and others have shown that pro-oxidative milieu can impair maturation of NGF leading to accumulation of proNGF [11,12]. ProNGF has high affinity to $p75^{NTR}$, the first identified member of the tumor necrosis factor (TNF) receptor superfamily that have the death domain [13]. Several reports demonstrated the action of proNGF mediating apoptosis [14-16] via activating $p75^{NTR}$ and forming a co-receptor with sortilin; a member of Vps10p-domain receptor family [17]. Nevertheless, its role in regulating cell survival and reparative angiogenesis is ill defined. We and others have shown that the precursor form of NGF; proNGF can drive the angiogenic behavior via activation of TrkA but not $p75^{NTR}$ in breast cancer cells [18] and retinal endothelial cells [19]. In the latter study, we observed that inhibition of $p75^{NTR}$ was associated with enhanced TrkA activation [19], suggesting a cross-talk between $p75^{NTR}$ expression and TrkA activation. Despite these significant findings, there is gap in knowledge about the interplay between NGF and proNGF and their receptors $p75^{NTR}$ and TrkA in response to hypoxia and angiogenesis.

In the current study using oxygen-induced retinopathy (OIR), a standard mouse model for ischemic retinopathy [20], we examined the impact of genetic deletion of $p75^{NTR}$ on vascular cell death as well as pathological neovascularization and reparative angiogenesis. Here, we share the findings that genetic deletion of $p75^{NTR}$ prevented vascular cell death, enhanced reparative central capillary growth and prevented pathological retinal neovascularization. The mechanisms involve restoring levels of NGF and BDNF, preserving VEGF level and enhancing TrkA-mediated survival and angiogenic signal. Thus, inhibition of $p75^{NTR}$ can provide potential therapeutic target for ischemic retinopathy.

Materials and Methods

Animals.

All animal experiments were conducted in agreement with Association for Research in Vision and Ophthalmology statement for use of animals in ophthalmic and vision research, and Charlie Norwood VA Medical Center Animal Care and Use Committee (ACORP#16–01–088). The $p75^{NTR}$, B6.129S4Ngfr^{tm1Jae} /J ($p75^{NTR+/-}$, exon III knockout mice [21]) were obtained from Jackson Laboratories (Bar Harbor, Maine, USA) and crossed with C57BL6/J mice (Jackson Laboratories). The WT and $p75^{NTR+/-}$ mice were crossed then back-crossed again to establish a colony of homozygous $p75^{NTR-/-}$ and WT breeders that produced the mice used here.

Oxygen-induced retinopathy (OIR) mouse model.

OIR model was performed as previously described by Smith et al. [22]. Briefly, on postnatal day 7 (p7), WT and $p75^{NTR-/-}$ mice pups were placed along with their dam into a custom-built chamber (Biospherix, Redfield, NY) in which the partial pressure of oxygen was maintained at 70% for 5 days. Pups were then returned to room air (21% oxygen, relative hypoxia) for 5 days (p12-p17). Central capillary drop-out (CDO) and retinal neovascularization (RNV) in hyperoxia-exposed mice pups were performed at P12 and P17, respectively, as described previously [22]; [23], whereas, biochemical assays; Western blotting and PCR were performed at p12 and p14. At the selected time points, mice pups were euthanized in carbon-dioxide chamber (2% flow rate for 5 min) followed by cervical dislocation. One eye was enucleated and fixed in 4% paraformaldehyde overnight to be flat-mounted for morphological studies. For the other eye, retinas were isolated and snap frozen for further analysis.

Intravitreal injection.

Mice pups were anesthetized by intraperitoneal injection of ketamine (237.7 g/mol, 100mg/ml) xylazine (220.3g/mol, 100mg/ml) mixture and complete anesthesia was confirmed by loss of reflex to sharp paw pinch. K252a (Sigma, MO, USA; 0.5 μg/μL/eye) or Dimethyl sulphoxide (DMSO) were injected intravitreally at p12 using a Hamilton syringe with 32-gauge glass capillary. Pups were left to recover (6-hours) from exposure to hyperoxia before intravitreal injection.

Morphological studies.

Retinas were dissected and permealized for 15 minutes with 0.3% Triton X-PBS then stained overnight at 4°C with isolectin; biotinylated griffonia (bandeiraea) simplicifolia lectin I (GSL I, BSL I), (Vector Labs, CA, USA; 1% in 5% normal goat serum in 0.3% Triton X-PBS) followed by incubation with secondary antibody; Texas red® avidin D (Vector labs, CA, USA; 0.5% in 5% normal goat serum in 0.3% Triton X-PBS). Retinal perfusion was assessed by intraperitoneal injection of FITC-dextran (Mol. WT. 2,000,000, Sigma, 50 mg/mL, 100μL/pup) 30 minutes prior to sacrificing mice pups as described previously [15]. Lectin-stained and/or FITC-perfused retinas were flat-mounted onto Superfrost/Plus microscope slides (Fisher Scientific, MA, USA) with the photoreceptor side facing down and imbedded in Vectashield mounting media for fluorescence (Vector Labs, CA, USA). Slides were photo-micrographed at 5X using a Zeiss AxioObserver.Z1. Images were assembled into a single file using photoshop software (CS6, Adobe system incorporated). CDO and RNV were quantified as previously described [24].

Western blot analysis.

Frozen retinas were placed into protein lysis buffer (Millipore) and briefly homogenized. Retinal lysates were centrifuged and 35-50µg were resolved on an SDS-PAGE gel (4-20% gradient Tris glycine precast gel, BioRad) and electro-blotted to nitrocellulose membranes. Membranes were blocked with 5% milk or BSA in PBS-tween and incubated overnight in 4°C with the following primary antibodies: $p75^{NTR}$ (kind gift from Dr. Bruce Carter, Department of Biochemistry, Vanderbilt University), Akt (#9272, Cell Signaling), p-Akt (#9275S, Cell Signaling), cleaved-PARP (#5625, Cell Signaling), total PARP (#9532, Cell Signaling), cleaved caspase-3 (#9664, Cell Signaling), TrkA (#76291, Abcam), phospho-TrkA (#1445, Abcam), NGF (# AN-240, Alomone), proNGF (#ANT-005, Alomone), sortilin (#16640, Abcam), BDNF and proBDNF (SC-546, Santa Cruz), VEGF (#ABS82, Millipore), VEGFR2 (#2472, Cell Signaling), phopho-VEGFR2 (#2474, Cell Signaling), then reprobed with the primary antibodies for the house-keeping proteins; Actin or tubulin to confirm equal loading. Membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-mouse or HRP-conjugated antirabbit secondary antibodies (Millipore) for 2 h at room temperature. The membranes were scanned with FluorChem™ FC3 (protein simple) and the band intensity was quantified using densitometry software version (Fiji) and expressed as relative optical density (OD).

Quantitative real-time (RT) PCR.

Retinas samples were processed using (mirVANA™ PARIS™ Kit) and RNA was purified and quantified as described previously [25] following the manufacturer's instructions. A one-step quantitative RT-PCR kit (Invitrogen) was used to amplify 10ng retinal mRNA as described previously [26,27]. PCR primers (listed in Table-1) were obtained from Integrated DNA Technologies (Coralville, IA, USA). Quantitative PCR was conducted using StepOnePlus

qPCR system (Applied BioSystems, Life Technologies). The percent expression of various genes was normalized to 18S and expressed relative to WT normoxic controls.

Statistical analysis:

All the data are expressed as mean ± SEM. Differences between 2 groups for morphological studies were detected using un-paired Student T-test. One-way ANOVA was used to assess significant differences between 3 groups. Two-way ANOVA was used to assess interaction between two variables; 2 genes (WT vs. $p75^{NTR-/-}$) X OIR exposure (normoxia vs. hyperoxia/hypoxia). Tukey-Kramer post-multiple comparisons was used for significant interactions among various groups. Significance for all tests was determined at $\alpha = 0.05$, Graphpad Prism, Ver.6.

Results

Deletion of $p75^{NTR}$ did not alter retinal vascular density under normoxic condition.

We aimed to examine the vascular protective effects of $p75^{NTR}$ in ischemic retinopathy using $p75^{NTR-/-}$, exon III knockout mice [21]. First, we examined vascular density in flat-mounted retina stained with isolectin at postnatal day (p12) and p17. As shown in supplementary Figure-1A-B, representative image and statistical analysis showed no difference in branching density between WT and $p75^{NTR-/-}$ pups at P12, whereas at p17, retinas from $p75^{NTR-/-}$ showed significant increase in branching density compared to WT (supplementary Figure-1C-D).

Hyperoxia triggered $p75^{NTR}$ expression and vaso-obliteration in WT, but not in $p75^{NTR-/-}$.

Exposing the developing retina of WT mice to hyperoxia (70% oxygen) from p7 to p12 resulted in significant (1.4-fold) increase in $p75^{NTR}$ expression compared to normoxic controls (Fig. 1A). These effects were associated with marked capillary dropout, illustrated by the central shaded

area (Fig. 1B) in WT mice pups. Statistical analysis showed significant reduction by 34% in central vascular cell death in $p75^{NTR-/-}$ pups compared to their WT littermates (Fig. 1C-D).

Deletion of $p75^{NTR}$ attenuated neovascularization and enhances reparative angiogenesis.

Next, we examined the expression of $p75^{NTR}$ receptor in WT mice during the hypoxic stage of OIR model (p12-p17). As shown in Fig. 2A-B, there was no significant difference in $p75^{NTR}$ expression neither at p14 or p17. These effects coincided with significant pathological retinal neovascularization in the mid-periphery of the retina, growing towards the vitreous (encircled in Fig. 2C by dotted white line) and impaired intra-retinal reparative angiogenesis towards the central avascular area (Fig. 2C, shaded area at the center). Deletion of $p75^{NTR}$ receptor ameliorated pathological neovascularization (Fig. 2D) by 42% and stimulated reparative angiogenesis as indicated by growth of capillaries and decreasing avascular area by ~32% during hypoxic stage of OIR model (Fig. 2 E-F).

Deletion of $p75^{NTR}$ preserved survival signal and attenuated hyperoxia-mediated apoptosis.

Exposure to hyperoxia tended to decrease the activation of the survival Akt by 30% (Fig. 3A) and increased total-PARP by 1.4-fold in WT pups by p12 (Fig. 3B). In contrast, $p75^{NTR-/-}$ retinas showed preserved level of Akt activation in response to hyperoxia and experienced ~50% lower levels of both total- and cleaved-PARP compared to their normoxic controls (Fig. 3A-B). 2X2 analysis showed significant impact of both hyperoxia and gene deletion effect on PARP expression. Interestingly, $p75^{NTR-/-}$ showed 40% lower basal level of cleaved PARP compared to their WT littermates under normoxic condition.

Deletion of p75^{NTR} attenuated hyperoxia-induced increase in proNGF and decrease in NGF.

We have previously shown that exposure to hyperglycemia disturbed homeostasis of NGF by accumulation of precursor, proNGF at the expense of its mature form NGF [12,28]. As shown in Fig. 4A-C, exposing WT pups to hyperoxia resulted in significant 1.7-fold increase in proNGF expression whereas; NGF showed mild decrease compared to WT normoxic controls. Deletion of p75^{NTR} reduced proNGF expression and increased NGF levels compared to their normoxic controls. As shown in Fig. 4D, hyperoxia resulted in reducing the ratio of NGF to proNGF levels by 50% in WT, while the ratio was preserved in p75$^{NTR-/-}$. These results suggest that hyperoxia shifted the homeostasis of NGF to toward the more apoptotic form at the expense of the survival.

Deletion of p75^{NTR} attenuated hypoxia-induced increase in proNGF and decrease in NGF.

During the hypoxic stage of OIR, alterations in NGF precursor continued in WT retinas; where they showed significant 1.35-fold increase in proNGF (Fig. 5A-B) and mild change in NGF levels (Fig. 5A, C). Deletion of p75^{NTR} prevented the increase in proNGF and significantly increased NGF levels (1.3-fold) compared to their normoxic controls (Fig. 5-A-C). NGF/proNGF ratio was decreased in hypoxic WT pups by 30%, which was preserved in p75$^{NTR-/-}$ pups (Fig. 5D).

Deletion of p75^{NTR} attenuated hypoxia-induced increase in proBDNF and decrease in BDNF.

We next examined whether alterations in NGF and its precursor form are observed in other neurotrophins. As shown in Fig. 5 E-F, WT retinas experienced similar changes in BDNF

and its precursor during hypoxic stage of OIR. Deletion of $p75^{NTR}$ receptor decreased proBDNF by ~25% and preserved BDNF that was decreased in WT pups by 15% during hypoxic stage of OIR (Fig. 5G). Consequently, hypoxic WT retinas showed 33% decrease in BDNF/proBDNF ratio that was normalized in hypoxic $p75^{NTR-/-}$ retinas (Fig. 5H). Although changes in homeostasis of BDNF did not reach statistical significance, the trend was similar to the prior observation in NGF.

Deletion of $p75^{NTR}$ prevented hyperoxia-induced decrease in TrkA expression and activation.

Exposure to hyperoxia resulted in significant decrease by ~75% in TrkA mRNA expression in WT retinas compared to normoxic controls (Fig. 6A). Exposure of $p75^{NTR-/-}$ retinas to hyperoxia resulted in significant 2-fold increase in TrkA mRNA and 1.63-fold in protein level compared to WT pups (Fig. 6A-B). Moreover, retinas from $p75^{NTR-/-}$, but not WT pups, showed significant 2.4-fold increase in TrkA activation compared to $p75^{NTR-/-}$ normoxic controls (Fig. 6C). Interestingly, basal level of TrkA mRNA (data not shown) and activation of TrkA receptor was lower in $p75^{NTR-/-}$ pups by ~ 43% compared to their WT littermates (Fig. 6D). During hypoxic stage of OIR, TrkA receptor mRNA tended to increase in WT and $p75^{NTR-/-}$ pups, yet, was not statistically significant (Fig. 6E). TrkA protein expression and its activation were not significantly altered in response to hypoxia or deletion of $p75^{NTR-/-}$ (Fig. 6F-H). These results indicate that exposure to hyperoxia rather than hypoxia, exerted stronger impact on reducing TrkA expression and activation, suggesting that TrkA signal is more pronounced in the text of vascular cell survival more than angiogenic signal.

Next, we examined impact of hypoxia and deletion of $p75^{NTR}$ on other neurotrophin receptors. As shown in Supplementary Fig. 2, sortilin expression was not affected in response to hyperoxia

at p12 (A), in response to hypoxia at p14 (B) in WT. In addition, sortilin expression was not altered due to deletion of $p75^{NTR}$ at normoxia or OIR. In contrast, TrkB expression was not detected from same retina lysates.

TrkA activity is required for the vasoprotective effects observed in $p75^{NTR-/-}$ but not WT.

In $p75^{NTR-/-}$, the decrease in central capillary dropout area (Fig.1) coincided with significant increase in expression and activation of TrkA at p12 (Fig.6). To examine the specific contribution of TrkA activation in $p75^{NTR-/-}$-vascular protection, we used a pharmacological inhibitor, compound K-252a, a staurosporine analog that is potent inhibitor of various Trk receptors [29] that was dissolved in dimethylsulfoxide (DMSO) and administered intravitreally (0.5μg/μl/eye) at p12. Animals were perfused with FITC-dextran to examine capillary perfusion and capillary dropout area (Fig.7A) and after sacrifice at p17, retinas were flat-mounted and stained with isolectin-GS-IB4 conjugate (Fig.7B). As shown in Fig.7C-D, treatment of WT with K-252a did not alter the retinal neovascularization or central capillary drop out area compared to DMSO-controls. As shown in Fig.7 C-D, treatment of $p75^{NTR-/-}$ with K-252a resulted in significant 2-fold increase in retinal neovascularization and 1.34-fold increase in central avascular area when compared to the DMSO-treated controls.

Deletion of $p75^{NTR}$ preserved hypoxia induced VEGF and VEGFR2 activation.

As expected, hypoxia resulted in significant 2.2-fold increase in VEGF mRNA expression (Fig. 8A) and 1.53-fold in VEGF protein expression compared to their normoxic controls (Fig. 8 B-C). Deletion of $p75^{NTR}$ receptor did not significantly impact basal expression of VEGF mRNA or protein, yet upon hypoxia exposure, VEGF mRNA expression tended to increase by 1.65-fold that was not statistically significant (Fig. 8A) and significant 1.55-fold

increase in protein compared to normoxic controls (Fig. 8B-C). Exposure to hypoxia resulted in increased activation of VEGFR2 in WT that was maintained in $p75^{NTR-/-}$ pups at both basal and hypoxic conditions, (Fig. 8D-F).

Deletion of $p75^{NTR}$ increased the number of tip cells during retinal angiogenesis.

Physiological vascular development is steered by specialized endothelial tip cells [30] that respond to VEGF by forming motile filopodia enriched in VEGFR2 and rich in guidance receptors that respond to directional cues [30]. Therefore, we examined the number of tip cells that guide normal retinal angiogenesis at postnatal day 5. As shown in Fig. 8G-H, overall number of tip cells per whole retina was significantly higher in $p75^{NTR-/-}$ pups compared to their WT littermates.

Discussion

Retinal ischemia and the subsequent secretion of angiogenic factors including VEGF and NGF are thought to be common precursors to retinal neovascularization. Although targeting VEGF has proven effective clinically in preventing macular edema and proliferative stage of diabetic retinopathy, the challenge remains to identify strategies to vascularize the ischemic retina and to maintain the homeostasis of the retina as neurovascular unit. The findings from this study support the vascular protective effects of targeting $p75^{NTR}$ as follows: 1. Deletion of the $p75^{NTR}$ reduced vaso-obliteration, pathological neovascularization and enhanced reparative angiogenesis in OIR model of ischemic retinopathy (**Fig.1,2**). 2. Under hyperoxia, deletion of $p75^{NTR}$ caused decrease in apoptotic signal, preserved survival signal by enhancing NGF and TrkA expression and activation (**Fig. 3,4,6**). 3. Under hypoxia, deletion of $p75^{NTR}$ restored NGF, maintained VEGF levels and enhanced VEGFR2 activation (**Fig.5,8**). 4. Vascular protection observed in $p75^{NTR-/-}$ was blunted by inhibition of Trk receptor but not in WT (**Fig.7**). These

results support that the neurotrophin receptor $p75^{NTR}$ and its cross-talk with TrkA receptor are viable therapeutic targets to combat retinal diseases with aberrant angiogenesis.

Growing body of evidence support the role of neurons in regulating angiogenesis by secreting growth factors and guidance cue (reviewed in [31]). NGF and BDNF are members of the neurotrophin family that can exert neuroprotective, vascular protective and angiogenic effects. As adaptive response to overcome ischemia, NGF will be released, initially as a precursor, proNGF that normally get cleaved to its mature form [13]. We and others have shown that pro-oxidative milieu can impair maturation of NGF leading to accumulation of proNGF and activation of $p75^{NTR}$ pathway [11,12]. Exposure of WT pups to hyperoxia, a well-documented condition of oxidative and nitrative stress [32,33] triggered the accumulation of proNGF compared to normoxic-controls. It is interesting that the ratio between NGF to proNGF was significantly reduced in hyperoxia that coincided with upregulation of its receptor $p75^{NTR}$ and vascular cell death at p12. The apoptotic action of $p75^{NTR}$ receptor via forming a co-receptor with sortilin is well-documented in both neuro- and microvascular degeneration (reviewed in [14]. While there was no change in sortilin expression in response to hyperoxia or gene deletion, we observed upregulation of $p75^{NTR}$ protein expression at p12, a time point for maximum central vascular death in the WT retina. Our results came in agreement with reports showing increased expression of $p75^{NTR}$ in ischemic retina models including ischemia induced by elevated intra-ocular pressure [34], and in an inherited retinal degeneration model, Royal College of Surgeons rats, that was associated with progressive capillary dropout and subretinal neovascularization [35]. While upregulation of proNGF/$p75^{NTR}$ was reported in cardiac ischemia-reperfusion resulting in microvascular damage [11] and after focal cerebral ischemia [36], we believe that

this is the first report that demonstrates involvement of proNGF/$p75^{NTR}$ in oxygen-induced retinopathy.

Deletion of $p75^{NTR}$ receptor significantly decreased vascular cell death as assessed by capillary dropout area at p12 (Fig. 1), reduced pathological retinal neovascularization (tufts) and stimulated reparative angiogenesis evident by smaller central avascular area by p17 (Fig. 2). These results lend further support to recent studies using molecular or pharmacological inhibitor of $p75^{NTR}$ [16,37]. Yet, both studies attributed the vascular protection mediated by modulating $p75^{NTR}$ receptor in OIR to either decreased stabilization of HIF-1α and its target genes including VEGF [37], or due to decreased secretion of Semaphorine-3A and TNF-α [16]. Our group has previously shown that vaso-obliteration at p12 is associated with increased expression of apoptotic markers including; cleaved caspase-3 and PARP as well as decreased activation of Akt [25,38]. In agreement, deletion of $p75^{NTR}$-attenuated vascular cell death coincided with preserved activation of the survival pAkt pathway as well as attenuated expression of apoptotic markers, cleaved and total-PARP (Fig. 3).

Interestingly, exposure to relative hypoxia resulted in significant increase in proNGF and modest increase in proBDNF in WT. Deletion of $p75^{NTR}$ receptor decreased the levels of proNGF and proBDNF and preserved the levels of mature NGF and BDNF at p14 (Fig. 5) that coincided with enhanced reparative angiogenesis (Fig. 2). In agreement, down regulation of neurotrophins; NGF, BDNF, NT-3 and GDNF was also reported in an ischemic rat retina model [39]. Clinically, preterm infants who experienced proliferative retinopathy had decreased serum levels of BDNF compared to full-term [40,41]. Exposure to relative hypoxia did not alter $p75^{NTR}$ expression at p14 or p17, a time point of maximum retinal pathological neovascularization. Our results came in agreement with a prior study that showed that hypoxia stimulates shedding of $p75^{NTR}$ rather

than modulate its expression [37]. In contrast, a recent study using similar OIR model showed that expression of $p75^{NTR}$ receptor did not change in response to hyperoxia (p8, p10 and p12), yet it showed significant increase to in response to hypoxia (p14 and p17) [16]. We observed increase in TrkA gene expression in $p75^{NTR-/-}$ pups as well as its activation during the vaso-obliteration phase of OIR. In addition, preserved ratio between mature versus premature forms of NGF was also observed. Expression as well as activation of TrkA receptor was significantly higher in $p75^{NTR-/-}$ pups by p14 in OIR (Fig. 6). Preserved NGF/TrkA signaling was maintained throughout the neovascularization phase of OIR (figures 4,5,6). Previous literature identified clear angiogenic effects of NGF via activation of TrkA in models of angiogenesis [8,42]. These findings support the hypothesis that vascular repair exerted by deletion of $p75^{NTR}$ receptor could be attributed to enhanced TrkA survival signaling (Fig. 3,4,6). Furthermore, blocking TrkA receptor using compound K-252a, a general and potent inhibitor of Trk receptors [29] at p12 prevented the amelioration of avascular area and retinal neovascularization in $p75^{NTR-/-}$, i.e. reversed vascular protection in the knockout mice but not in WT (Fig. 7). We and others have shown upregulation of $p75^{NTR}$ expression concurred with inhibition of Trk-Y490 phosphorylation [26,27,43]. Treatment that restored TrkA-Y490 phosphorylation in diabetic animals was associated with reduction of $p75^{NTR}$ expression [26]. In 2013, we have reported that inhibition of $p75^{NTR}$ was associated with enhanced TrkA activation in endothelial cell cultures [19]. Here, we report for the first time that deletion of $p75^{NTR}$ can impact TrkA expression and activation, suggesting that the cross talk between these two receptors can be utilized not only to downregulate one of them but also to upregulate the other receptor endogenously.

VEGF could be a potential contributor to vascular protection observed in $p75^{NTR-/-}$ mice pups. During the hypoxic stage of OIR, we have seen significant increase in gene and protein

expression of VEGF (Fig. 8A,B). This corroborates with previous findings [15,37,44]. Deletion of $p75^{NTR}$ sustained hypoxia-induced VEGF and VEGFR2 activation. These results support prior findings that gene delivery of $p75^{NTR}$ impaired neovascularization and blood flow recovery in diabetic mouse with hind limb ischemia through depression of VEGF [45], however our results came in contrast to results showing that genetic deletion of $p75^{NTR}$ receptor resulted in decreased stabilization of HIF-1α and VEGF expression at p17 [37]. Our results are further supported by prior work showing that treatments that sustain VEGF/VEGFR2 activation stimulate reparative angiogenesis in addition to preventing retinal neovascularization in OIR model [46]. This increase was associated with significant increase in number of tip cells. Indeed, our results showed that deletion of $p75^{NTR}$ resulted in significant increase in the number of tip cells in the developing retina at p5 compared to WT. Development of the intraretinal vasculature in mice occurs in a tightly regulated pattern [47]. Of note, although the number of tip cells were much higher at p5, the vascular density in retinas from p12 of $p75^{NTR-/-}$ were not different from their age-matched WT. However, at p17, branching density of $p75^{NTR-/-}$ were significantly higher than WT. This could be explained by that fact that the deep plexus, located in the outer plexiform layer, forms rapidly and reaches the retinal periphery at approximately p12, followed by the intermediate plexus in the inner plexiform layer between p12 and p15 [23].

Here, we demonstrate a number of unexplored pathways that unraveled novel pathways by which the $p75^{NTR}$ can contribute to vascular protection including preserving NGF and BDNF levels, TrkA survival signal and sustaining VEGF/VEGFR2 activation that together maintain healthy retinal vasculature.

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Conflict of interest

The authors have no conflict of interest to declare.

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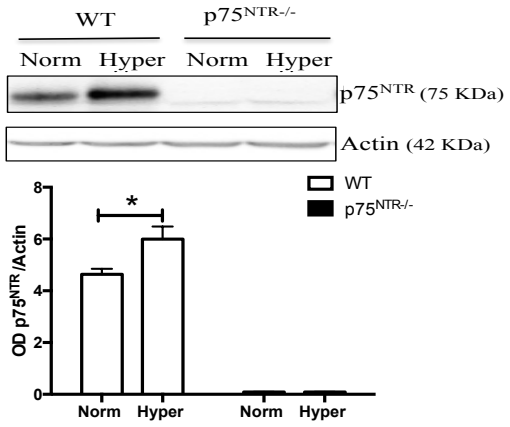
Figure 2.1 (A-D)

(A)



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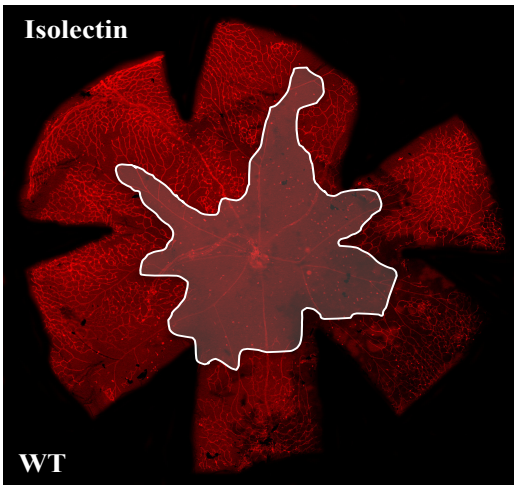

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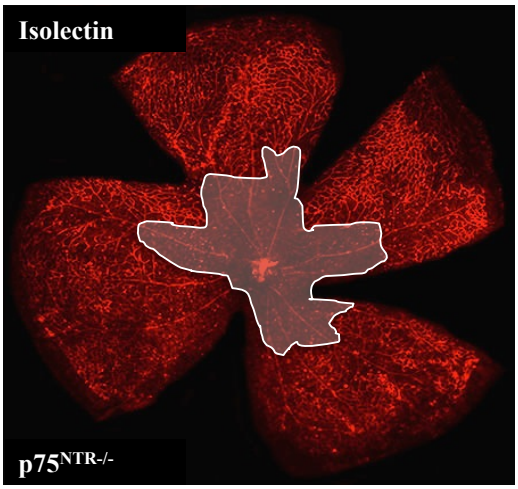

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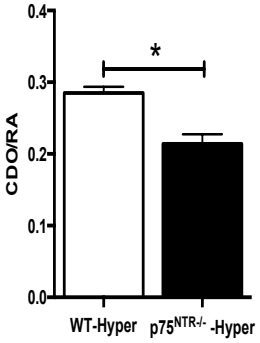


Figure 2.1. Hyperoxia triggered $p75^{NTR}$ expression and vaso-obliteration in WT, but not in $p75^{NTR-/-}$.

(A) Exposing WT pups to 70% oxygen from p7-p12 significantly increase p75NTR expression (*, significant compared to WT-controls using two-way ANOVA, $p<0.05$, n=3-4). (B, C) Isolectin GS-stained retinal flat mounts of WT and $p75^{NTR-/-}$ p12 pups respectively, showing marked decrease in central vascular cell death in $p75^{NTR-/-}$ pups. Central capillary drop-out (CDO) is illustrated by shaded area at the center. (D) Bar graph for CDO of hyperoxic WT and $p75^{NTR-/-}$ pups (*, significant compared to WT-hyperoxic group using unpaired student T-test, $p<0.05$, n=8-12). (Hyper) hyperoxia-exposed group, (Norm) normoxic controls, (CDO): central capillary dropout.

Figure 2.2 (A-D)

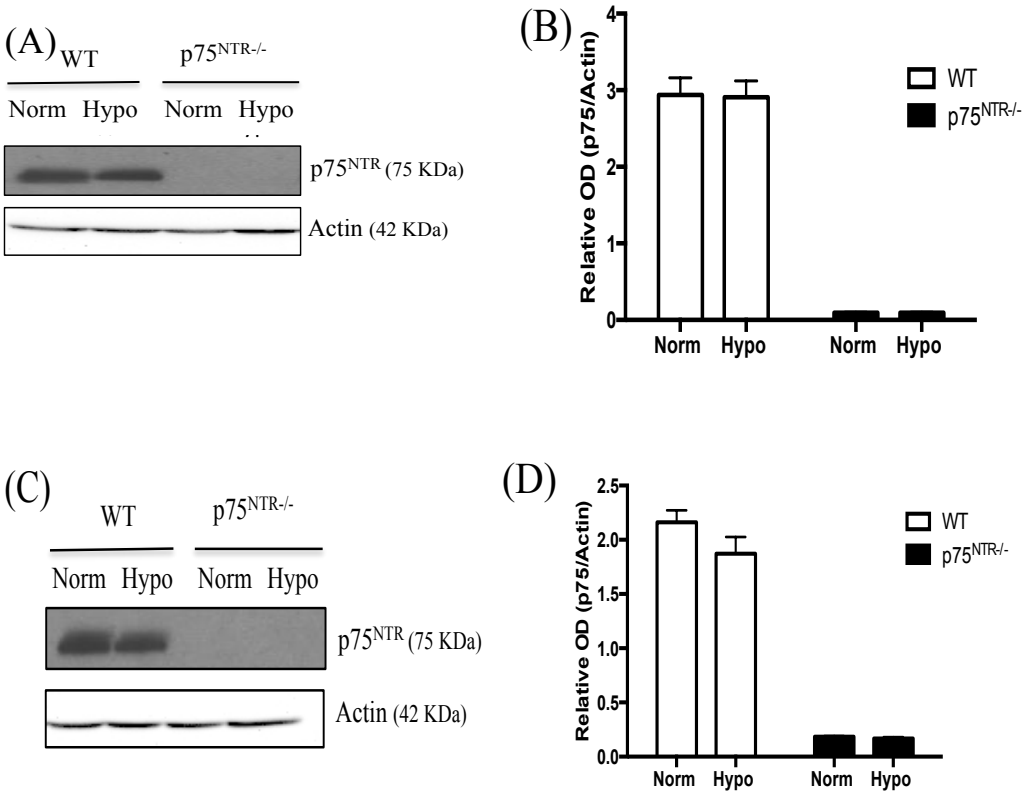
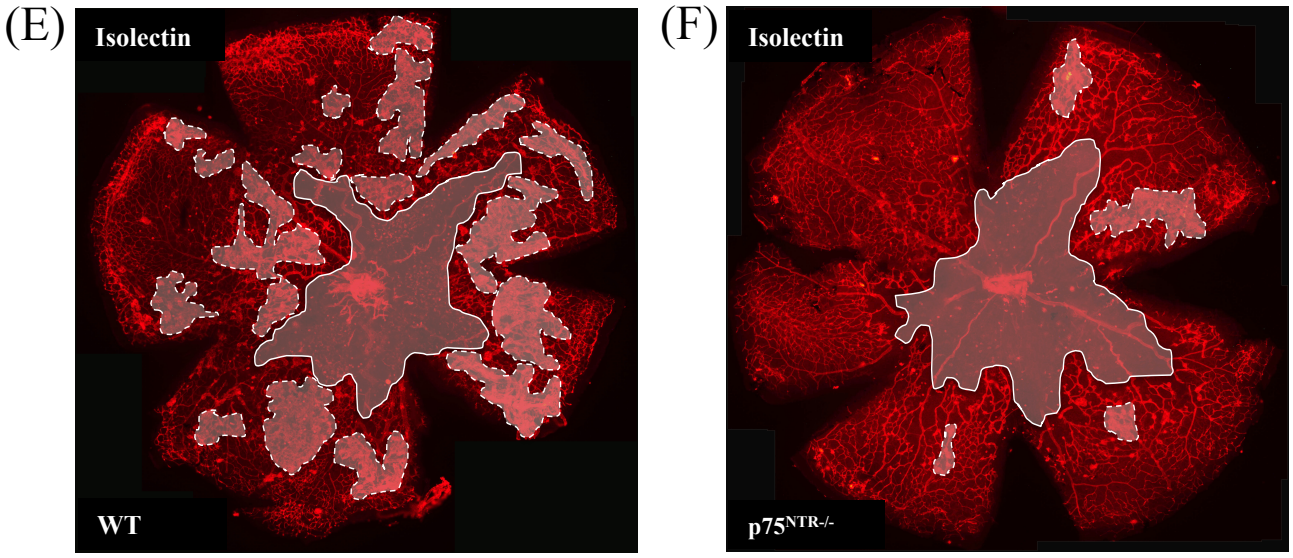
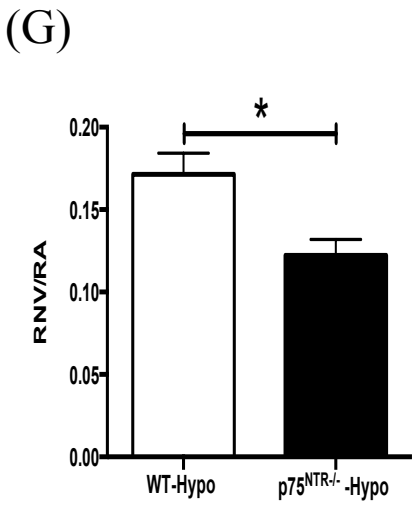

(A)
WT
p75NTR-/-
Norm Hypo Norm Hypo
p75NTR (75 KDa)
Actin (42 KDa)
(B)
Relative OD (p75/Actin)
WT
p75NTR-/-
Norm Hypo Norm Hypo
(C)
WT
p75NTR-/-
Norm Hypo Norm Hypo
p75NTR (75 KDa)
Actin (42 KDa)
(D)
Relative OD (p75/Actin)
WT
p75NTR-/-
Norm Hypo Norm Hypo

Figure 2.2 (E-H)


(E)
Isolectin
WT
(F)
Isolectin
p75NTR-/-


(G)
RNV/RA
0.20
0.15
0.10
0.05
0.00
*
WT-Hypo
p75NTR-/- -Hypo

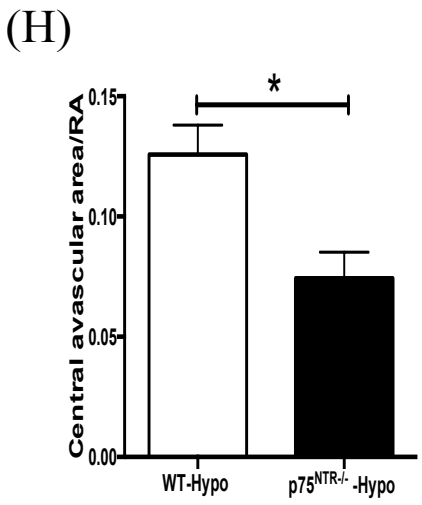

(H)
Central avascular area/RA
0.15
0.10
0.05
0.00
*
WT-Hypo
p75NTR-/- -Hypo

Figure 2.2. Deletion of $p75^{NTR}$ attenuated hypoxia-induced neovascularization and enhances reparative angiogenesis.

(A, B) Western blotting representative and bar graph of $p75^{NTR}$ receptor expression in WT pups by p14 showing no alteration during hypoxic stage of OIR (n= 4-6). (C, D) Western blotting representative and bar graph of $p75^{NTR}$ receptor expression in WT pups by p17 showing a trend for decreased $p75^{NTR}$ expression that was not statistically significant (n= 4). (E, F) Representative images of isolectin GS-stained retinal flat mounts of p17 WT and $p75^{NTR-/-}$ pups, showing pathological retinal neovascularization (RNV, encircled by dotted white line) and central avascular area (shaded area at the center). Images were taken on 5X magnification. (G, H) Bar graph of RNV and central avascular area showing significant decrease in both manifestations in $p75^{NTR-/-}$ pups by p14 during hypoxic stage of OIR (*, significant using unpaired student T-test, $p<0.05$, n= 7-14). Norm: normoxic controls, Hypo: hypoxia-exposed groups, IL B4: isolectin B4, RNV: retinal neovascularization, RA: total retinal area.

Figure 2.3.

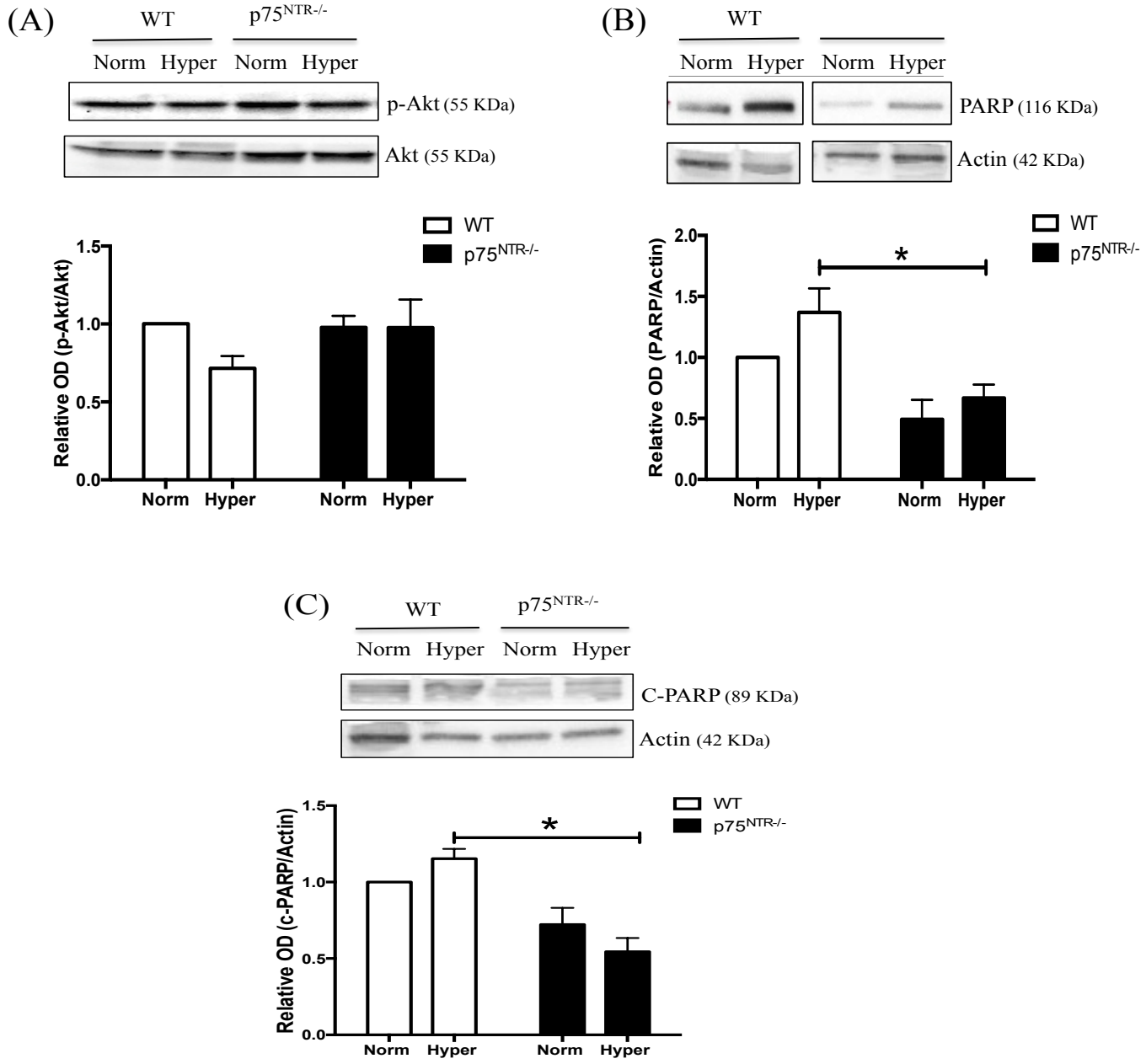

(A)
WT
p75NTR-/-
Norm Hyper Norm Hyper
p-Akt (55 KDa)
Akt (55 KDa)
WT
p75NTR-/-
Relative OD (p-Akt/Akt)
1.5
1.0
0.5
0.0
Norm Hyper Norm Hyper
(B)
WT
Norm Hyper Norm Hyper
PARP (116 KDa)
Actin (42 KDa)
WT
p75NTR-/-
Relative OD (PARP/Actin)
2.0
1.5
1.0
0.5
0.0
*
Norm Hyper Norm Hyper
(C)
WT
p75NTR-/-
Norm Hyper Norm Hyper
C-PARP (89 KDa)
Actin (42 KDa)
WT
p75NTR-/-
Relative OD (c-PARP/Actin)
1.5
1.0
0.5
0.0
*
Norm Hyper Norm Hyper

Figure 2.3. Deletion of $p75^{NTR}$ preserved survival signal and attenuated hyperoxia-mediated apoptosis.

(A) Representative Western blotting and bar graph of Akt activation (Y308) in p12 retinal lysates of WT and $p75^{NTR-/-}$ mice (n=5-7). (B) Representative Western blotting and bar graph of total PARP expression in p12 retinal lysates of WT and $p75^{NTR-/-}$ mice (*, significant using two-way ANOVA, $p<0.05$, n= 7). (C) Representative Western blotting and bar graph of cleaved PARP expression in p12 retinal lysates of WT and $p75^{NTR-/-}$ mice (*, significant using two-way ANOVA, $p<0.05$, n=4-6).

Figure 2.4.

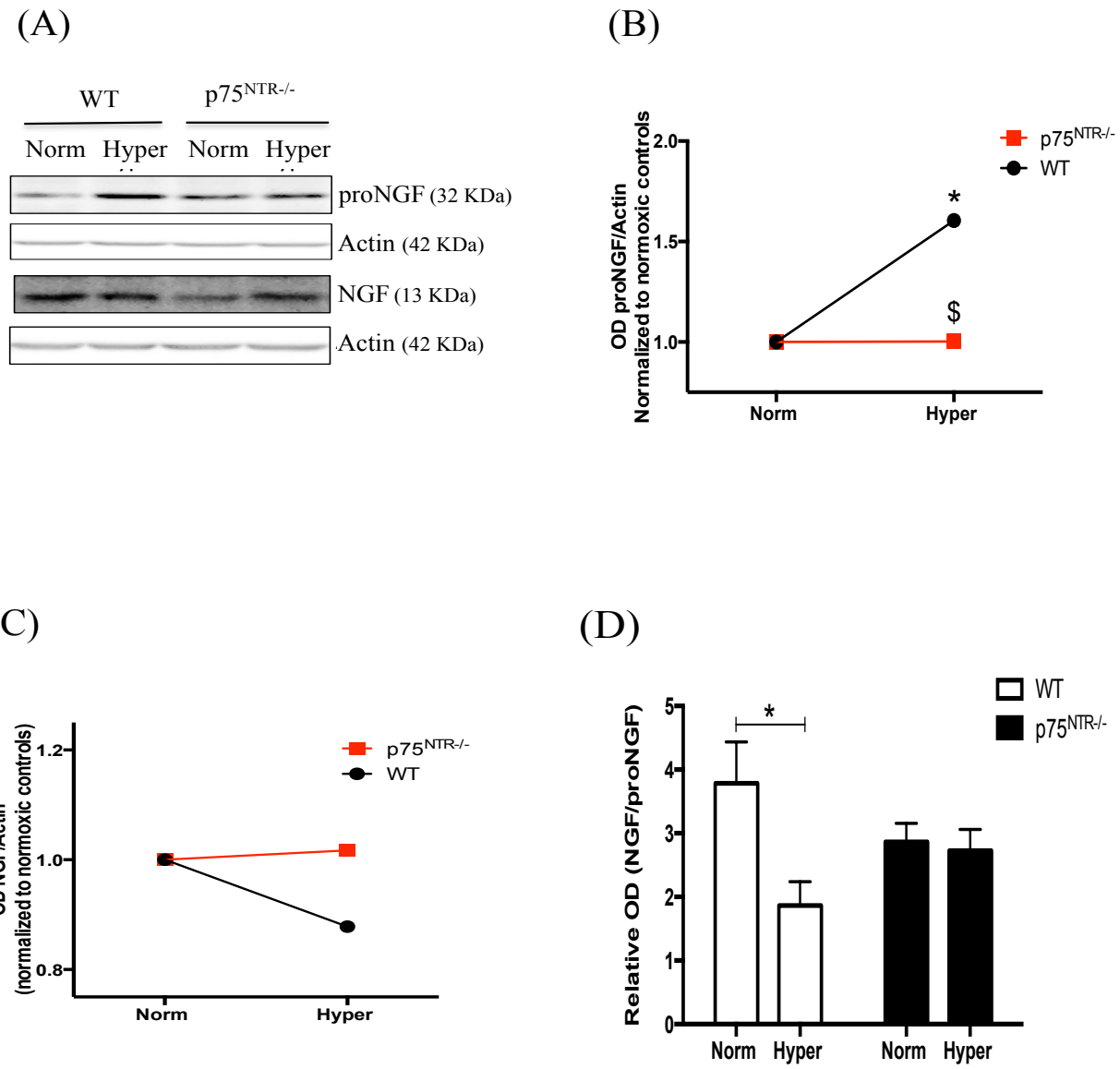

(A)
WT
p75NTR-/-
Norm
Hyper
Norm
Hyper
proNGF (32 KDa)
Actin (42 KDa)
NGF (13 KDa)
Actin (42 KDa)
(B)
OD proNGF/Actin
Normalized to normoxic controls
2.0
1.5
1.0
p75NTR-/-
WT
*
$
Norm
Hyper
(C)
OD NGF/Actin
(normalized to normoxic controls)
1.2
1.0
0.8
p75NTR-/-
WT
Norm
Hyper
(D)
Relative OD (NGF/proNGF)
5
4
3
2
1
0
*
WT
p75NTR-/-
Norm
Hyper
Norm
Hyper

Figure 2.4. Deletion of $p75^{NTR}$ attenuated hyperoxia-induced increase in proNGF and decrease in NGF.

(A, B) Representative Western blotting and bar graph for proNGF showing significant increase in WT but not $p75^{NTR-/-}$ during hyperoxia. (A, C) Representative Western blotting and bar graph for NGF showing decreased level in WT but preserved level in $p75^{NTR-/-}$during hyperoxia. (D) Bar graph of NGF/proNGF ratio showing significant decrease in WT during hyperoxia but not $p75^{NTR-/-}$ pups (*, $, significant compared to WT normoxic and hyperoxic groups respectively, using two-way ANOVA, $p<0.05$, n=6-8).

Figure 2.5 (A-D)

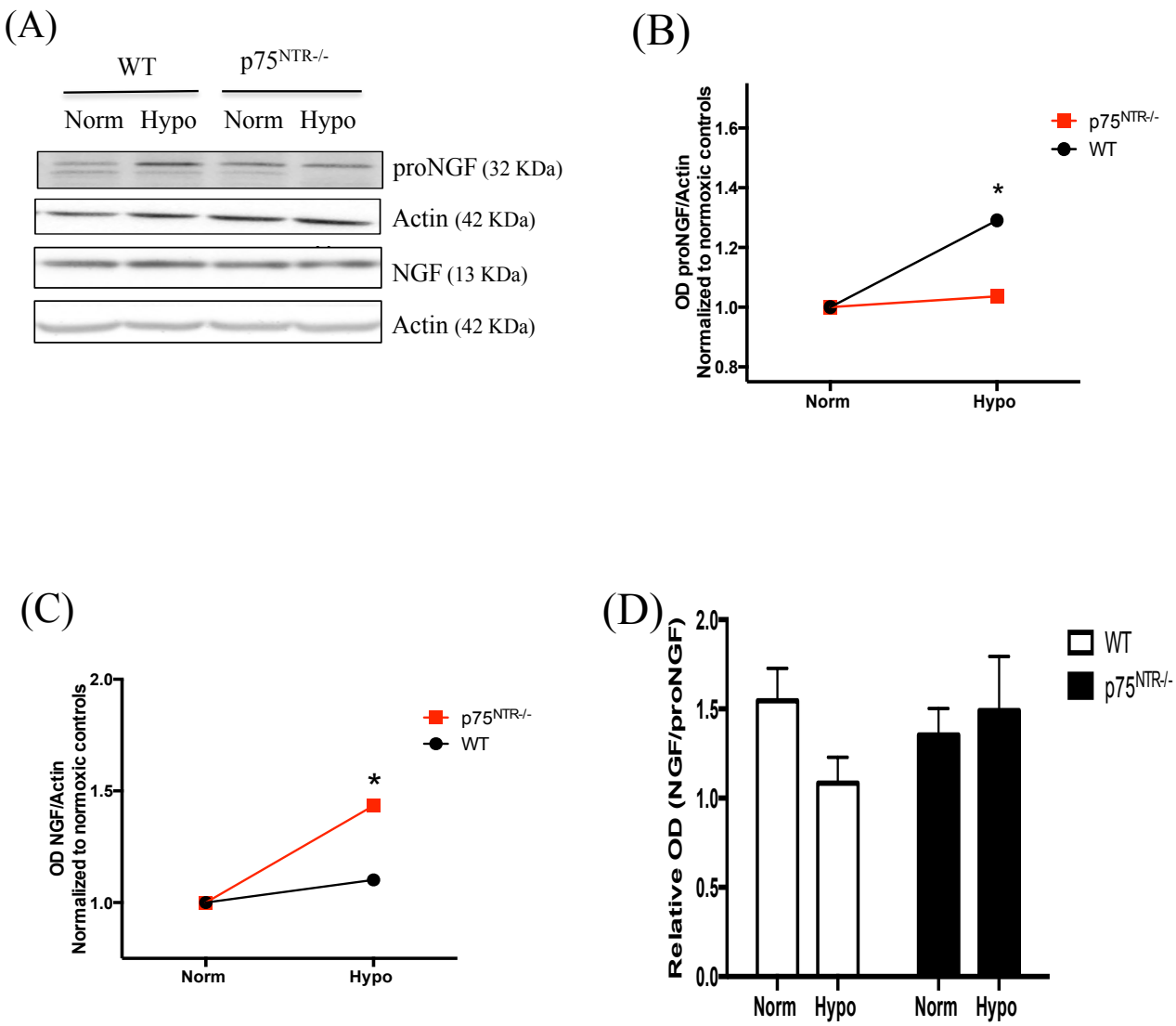

(A)
WT
p75NTR-/-
Norm Hypo Norm Hypo
proNGF (32 KDa)
Actin (42 KDa)
NGF (13 KDa)
Actin (42 KDa)
(B)
OD proNGF/Actin
Normalized to normoxic controls
1.6
1.4
1.2
1.0
0.8
*
Norm
Hypo
p75NTR-/-
WT
(C)
OD NGF/Actin
Normalized to normoxic controls
2.0
1.5
1.0
*
Norm
Hypo
p75NTR-/-
WT
(D)
Relative OD (NGF/proNGF)
2.0
1.5
1.0
0.5
0.0
Norm Hypo Norm Hypo
WT
p75NTR-/-

Figure 2.5 (E-H)

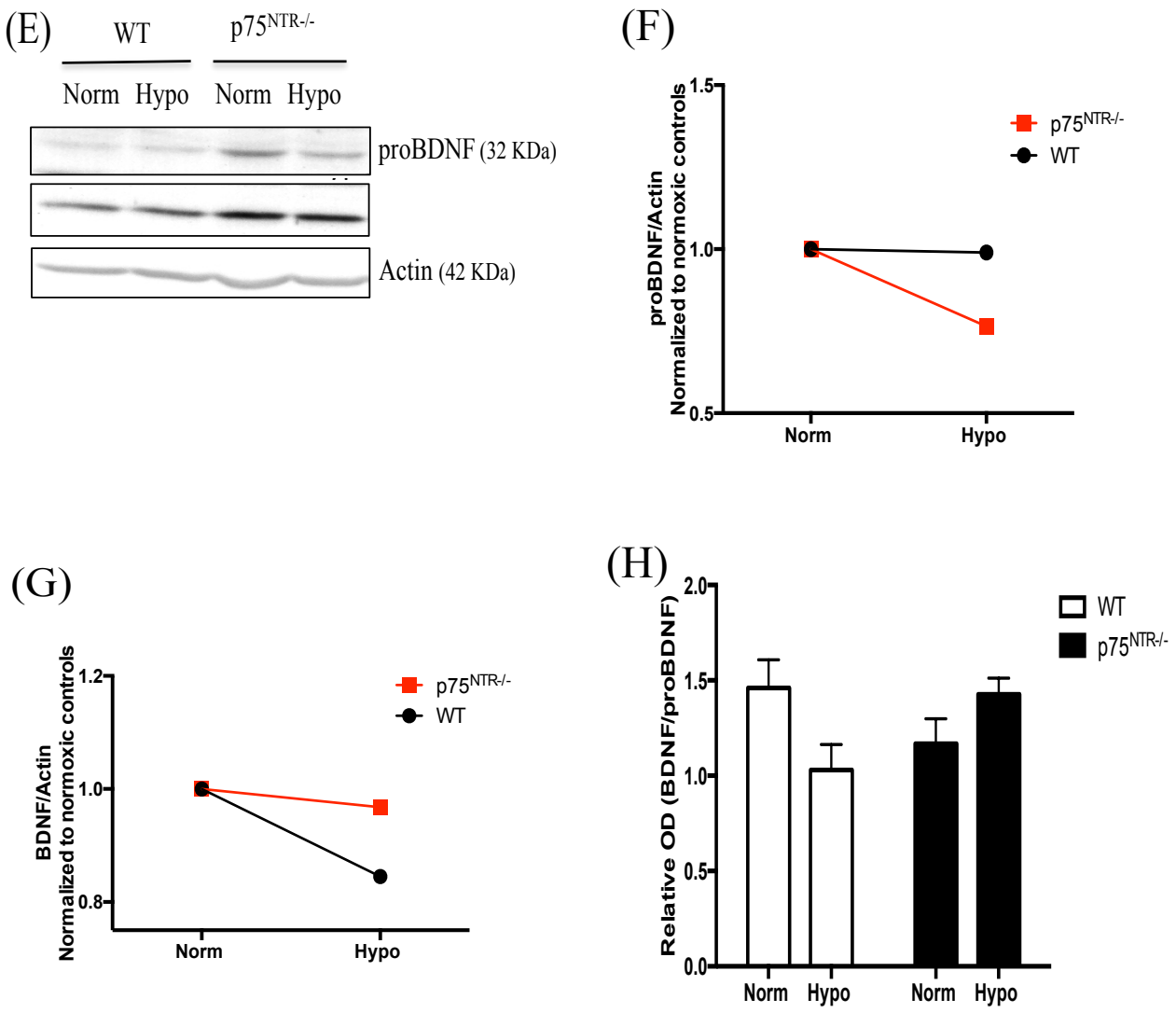

(E)
WT
p75NTR-/-
Norm Hypo Norm Hypo
proBDNF (32 KDa)
Actin (42 KDa)
(F)
proBDNF/Actin
Normalized to normoxic controls
1.5
1.0
0.5
Norm
Hypo
p75NTR-/-
WT
(G)
BDNF/Actin
Normalized to normoxic controls
1.2
1.0
0.8
Norm
Hypo
p75NTR-/-
WT
(H)
Relative OD (BDNF/proBDNF)
2.0
1.5
1.0
0.5
0.0
Norm Hypo Norm Hypo
WT
p75NTR-/-

Figure 2.5. Deletion of $p75^{NTR}$ attenuated hypoxia-induced increase in proNGF and decrease in NGF.

(A, B) Representative Western blotting and bar graph for proNGF showing significant increase in WT but not $p75^{NTR-/-}$ during hypoxia. (A, C) Representative Western blotting and bar graph for NGF showing significant increase in $p75^{NTR-/-}$ during hypoxia. (D) Bar graph of NGF/proNGF ratio showing significant decrease in WT during hypoxia but not $p75^{NTR-/-}$ pups (*, significant compared to WT normoxic group using two-way ANOVA, $p<0.05$, n=5-7). (E, F) Representative Western blotting and bar graph for proBDNF by p14 showing decreased level in $p75^{NTR-/-}$ pups during hypoxic stage of OIR. (E, G) Representative Western blotting and bar graph for BDNF expression by p14 showing preserved level in $p75^{NTR-/-}$ pups during hypoxic stage of OIR. (H) Bar graph of BDNF/proBDNF ratio showing decreasing trend in WT pups during hypoxia but not $p75^{NTR-/-}$ pups (n=4).

Figure 2.6 (A-D)

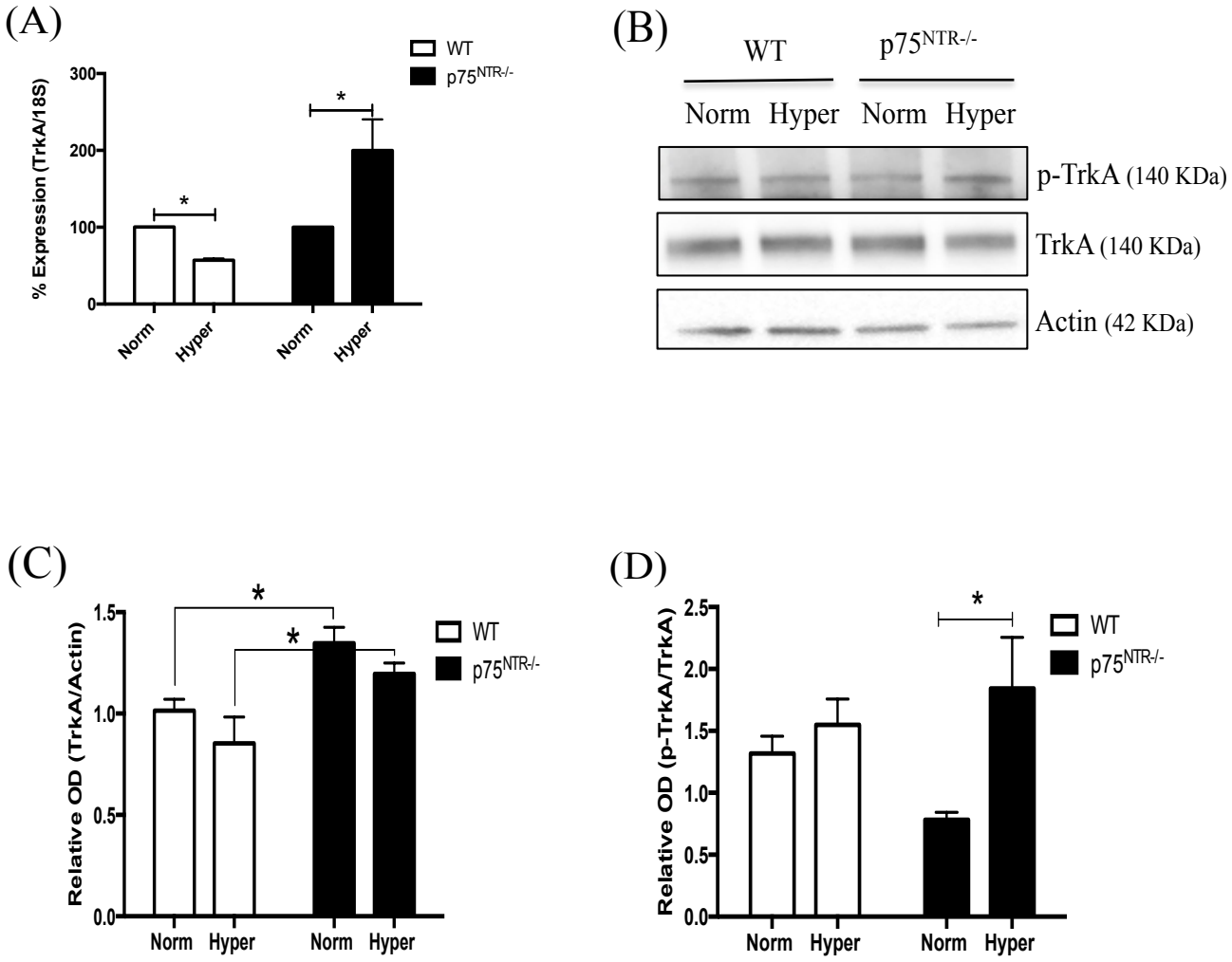

(A)
WT
p75NTR-/-
% Expression (TrkA/18S)
300
200
100
0
*
*
Norm
Hyper
Norm
Hyper
(B)
WT
p75NTR-/-
Norm
Hyper
Norm
Hyper
p-TrkA (140 KDa)
TrkA (140 KDa)
Actin (42 KDa)
(C)
Relative OD (TrkA/Actin)
1.5
1.0
0.5
0.0
*
*
WT
p75NTR-/-
Norm
Hyper
Norm
Hyper
(D)
Relative OD (p-TrkA/TrkA)
2.5
2.0
1.5
1.0
0.5
0.0
*
WT
p75NTR-/-
Norm
Hyper
Norm
Hyper

Figure 2.6 (E-H)

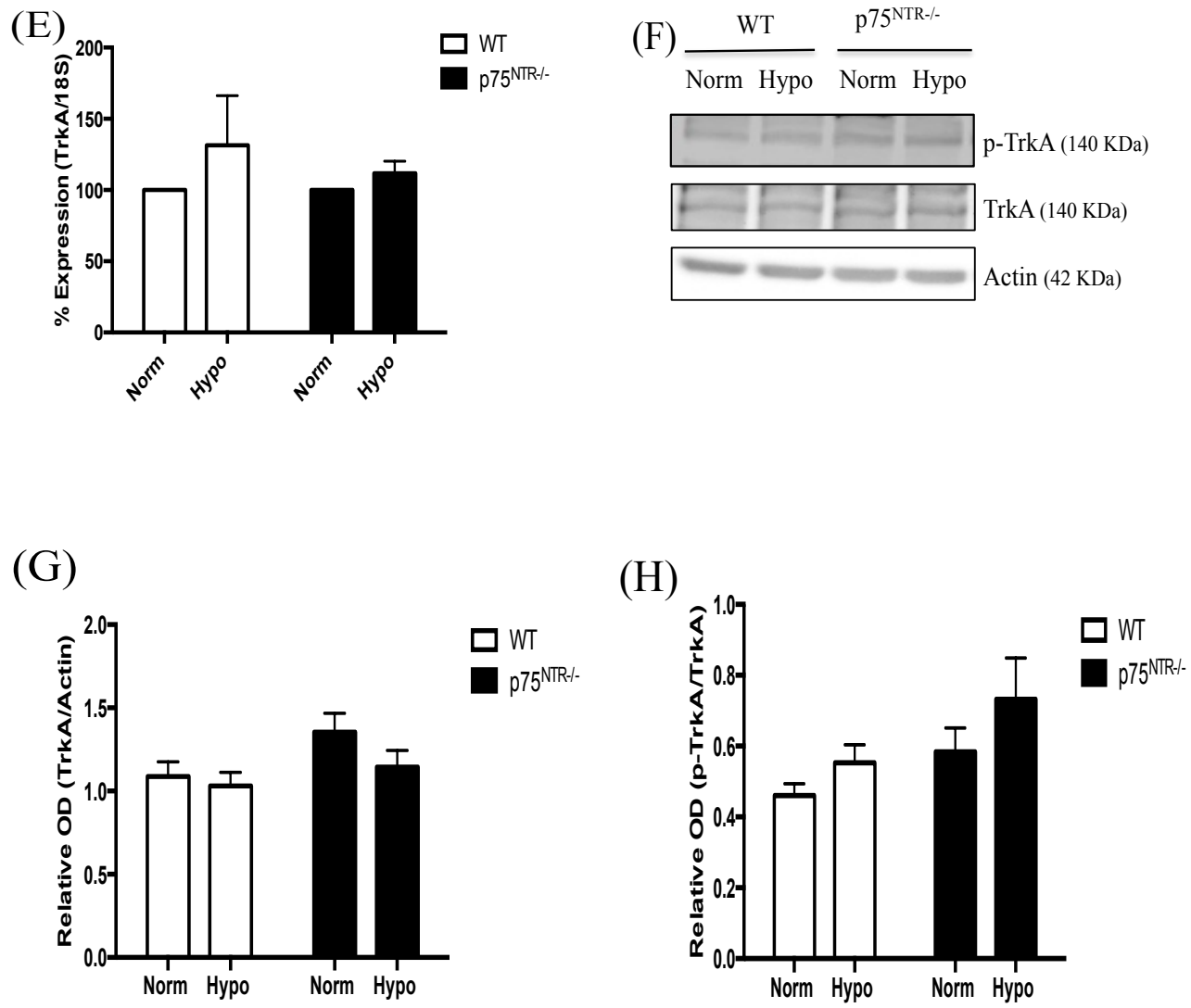

(E)
% Expression (TrkA/18S)
200
150
100
50
0
Norm
Hypo
Norm
Hypo
WT
p75NTR-/-
(F)
WT
p75NTR-/-
Norm Hypo Norm Hypo
p-TrkA (140 KDa)
TrkA (140 KDa)
Actin (42 KDa)
(G)
Relative OD (TrkA/Actin)
2.0
1.5
1.0
0.5
0.0
Norm Hypo Norm Hypo
WT
p75NTR-/-
(H)
Relative OD (p-TrkA/TrkA)
1.0
0.8
0.6
0.4
0.2
0.0
Norm Hypo Norm Hypo
WT
p75NTR-/-

Figure 2.6. Deletion of $p75^{NTR}$ prevented hyperoxia-decrease in TrkA expression and activation.

(A) Quantitative real-time PCR of TrkA gene expression in p12 WT and $p75^{NTR-/-}$ exposed to OIR showing significant decrease in WT but increase in $p75^{NTR-/-}$ (*, significant using unpaired T-test, $p<0.05$, n=6). (B, C) Representative western blotting and bar graph analysis of TrkA expression by p12 showing significant increase in TrkA expression in $p75^{NTR-/-}$ pups (*, significant using two-way ANOVA, $p<0.05$, n=6-9). (B, D) Representative western blotting and bar graph analysis of TrkA activation by p12 showing significant increase in TrkA activation in hyperoxic $p75^{NTR-/-}$ pups (*, significant using two-way ANOVA, $p<0.05$, n=6-9). (E) Quantitative real-time PCR of TrkA gene expression in p14 WT and $p75^{NTR-/-}$ exposed to OIR showing no significant difference in TrkA gene expression during hypoxic stage of OIR (n=3). (F, G) Representative western blotting and bar graph analysis of TrkA expression by p14 showing no alteration in TrkA expression in $p75^{NTR-/-}$ pups (n=6-9). (F, H) Representative Western blotting and bar graph analysis of TrkA activation by p14 showing an increasing trend in $p75^{NTR-/-}$ pups that was not significant (n=6-9).

Figure 2.7(A, B)

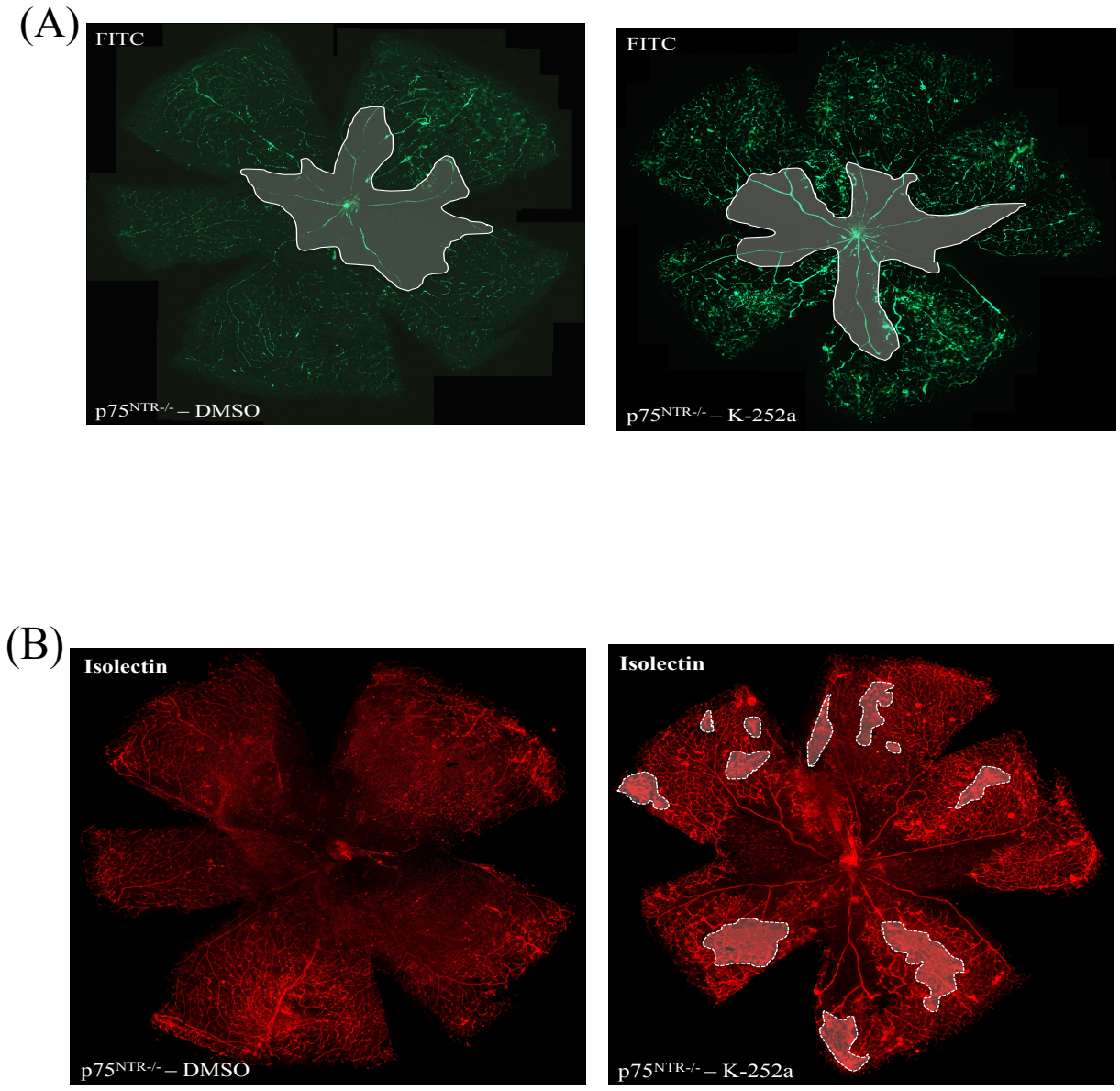

(A)
FITC
p75NTR-/- – DMSO
FITC
p75NTR-/- – K-252a
(B)
Isolectin
p75NTR-/- – DMSO
Isolectin
p75NTR-/- – K-252a

Figure 2.7(C-F)

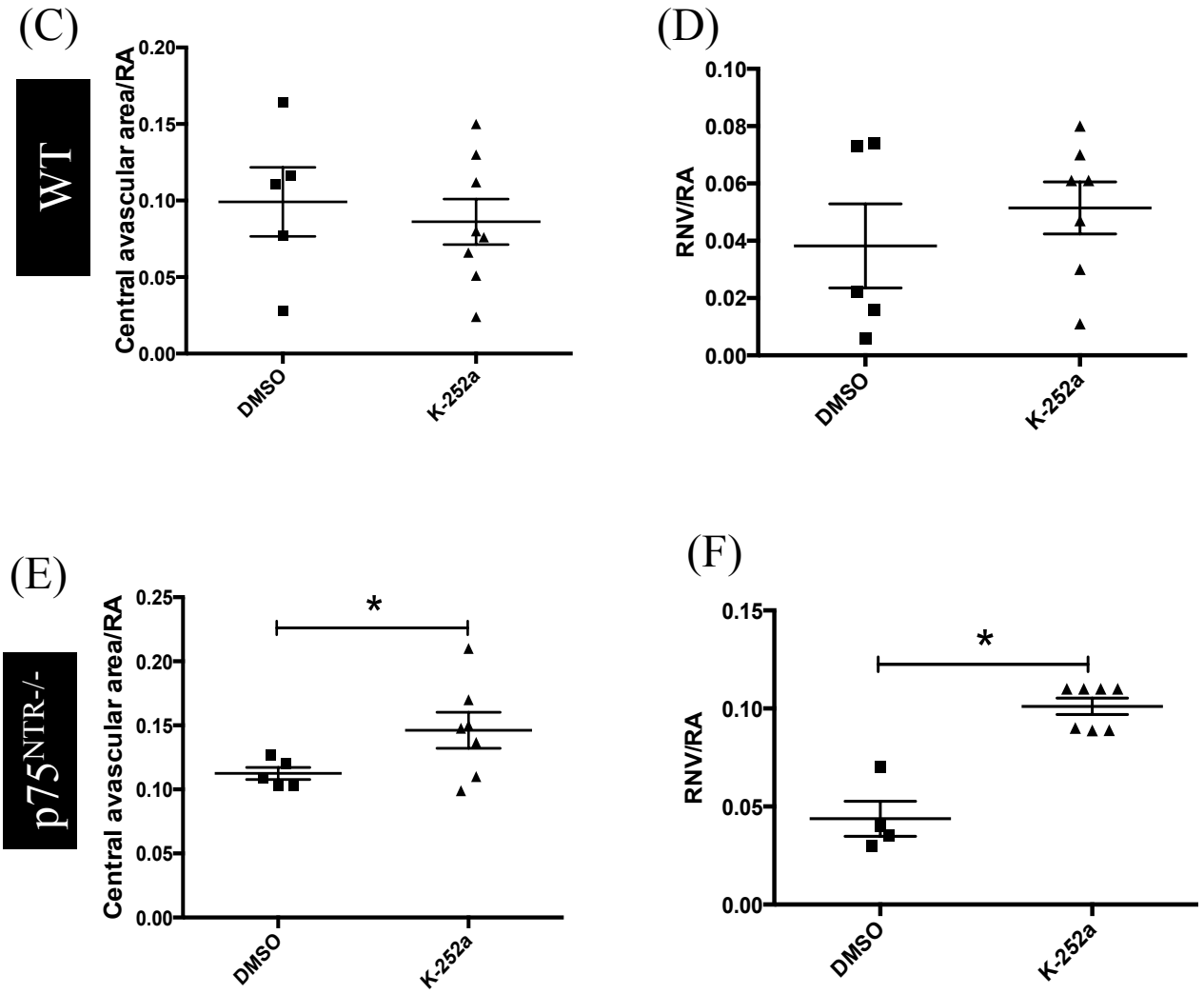

(C)
WT
Central avascular area/RA
0.20
0.15
0.10
0.05
0.00
DMSO
K-252a
(D)
RNV/RA
0.10
0.08
0.06
0.04
0.02
0.00
DMSO
K-252a
(E)
p75NTR-/-
Central avascular area/RA
0.25
0.20
0.15
0.10
0.05
0.00
*
DMSO
K-252a
(F)
RNV/RA
0.15
0.10
0.05
0.00
*
DMSO
K-252a

Figure 2.7. TrkA activity is required for the vasoprotective effects observed in $p75^{NTR-/-}$ but not WT.

(A) Statistical representation of central avascular area by p17 in WT pups receiving K252a or DMSO (0.5μg/μL/eye) showing no significant alteration (n=5-8). (B) Statistical representation of retinal neovascularization (RNV) in p17 WT pups receiving K-252a or DMSO (0.5μg/μL/eye) showing significant decrease in RNV upon DMSO or K-252a injection (*, significant using one-way ANOVA, $p<0.05$, n=5-7). (C) Statistical representation of central avascular area in p17 $p75^{NTR-/-}$ pups receiving K-252a or DMSO (0.5 μg/μL/eye) showing increased central avascular area upon K-252a injection (*, significant using one-way ANOVA, $p<0.05$, n=5-7). (D) Statistical representation of RNV in p17 $p75^{NTR-/-}$ pups receiving K-252a or DMSO (0.5μg/μL/eye) showing increased RNV upon K252a injection (*, significant using one-way ANOVA, $p<0.05$, n=4-7). (E) Representative FITC-perfused $p75^{NTR-/-}$ retinal flat mounts showing increased central avascular area by K252a injection (shaded area at the center, 5X magnification). (F) Representative Isolectin GS-stained $p75^{NTR-/-}$ retinal flat mounts showing increased RNV by K252a injection (encircled by dotted white line, 5X magnification).

Figure 2.8(A-D)

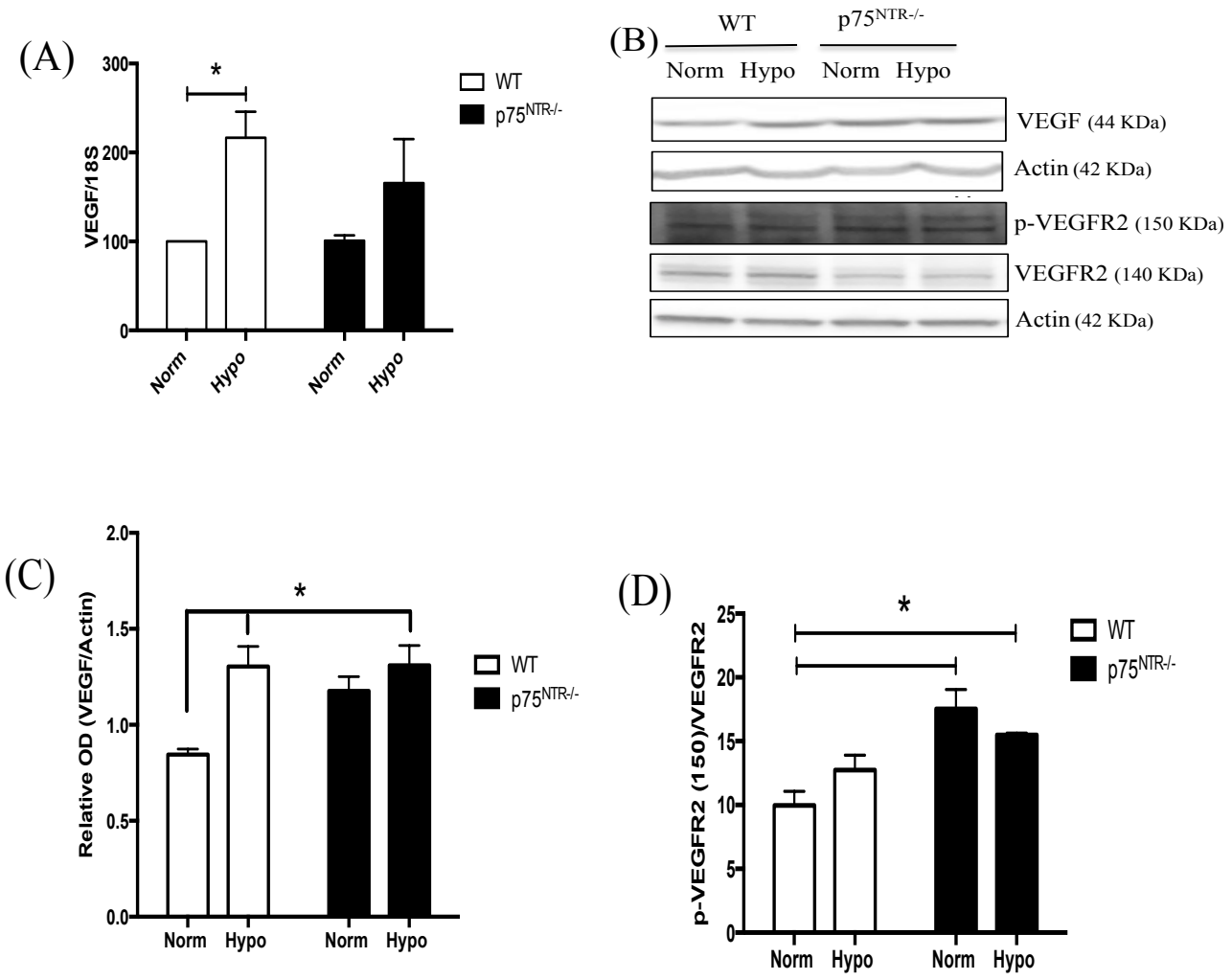
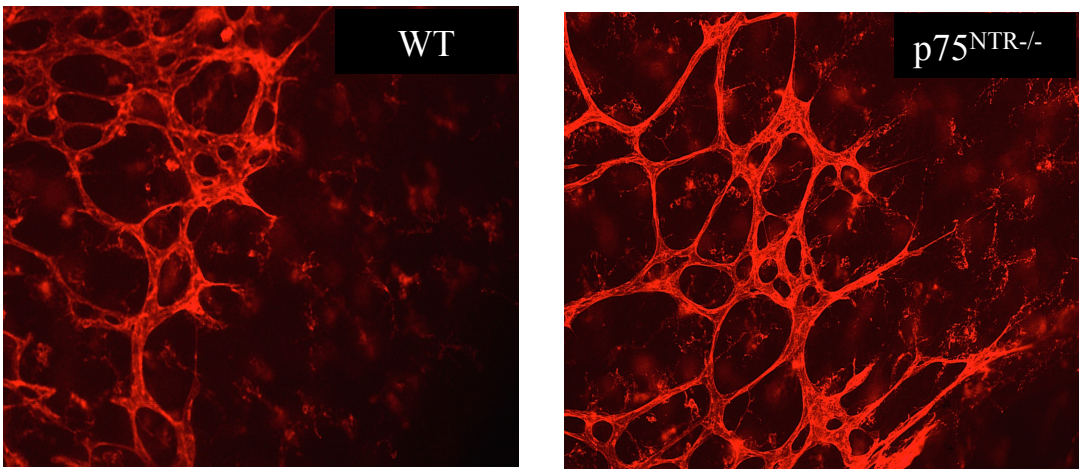

(A)
VEGF/18S
300
200
100
0
*
WT
p75NTR-/-
Norm
Hypo
Norm
Hypo
(B)
WT
p75NTR-/-
Norm Hypo Norm Hypo
VEGF (44 KDa)
Actin (42 KDa)
p-VEGFR2 (150 KDa)
VEGFR2 (140 KDa)
Actin (42 KDa)
(C)
Relative OD (VEGF/Actin)
2.0
1.5
1.0
0.5
0.0
*
WT
p75NTR-/-
Norm Hypo Norm Hypo
(D)
p-VEGFR2 (150)/VEGFR2
25
20
15
10
5
0
*
WT
p75NTR-/-
Norm Hypo Norm Hypo

Figure 2.8(E)

(E)



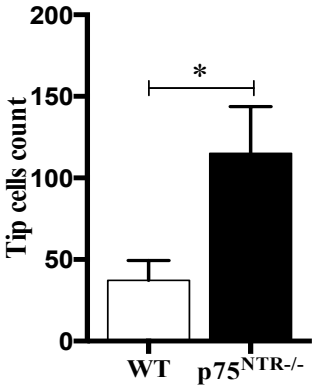
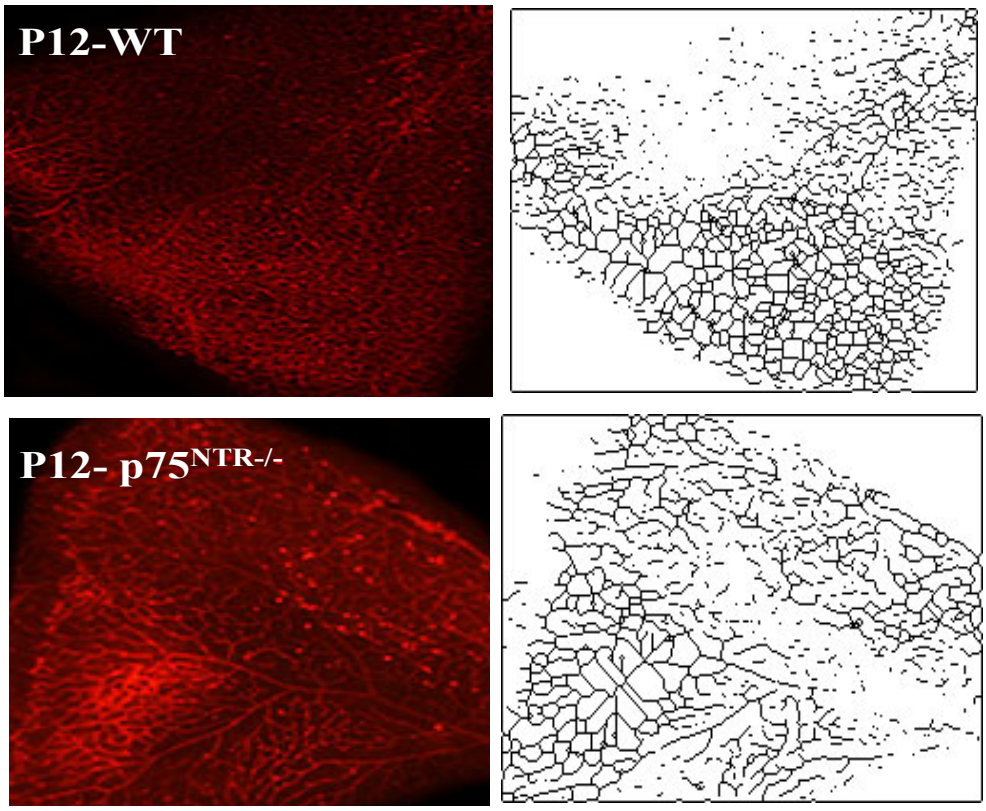


Figure 2.8. Deletion of $p75^{NTR}$ preserved hypoxia induced VEGF expression and VEGFR2 activation.

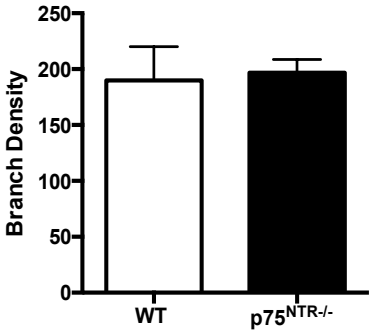
(A) Quantitative real-time PCR of VEGF gene expression for p14 WT and $p75^{NTR-/-}$ pups exposed to OIR (*, significant compared to WT normoxic group using two-way ANOVA, $p<0.05$, n=6). (B, C) Representative Western blotting and bar graph of VEGF protein expression in p14 WT and $p75^{NTR-/-}$ pups exposed to OIR (*, significant compared to WT normoxic group using two-way ANOVA, $p<0.05$, n=6-15). (B, D) Representative Western blotting and bar graph of VEGFR2 activation showing significant decrease in VEGFR2 expression but increases in VEGFR2 activation in $p75^{NTR-/-}$ pups (*, significant compared to WT normoxic group using two-way ANOVA, $p<0.05$, n=4-5). (E) Representative and bar graph of tip cells count in WT and $p75^{NTR-/-}$ p5 pups showing significantly higher number in $p75^{NTR-/-}$ pups. Retinal flat mounts were stained with Isolectin GS (20X magnification, *, significant using unpaired student T-test, $p<0.05$, n= 5-6).

Supplementary Figure 1 (A, B)

(A)

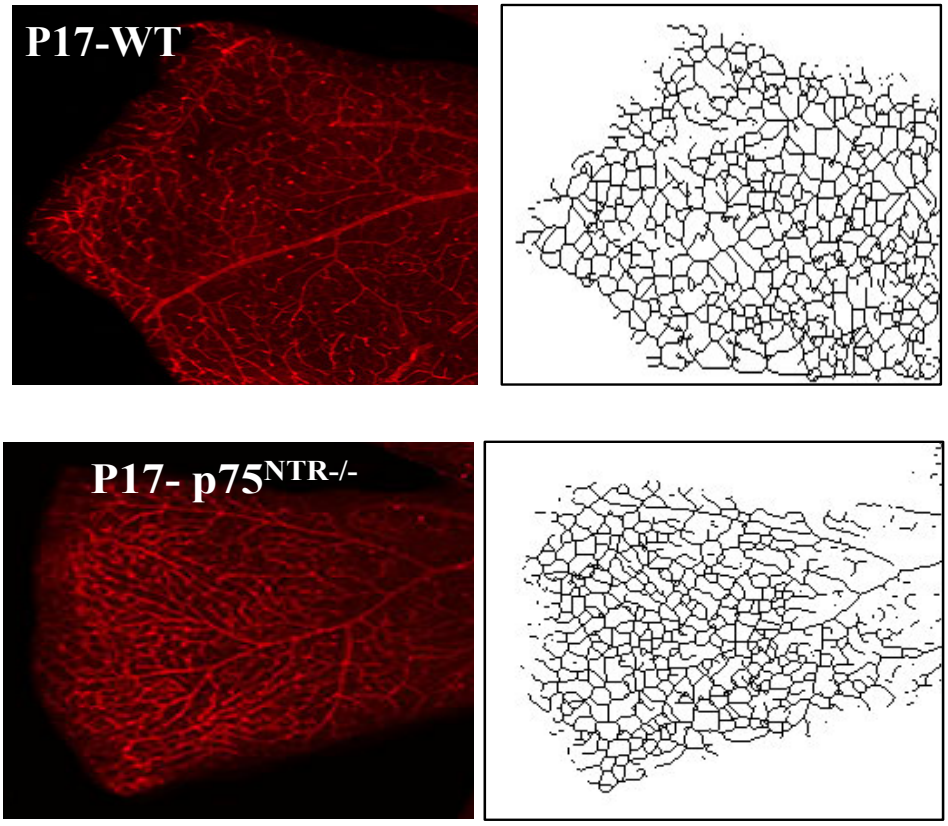


(B)

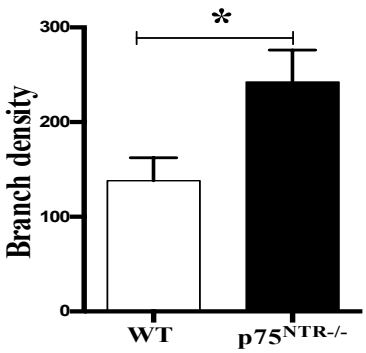


Supplementary Figure 2.1 (C, D)

(C)



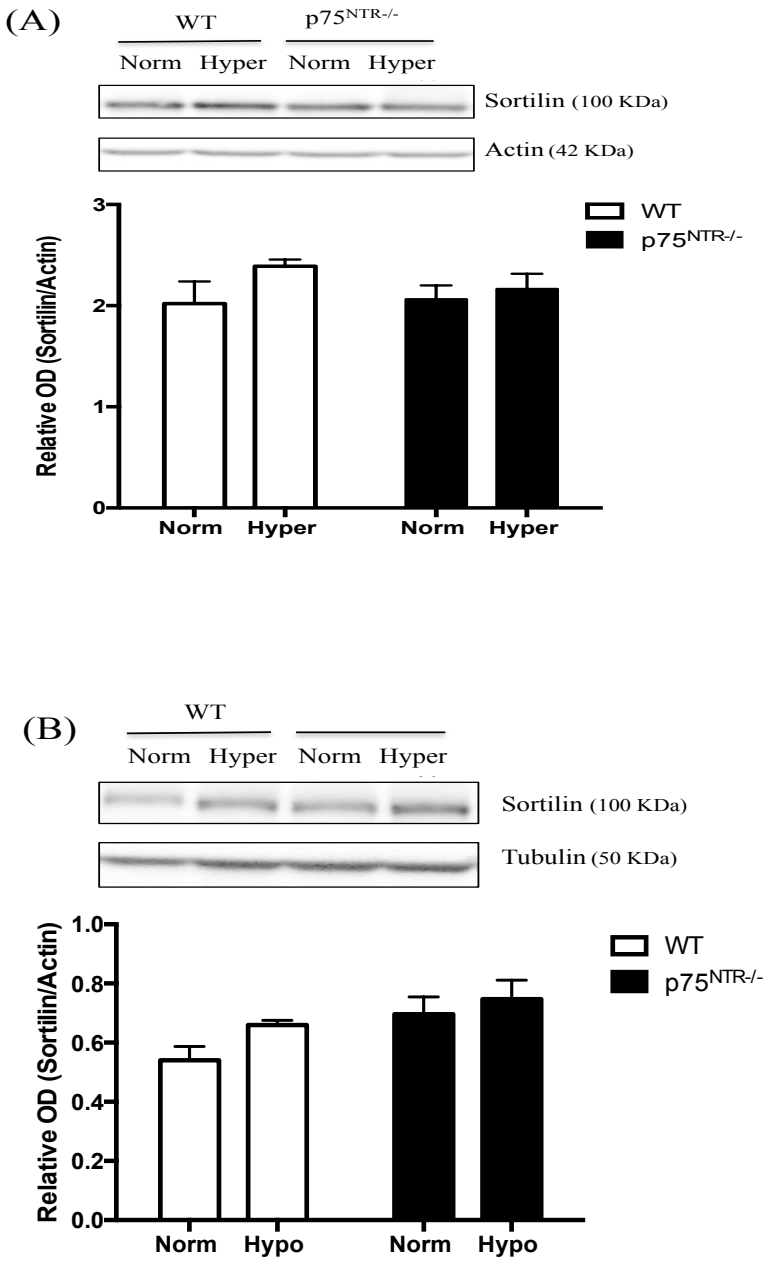
(D)



Supplementary Figure 2.1. Effect of deleting $p75^{NTR}$ on basal branching density of the retina.

(A, B) Representative Isolectin GS-stained retinal flat mounts, binary images and bar graph of branching density in WT and $p75^{NTR-/-}$ pups showing no significant alteration at p12 time point (n=4-5, 5X magnification). (C, D) Representative Isolectin GS-stained retinal flat mounts, binary images and bar graph of branching density in WT and $p75^{NTR-/-}$ pups showing significant increase at p17 time point (*, significant using unpaired student T-test, $p<0.05$, n=7-9).

Supplementary Figure 2.2



Supplementary Figure 2.2 Deletion of $p75^{NTR}$ receptor did not impact sortilin receptor expression during vaso-obliteration and neo-vascularization stages of OIR.

Representative Western blotting and bar graph analysis of sortilin expression in WT and $p75^{NTR-/-}$ pups during hyperoxic stage (A) and hypoxic stage (B) of OIR. Deletion of $p75^{NTR}$ receptor did not significantly alter sortilin expression during both stages (n=4).

Table 1. Primer sequences

Gene-mouse	Forward primer	Reverse primer
TrkA	5`-TGGGCAGAGAATGATGTGGG-3`	5`-CGAGAAGGGGATGCACCAAT-3`
VEGF	5`-CACGACAGAAGGAGAGCAGAA-3`	5`-CGCTGGTAGACGTCCATG-3`
NGF	5`-AGGCCCATGGTACAATCCCTTTCA-3`	5`-ATCTCCAACCCACACACTGACACT-3`
18S	5`-CGCGGTTCTATTTTGTTGGT-3`	5`-AGTCGGCATCGTTTATGGTC-3`

CHAPTER 4

MODULATION OF P75^{NTR} ON MESENCHYMAL STEM CELLS INCREASES THEIR VASCULAR PROTECTION IN RETINAL ISCHEMIA-REPERFUSION MOUSE MODEL. [1]

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[1] Sally L. Elshaer, William D. Hill and Azza B. El-Remessy
To be submitted to Journal of Stem cells.

Abstract

Proliferative diabetic retinopathy (PDR) is characterized by accelerated cell death, impaired vascular repair and pathological retinal neovascularization (RNV). Mesenchymal stem cells (MSCs) are promising therapy to improve vascular repair, yet their role in PDR is not fully understood. We have shown that genetic deletion of neurotrophin receptor; $p75^{NTR}$ enhanced vascular repair and ameliorated RNV in ischemic retinopathy model. Furthermore, its deletion preserved the expression of angiogenic markers; SDF-1, CXCR-4 and CXCR-7 responsible for MSCs homing to vasculature in diabetic ischemic limbs. The aim of this study is to investigate the contribution of MSCs to vascular protection associated with $p75^{NTR}$ deletion in ischemic retina. **Methods** : WT and $p75^{-/-}$ mice were subjected to ischemia reperfusion (I/R) injury by increasing intra-ocular pressure to 120 mmHg for 45-60 minutes followed by perfusion. Murine GFP-labeled MSCs (100,000 cells/eye) were injected intravitreally 2 days-post-surgery and vascular homing was assessed 1-week later. Acellular capillaries were counted using trypsin digest 10-days post IR. In vitro, MSCs were treated with a specific pharmacological inhibitor against $p75^{NTR}$ receptor (LM11A-31, 200 nM, Stanford University) and conditioned media was co-cultured with human retinal endothelial (HREs) to examine the angiogenic response. **Results**: I/R increased number of acellular capillaries (~ 2.3 fold) in WT mice, but not in p75KO compared to sham-controls. GFP-MSCs showed better incorporation into retinal vasculature in p75KO mice compared to WT. Administration of MSCs decreased number of acellular capillaries in all groups (~ 2.3-fold decrease in WT-sham, 2.7-fold decrease in WT-IR, 3-fold decrease in KO-Sham and 2.5 fold decrease in KO-IR). In vitro, inhibition $p75^{NTR}$ in MSCs enhanced the angiogenic response of their conditional media in HREs, where HREs showed enhanced migration (~ 1.3 fold) and tube formation (~ 2.7 fold) compared to controls.

Conclusions: Our results showed that deletion of $p75^{NTR}$ protects against retinal ischemia by mechanisms that partially involve improved MSCs homing to ischemic vasculature to prevent vascular degeneration and support newly grown blood vessels. Thus, combination of MSCs injection and $p75^{NTR}$ inhibitor can serve as potential therapeutic strategy to harness vascular repair in ischemic retinopathy diseases.

Introduction

Vision loss associated with ischemic diseases such as retinopathy of prematurity and diabetic retinopathy are often due to abnormal blood vessel growth, often in response to retinal ischemia [1]. Significant progress has been made in the development of compounds that combat abnormal vascular proliferation. Laser photocoagulation as well as anti-angiogenic compounds including VEGF inhibitors, angiostatic steroids, integrin antagonists and others have been developed [2-5]. Nevertheless, current approaches fail to treat ischemic areas with large degree of tissue injury in addition to increased risk of local and systemic complications [6]. Moreover, hypoxia that stimulates the observed mal-functional vascular growth still not resolved. Therefore, there is urgent need to develop new therapeutic alternatives to combat retinal ischemia and thus diminish subsequent destructive angiogenesis.

Stem cells have been shown to be an attractive candidate for retinal regeneration [7]. Among, embryonic stem cells, neural stem cells, retinal stem cells and adult stem cells, Mesenchymal stem cells (MSCs) gained big interest because they can be obtained from the patients' bone marrow in quantities appropriate for clinical application. MSCs are multipotent stem cells present in adult marrow and have the potential to differentiate into lineages of mesenchymal tissues, including bone, cartilage, fat, tendon, muscle and marrow stroma [8]. Several studies have shown that MSCs differentiate into retinal neurons in vivo and in vitro [9].

Animal studies have also demonstrated that subretinal transplantation of MSCs delays retinal degeneration and preserves retinal function [10]. Nevertheless, vascular protection of MSCs transplantation in ischemic retina has not been fully elucidated.

The $p75^{NTR}$ has been described for its signaling role as a common receptor for all neurotrophins along with their precursor forms, as such, $p75^{NTR}$ receptor can exert multiple functions according to cell context [11,12]. It is mainly implicated in regulation of cell death and its role is ischemic vascular diseases, including retinal diseases, is extensively demonstrated (as reviewed in [13]. Among increasing prospective stem cells markers, $p75^{NTR}$ receptor, also known as CD271, enriches several progenitor/stem cells subtypes [14]. $p75^{NTR}$ was first identified as a genuine neural crest stem cell marker [15]. Since then, it has been widely used to isolate putative stem cells from neural crest derived tissues. MSCs were stained positive for $p75^{NTR}$ as early as 1988 in a study done by Thomson et al [16]. $p75^{NTR}$ was shown to directly inhibit differentiation of MSCS into multiple cell types through inhibition of transcription factors, including Runx2 and OSX, which are essential for osteoblast differentiation and for expression of chondrogenesis marker Sox9 and the myogenic marker Myf5 as reported by Mikami et al [17]. Nonetheless, $p75^{NTR}$ expression and function in vivo as well as its underlying mechanisms in MSCs biology, have not been sufficiently addressed. In the current study, we tried to evaluate the role of pharmacological inhibition of $p75^{NTR}$ on the surface of MSCs in increasing their vascular homing and repair in ischemic retina using ischemia-reperfusion (I/R) mouse model.

Materials and Methods

Animals

All animal experiments were conducted in agreement with Association for Research in Vision and Ophthalmology statement for use of animals in ophthalmic and vision research, and

Charlie Norwood VA Medical Center Animal Care and Use Committee (ACORP#16–01–088). The $p75^{NTR}$, B6.129S4Ngfrtm1Jae /J ($p75^{NTR-/-,}$ exon III knockout mice [18] were obtained from Jackson Laboratories (Bar Harbor, Maine, USA) and crossed with C57BL6-J mice (Jackson Laboratories). These mice were crossed and back-crossed to establish a colony of homozygous $p75^{NTR-/-}$ and WT breeders that produced the mice used in the current study.

Retinal ischemia-reperfusion (I/R).

For surgeries, mice were anesthetized with intraperitoneal ketamine (50 mg/kg; Hospira, Inc., Lake Forest, IL, USA) and xylazine (10 mg/kg; Akorn, Decatur, IL, USA). Retinal ischemia-reperfusion was performed as described previously by Zheng et al. [19]. Briefly, pupils were dilated with 1% Atropine Sulfate (Akorn, Inc., Lake Forest, IL). The anterior chamber was cannulated with a 32-gauge needle (company) attached to a line from a saline reservoir at a height calibrated to yield 120 mmHg. The IOP was elevated to 120 mm Hg for 45-60 minutes; I/R injury and choroidal non-perfusion was evident by whitening of the anterior segment of the globe and blanching of the episcleral veins [20]. During infusion, topical anesthesia (0.5% tetracaine HCL) was applied to the cornea. After ischemia, the needle was immediately withdrawn, allowing for rapid reperfusion; IOP was normalized, and reflow of the retinal vasculature was confirmed by observation of the episcleral veins. Topical antibiotic (Neo-Plycin, Perrigo) was applied to the cornea to minimize infection. I/R injury was performed in one eye with the other undergoing sham surgery, in which the needle was inserted into the anterior chamber without elevating the IOP. Mice were killed 10 days post I/R and eyes were processed as described below.

Mesenchymal stem cells (MSCs) culture

GFP-labeled mouse MSCs were obtained as a kind gift from Dr. William D. Hill (Augusta University) [21] and used between passages 5-9. MSCs cells were cultured in high glucose DMEM (4.5 g/L D-glucose, Life technologies^{TM}) supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C and 5% CO_2.

Intravitreal injection of MSCs.

Mice were anesthetized by intraperitoneal injection of ketamine (237.7 g/mol, 100 mg/ml)-xylazine (220.3 g/mol, 100 mg/ml) mixture and complete anesthesia was confirmed by loss of reflex to sharp paw pinch. MSCs (100,000 cells/ 2 μL sterile PBS/ eye) were injected intravitreally 48 hours after I/R using a Hamilton syringe with 32 gauge glass capillary.

Vascular localization of MSCs

7-days post intravitreal injection of MSCs, mice were euthanized in CO_2 chamber (2% flow rate for 5 min) followed by cervical dislocation. Eyes were enucleated and fixed in 2% paraformaldehyde overnight. Retinas were dissected and permealized for 15 minutes with 0.3% Triton X-PBS then stained overnight at 4°C with isolectin GS; biotinylated griffonia (bandeiraea) simplicifolia lectin I (GSL I, BSL I), (Vector Labs, CA, USA; 1% in 5% normal goat serum in 0.3% Triton X-PBS) followed by incubation with secondary antibody; Texas red® avidin D (Vector labs, CA, USA; 0.5% in 5% normal goat serum in 0.3% Triton X-PBS). Lectin-stained retinas were flat-mounted onto Superfrost/Plus microscope slides (Fisher Scientific, MA, USA) with the photoreceptor side facing down and imbedded in Vectashield mounting media for fluorescence (Vector Labs, CA, USA). Slides were photo-micrographed at 20X using a Zeiss AxioObserver.Z1. For biochemical assays, separate I/R and intra-vitreal injection were

performed and retinas were isolated and snap frozen for Western blotting and quantitative PCR analysis.

Isolation of retinal vasculature and determination of acellular capillaries.

The retinal vasculature was isolated as described previously [22]. Freshly enucleated eyes were fixed with 2% paraformaldehyde overnight. Retinal cups were dissected, washed in phosphate-buffered saline, then incubated with 3% Difco-trypsin 250 (BD Biosciences, San Jose, CA) in 25 mmol/l Tris buffer, pH 8, at 37 °C for 2 hours. Vitreous and nonvascular cells were gently removed from the vasculature, which was soaked in several washes of 0.5% Triton X-100 to remove the neuronal retina. Trypsin-digested retinas were stained with periodic acid–Schiff and hematoxylin. Numbers of acellular capillaries were quantified in six different areas of the mid-retina under the microscope (×20) in a masked manner by two different researchers. Acellular capillaries were identified as capillary-sized blood vessel tubes with no nuclei along their length.

Transfection of MSCs

Knocking down $p75^{NTR}$ expression was performed according to manufacturer's instructions (Santa Cruz). Briefly, MSCs were shifted to antibiotic-free medium over night when 60-80% confluent. Cells were transfected with Si-RNA against $p75^{NTR}$ receptor (sc-37268) or scrambled (sc-37007) with the aid of lipofectamine transfection reagent (Santa Cruz, sc-29528) for 6 hours. DMEM medium containing 2X of FBS and antibiotic was added on top of pure transfection medium for an extra 12 hours. Next, transfection medium was removed and cells were allowed to recover in 1X DMEM medium for 6 hours. For in vivo studies, cells were collected in sterile PBS (50,000 cells/uL) for intra-vitreal injection 48 hours after IR. For

biochemical assays, conditional medium was collected as well as cell lysates using mirVANA^{TM} PARIS^{TM} Kit (Ambion), according to manufacturer's instructions.

HRECs cultures and in vitro angiogenic assays.

MSCs cultures were treated with pharmacological inhibitors against p75^{NTR} when 80-90% confluent. 2 different inhibitors were used; LM11A-31 (200 nM, kind gift from Dr. Frank Longo, Stanford University) and compound-A (20 uM, kind gift from Dr. Uri Saragovi, McGill University). At 6 or 12 hours after treatment, conditional media was collected as well as cell lysates using mirVANA^{TM} PARIS^{TM} Kit (Ambion), according to manufacturer's instructions. Conditional medium was used for in vitro angiogenic assays of human retinal endothelial cell (HRECs). All HRECs studies were in accordance with Association for Research in Vision and Ophthalmology and Charlie Norwood Veterans Affairs Medical Center, research and ethics committee. HREs and supplies were purchased from Cell Systems Corporations (Kirkland, WA) and VEC Technology (Rensselaer, NY) as described previously [23].

Cell Migration assay

HRECs were grown to confluence and then were wounded with a single sterile cell scraper of constant diameter as described previously [24]. Cells were treated with conditional medium of treated MSCs (10uL/mL SFM) with VEGF (20 ng/mL) serving as positive control. Images of wounded areas were taken immediately after adding the treatment and after 18 hours using a Zeiss AxioObserver.Z1and % cell migration was calculated. Each condition was verified in triplicate and was repeated using independent cultures.

Tube formation assay

Tube formation assay was performed using growth factor-reduced Matrigel (BD Biosciences) as described previously [24,25]. HRECs were counted and plated at 50,000 cells

with Matrigel and conditional medium of treated MSCs in a ratio of (50:25:25) in a 96-well plate. VEGF (20 ng/mL) was used as positive control. 18 hours later, images of the tube-like structures were captured and analyzed using a Zeiss AxioObserver.Z1. Each condition was verified in triplicate and was repeated using independent cultures.

Western blotting

Cell lysates were processed using (mirVANA^{TM} PARIS^{TM} Kit) and briefly homogenized. Lysates were centrifuged and 35-50 µg were resolved on an SDS-PAGE gel (4-20% gradient Tris glycine precast gel, BioRad) and electro-blotted to nitrocellulose membranes (BioRad). Membranes were blocked with 5% milk or BSA in PBS-tween and incubated overnight in 4°C with the following primary antibodies: p75^{NTR} (kind gift from Dr. Bruce Carter, Department of Biochemistry, Vanderbilt University), CXCR-7 (# 106-117,Thermo fisher) then reprobed with the primary antibodies for the house-keeping protein; Actin to confirm equal loading. Membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-mouse or HRP-conjugated antirabbit secondary antibodies (Millipore) for 2 h at room temperature. The films were scanned with FluorChem™ FC3 (protein simple) and the band intensity was quantified using densitometry software version (Fiji) and expressed as relative optical density (OD).

Real-time quantitative PCR.

Cell lysates were processed using (mirVANA^{TM} PARIS^{TM} Kit) and RNA was purified and quantified as described by the manufacturer's instructions. A one-step quantitative RT-PCR kit (Invitrogen) was used to amplify 10ng retinal mRNA as described previously {Al-Gayyar, 2011 #2;Ali, 2008 #3}. PCR primers (listed in Table-1) were obtained from Integrated DNA Technologies (Coralville, IA, USA). Quantitative PCR was conducted using StepOnePlus qPCR system (Applied BioSystems, Life Technologies). The percent expression of various genes

($p75^{NTR}$, CXCR-4, CXCR-7) was normalized to 18S and expressed relative to WT sham controls.

Statistical analysis.

All the data are expressed as mean ± SEM. Differences between 2 groups were detected using un-paired Student T-test. One-way ANOVA was used to assess significant differences between 3 groups. Two-way ANOVA was used to assess interaction between two variables; 2 genes (WT vs. $p75^{NTR-/-}$) X I/R exposure (IR vs Sham). Tukey-Kramer post-multiple comparisons was used for significant interactions among various groups. Significance for all tests was determined at $\alpha = 0.05$, Graphpad Prism, Ver.6.

Results

MSCs localized to ischemic vasculature, one week post intra-vitreal injection in WT eyes subjected to ischemia-reperfusion

Isolectin GS-stained retinal flat mounts showed vascular incorporation of GFP-labeled MSCs, one-week post intravitreal injection in ischemic eyes (Fig. 1, lower panel). Earlier time point; 3 days post intravitreal injection did not show any vascular incorporation of MSCs (Fig. 1, upper panel).

Deletion of $p75^{NTR}$ decreased number of acellular capillaries, an effect enhanced by MSCs injection.

I/R results in degeneration of retinal capillaries identified by presence of acellular capillaries. Morphologically, degenerated capillaries in retinal I/R seem comparable to acellular capillaries found in diabetic retinopathy. Degenerated, acellular capillaries are not perfused, and are believed to represent a discrete event that progressively contributes to the development of retinal ischemia, and ultimately, to neovascularization [26,27]. Retinas from rodents after I/R

show biochemical alterations that also are seen in diabetes, including increased expression of iNOS [28] and ICAM-1 [29] and activation of caspase-1 [30]. Based on this similarity between I/R and diabetic retinopathy, it was postulated that retinal I/R model may be used as an acute model for screening therapeutic approaches to inhibit capillary degeneration in diabetic retinopathy and other retinopathies [31].

Trypsin-digested retinas showed that I/R resulted in significant increase in number of acellular capillaries (3-Fold) in WT mice compared to their sham-operated controls (Figure 2 A, C). Deletion of $p75^{NTR}$ receptor reduced number of acellular capillaries by 25% compared to ischemic WT retinas that did not reach statistical significance (Figure 2 A, C). MSCs showed enhanced vascular protection in all groups where MSCs injection decreased number of acellular capillaries in WT mice by 40% and 54% in sham-operated and IR-operated groups, respectively (Figure 2 B, C). Nevertheless, number of acellular capillaries was still significantly higher (2-Folds) in IR-operated WT compared to their sham-operated after injection of MSCs (Figure 2C). On the other hand, injection of MSCs additionally protected retinal vasculature against ischemia in $p75^{NTR-/-}$ mice where number of acellular capillaries was decreased by 60% compared to non-inj $p75^{NTR-/-}$ ischemic retinas (Figure 2 B, C).

Knocking down the expression of $p75^{NTR}$ receptor in MSCs increased vascular incorporation into WT ischemic retinas.

MSCs cultures were treated with different concentrations of Si-RNA against $p75^{NTR}$; 100, 200, 300 and 500 nM where both 100 and 300 nM showed significant decrease in $p75^{NTR}$ mRNA with no significant difference in mRNA of $p75^{NTR}$ observed between 100 and 300 nM, thus 100 nM concentration was selected for further studies (Figure 3A, data not shown for 200 and 500 nM concentrations). Next, we confirmed down-regulation of $p75^{NTR}$ protein in MSCs lysates,

where 100 nM Si-RNA against $p75^{NTR}$ resulted in 30% decrease (Figure 3B). To assess change in vascular homing upon knocking-down $p75^{NTR}$ expression, Scr- or Si-100-treated GFP-labeled MSCs were injected intravitreally, 2 days post I/R injury in WT mice. Isolectin GS-stained retinal flat mounts showed enhanced vascular incorpotation of GFP-labeled MSCs after knocking down $p75^{NTR}$ expression on their surface (Figure 3C).

Genetic deletion of $p75^{NTR}$ enhanced SDF-1α/CXCR-4 & -7 signaling axis

Knocking down the expression of $p75^{NTR}$ on MSCs was shown to increase mRNA expression of SDF-1α receptors; CXCR-4 by 30% (Figure 4A) and mRNA and protein expression of CXCR-7 (Figure 4B, C) by 50%, yet the increase did not reach statistical significance. On the other hand, lysates of I/R-operated WT retinas showed 30% decrease in mRNA expression of CXCR-7 (Figure 4C). Deletion of $p75^{NTR}$ did not alter basal gene expression of CXCR-7 in retinal lysates, meanwhile, its deletion preserved the gene expression of CXCR-7 in I/R-operated eyes (Figure 4D).

Pharmacological inhibition of MSCs-$p75^{NTR}$ increased angiogenic behavior of their conditional media on endothelial cells.

Conditional medium of MSCs treated with pharmacological inhibitors against $p75^{NTR}$ for 6 hours significantly increased percentage of HRECs migration by 30% compared to non-treated HRECs cultures (Figure 5A (upper panel), B (open bars)). Conditional medium of MSCs treated with pharmacological inhibitors against $p75^{NTR}$ for 12 hours tended to increase percentage of HRECs migration, yet the increase did not reach statistical significance (Figure 5A (lower panel), B (closed bars)). Tube formation showed similar results where, HRECs cultures tended to form higher number of tubes when subjected to conditional medium of MSCs treated with

pharmacological inhibitors against $p75^{NTR}$ receptor at both 6 hours (Figure 5C (upper panel), D (open bars)) and 12 hours (Figure 5C (lower panel), D (closed bars)) treatment.

Discussion

The main findings of the current study are: 1) Deletion of $p75^{NTR}$ receptor protected against retinal ischemia, 2) increased vascular homing of MSCs and 3) increased paracrine angiogenic behavior in endothelial cells, 4) through SDF-1/CXCR-4&7 axis. Retinal ischemia is a common underlying pathology for multiple retinal diseases including; acute closed-angle glaucoma [32] and diabetic retinopathy [33]. One of the most frequently used models for the investigation of molecular mechanisms and potential therapeutic strategies for retinal ischemia has been a rodent model of acute elevation of intraocular pressure (IOP) followed by reperfusion; I/R [19]. The surrogate marker for retinal ischemia is degenerated capillaries; acellular capillaries.

We have seen significant increase in acellular capillaries formation in WT mice exposed to I/R (Figure 2). There are several possibilities for the cause of the capillary degeneration seen in I/R model. First, the release of toxic factors or loss of trophic factors from the damaged neuroglial cells of the retina may contribute to capillary degeneration. Second, activated leukocytes or other inflammatory cells such as infiltrated macrophages may stimulate cell death signaling in capillary cells, leading to capillary degeneration. Third, capillary degeneration may be due to failure of endothelial hematopoietic stem cells to maintain normal retinal vasculature or to repair damaged vasculature. Finally, direct damage to the vasculature as result of thrombosis, pressure-induced damage, or reactive oxygen species all could contribute to capillary degeneration in this I/R model [31]. Genetic deletion of $p75^{NTR}$ receptor ameliorated number of acellular capillaries (Figure 2) that was not statistically significant compared to ischemic WT

retinas. $P75^{NTR}$ is a main regulator of vascular cell death and it was shown, elsewhere, to contribute to retinal ischemia where it upregulated, in addition to its co-receptor sortilin at 3, 5 and 7 days after retinal ischemia [34].

Intravitreal injection of MSCs into ischemic retinas showed maximum vascular homing and integration one-week post injection (Figure 1) and exerted additional vascular protection in both WT and $p75^{NTR-/-}$ mice subjected to I/R (Figure 2). To our knowledge, this is the first report examining vascular protection of MSCs in ischemic retina. Meanwhile, neuroprotection has been described elsewhere. Li et al., reported their neuroprotection in ischemic rat retina subjected to I/R [35], where 2-4 weeks after transplantation, MSCs expressed markers of neurons; neurone specific enolase, neurofilament and various neurotrophic factors. The MSCs-injected I/R rat eyes experienced preserved number of RGCs compared to PBS-injected controls [35]. Another study showed that, transplantation of bone marrow-derived MSCs rescued photoreceptor cells in dystrophic retina of a mouse model of retinitis pigmentosa [36].

To further dissect the role of $p75^{NTR}$ receptor on the surface of MSCs, we aimed at transiently knock down its expression on MSCs and examine vascular consequences in ischemic retinas. $p75^{NTR}$ receptor, also known as CD271, enriches several progenitor/stem cells subtypes including MSCs [14]. As shown in Figure 3, we transiently knocked down MSCs-$p75^{NTR}$ expression by ~30% on both mRNA (Figure 3A) and protein (Figure 3B) levels. Previously, $p75^{NTR}$ was shown to be involved in MSCs differentiation where expression of $p75^{NTR}$ was shown to rapidly down-regulate upon differentiation of MSCs in vitro [37]. Moreover, $p75^{NTR}$ was shown to directly inhibit differentiation of MSCS into multiple cell types through inhibition of transcription factors including, Runx2 and OSX, which are essential for osteoblast differentiation and for expression of chondrogenesis marker, Sox9 and the myogenic marker,

Myf5 as reported by Mikami et al [17]. We are the first to report increased vascular homing of MSCs upon knocking down $p75^{NTR}$ on their surface (Figure 3C). Moreover, pharmacological inhibition of $p75^{NTR}$ on the surface of MSCs increased the paracrine angiogenic behavior of their conditional media on endothelial cells in vitro (Figure 5 A, B). Further studies are warranted to assess effect of deletion of MSCs-$p75^{NTR}$ on their vascular protection in ischemic retina in terms of number of acellular capillaries.

Next, we wanted to investigate the underlying mechanism of increased vascular protection observed after knocking down MSCs-$p75^{NTR}$. We have seen increased mRNA expression of SDF-1α receptors; CXCR-4 (Figure 4A) as well as increased mRNA and protein expression of CXCR-7 (Figure 4B, C) in MSCs lysates upon knocking down their $p75^{NTR}$ expression. Moreover, genetic deletion of $p75^{NTR}$ receptor was also shown to preserve mRNA expression of CXCR-7 in ischemic retinal lysates (Figure 4D). Further studies are warranted to assess the relationship between genetic deletion of $p75^{NTR}$ receptor and the expression of SDF-1α. SDF-1 is a classical chemo-attractant stimulus of cells of endothelial lineage [38] that is known to upregulate following ischemic insult [39]. SDF-1/CXCR-4 is a known signaling axis regulating physiological retinal angiogenesis [40]. Moreover, SDF-1/CXCR-7 was reported to enhance migration of MSCs in a transient cerebral I/R rat hippocampus model [41]. SDF-1/CXCR-4 signaling axis was also shown to play an important role in directing the migration of sensory neuron progenitors from neural crest to the dorsal root ganglia in a previous report by Belmadani A et al. [42]. Contradictory to our results, inhibition of $p75^{NTR}$ was shown to restore the function of retinal pigment epithelium (RPE) through down-regulation of pro-angiogenic factors including SDF-1, IL-1β and IL-18, thus, ameliorating choroidal neovascularization [43].

The overall conclusion from our study is that, deletion of $p75^{NTR}$ from MSCs surface potentiates their vascular protective effect in vivo and in vitro. The underlying mechanism can involve, at least in part, activation of SDF-1α and its receptors. Further studies are warranted to fully understand role of $p75^{NTR}$ receptor in regulating MSCs-mediated vascular repair in ischemic retinal diseases.

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Fig.ure 3.1.

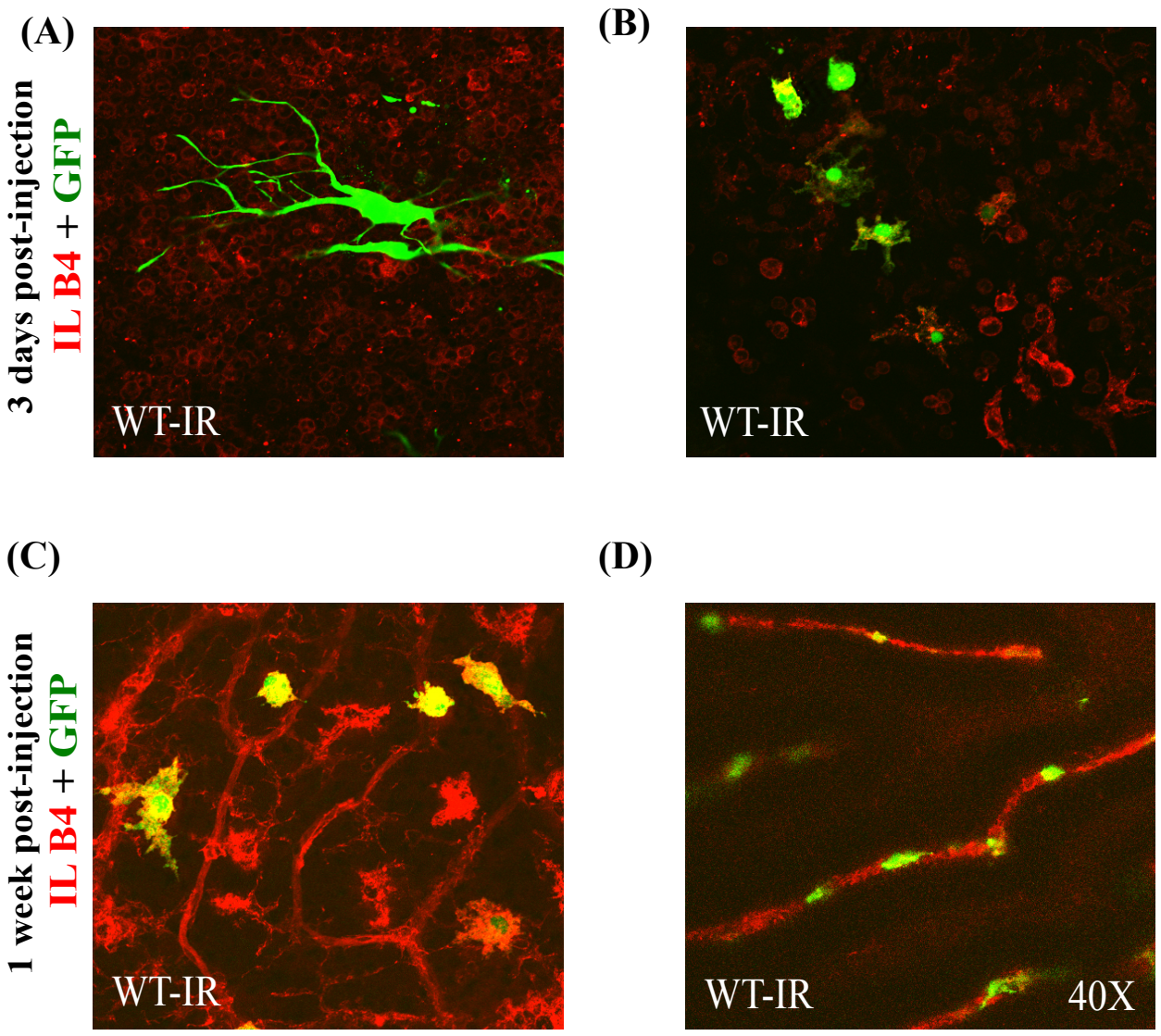

(A)
3 days post-injection
IL B4 + GFP
WT-IR
(B)
WT-IR
(C)
1 week post-injection
IL B4 + GFP
WT-IR
(D)
WT-IR
40X

Figure 3.1. GFP-labeled MSCs localization to ischemic retinal vasculature.

Confocal images of retinal flat mounts stained with isolectin (red), showing incorporation of GFP-labeled MSCs into WT retinas subjected to I/R. Cells showed minimal incorporation 3 days post I/R (A, B), whereas, they showed better homing (C), and complete integration into retinal vasculature one week post-surgery (D). Images were taken on 40X magnification.

Figure 3.2.

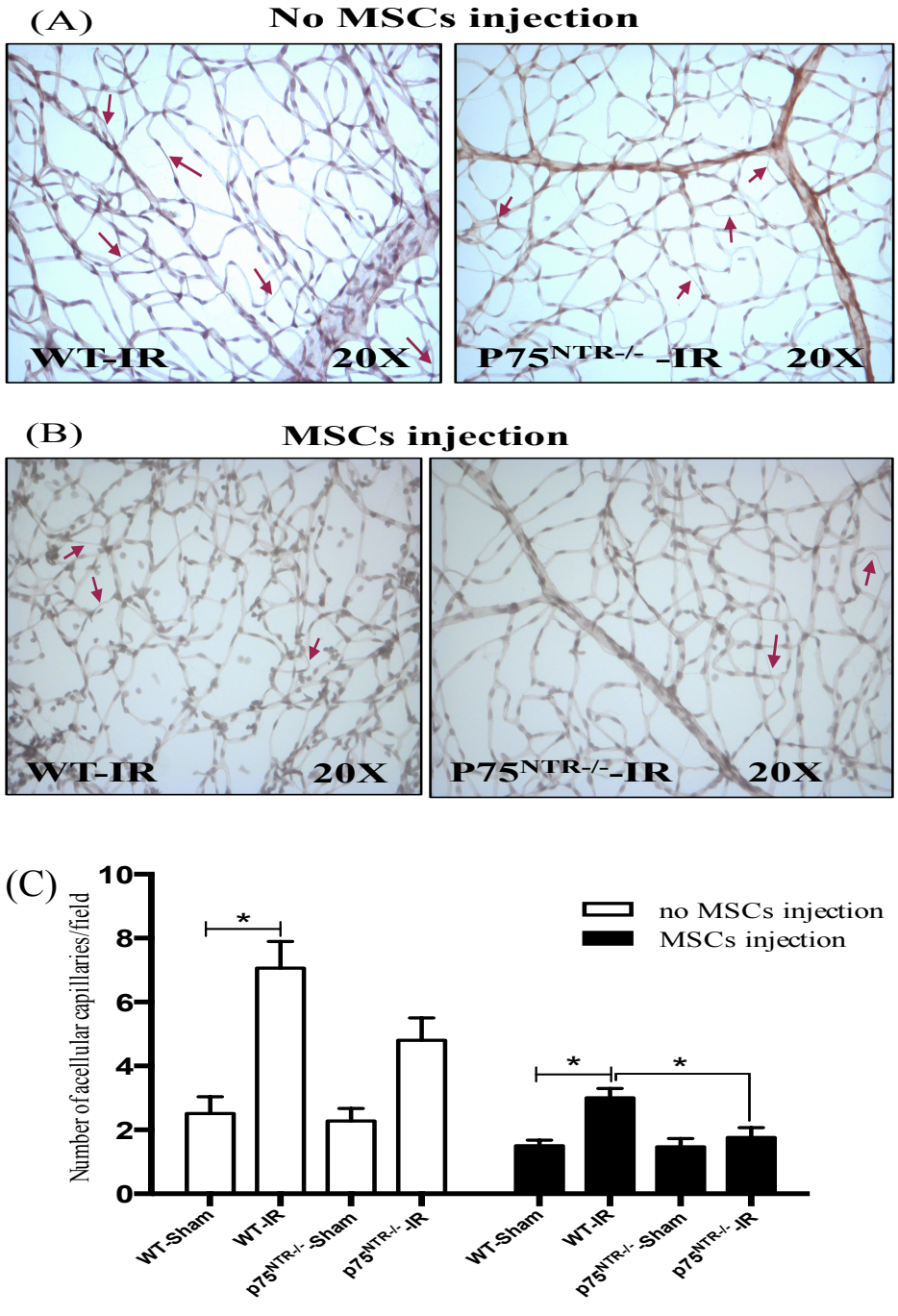
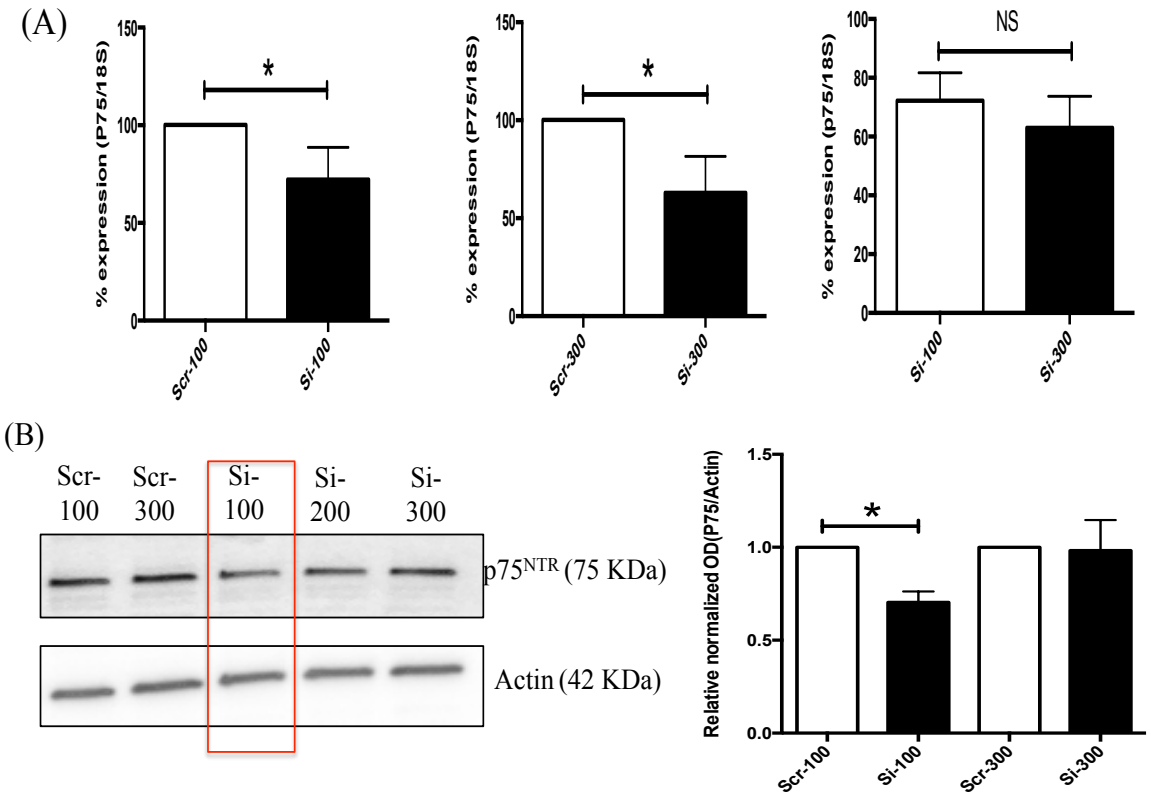

(A)
No MSCs injection
WT-IR
20X
P75NTR-/- -IR
20X
(B)
MSCs injection
WT-IR
20X
P75NTR-/--IR
20X
(C)
Number of acellular capillaries/field
10
8
6
4
2
0
*
*
*
no MSCs injection
MSCs injection
WT-Sham
WT-IR
p75NTR-/- -Sham
p75NTR-/- -IR
WT-Sham
WT-IR
p75NTR-/- -Sham
p75NTR-/- -IR

Figure 3.2. Deletion of $p75^{NTR}$ decreased number of acellular capillaries, an effect enhanced by MSCs injection.

(A) Representative trypsin-digested and PASH stained retinas of WT and $p75^{NTR-/-}$ mice subjected to I/R. Bright field imaging showed increased acellular capillaries (red arrows) in WT that was attenuated in $p75^{NTR-/-}$ mice. (B) Representative trypsin-digested and PASH stained retinas of WT and $p75^{NTR-/-}$ mice subjected to I/R and receiving GFP-labeled MSCs. Bright field imaging showed enhanced vascular protection in both WT and $p75^{NTR-/-}$ mice upon MSCs injection. (C) Statistical analysis of acellular capillaries count in WT and $p75^{NTR-/-}$ mice subjected to I/R and/or receiving intra-vitreal injection of GFP-labeled MSCs (*, significant using two-way ANOVA, n=4-5 (no MSCs injection), n=7-10 (MSCs injection)).

Figure 3.3.


(A)
% expression (P75/18S)
150
100
50
0
*
Scr-100
Si-100
% expression (P75/18S)
150
100
50
0
*
Scr-300
Si-300
% expression (p75/18S)
100
80
60
40
20
0
NS
Si-100
Si-300
(B)
Scr-100
Scr-300
Si-100
Si-200
Si-300
p75NTR (75 KDa)
Actin (42 KDa)
Relative normalized OD(P75/Actin)
1.5
1.0
0.5
0.0
*
Scr-100
Si-100
Scr-300
Si-300

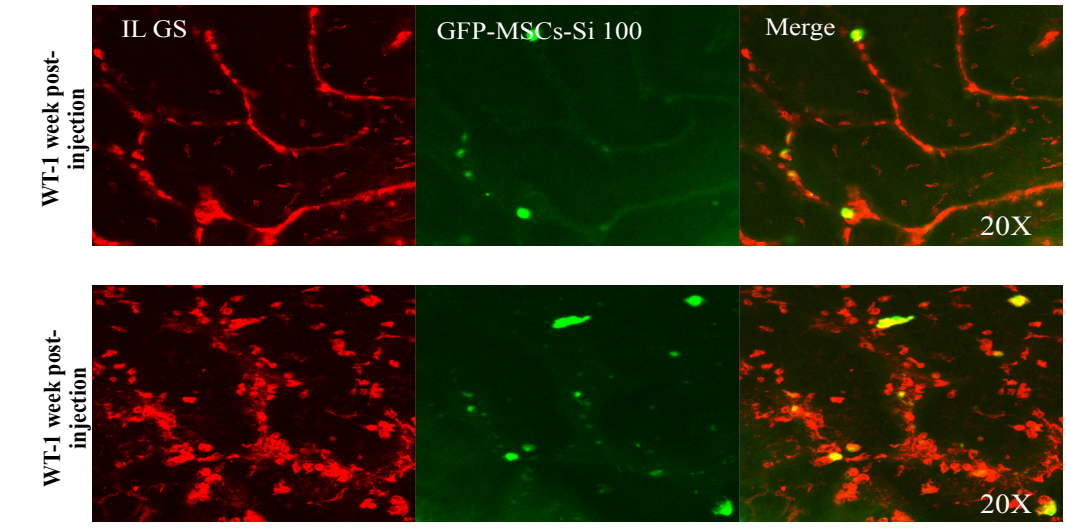

(C)
WT-1 week post-injection
IL GS
GFP-MSCs-Si 100
Merge
20X
WT-1 week post-injection
20X

Figure 3.3. Knocking down the expression of $p75^{NTR}$ receptor in MSCs increased vascular incorporation into WT ischemic retinas.

(A) Quantitative real-time PCR of $p75^{NTR}$ mRNA expression in GFP-labeled MSCs lysates treated with different concentrations of Si-RNA against $p75^{NTR}$ receptor (*, significant using un-paired Student T-test, n=3). (B) Western blotting representative and bar graph of $p75^{NTR}$ protein expression in GFP-labeled MSCs lysates treated with different concentrations of Si-RNA against $p75^{NTR}$ receptor (*, significant using un-paired Student T-test, n=4-5). (C) Representative confocal imaging of WT ischemic retinal flat-mounts receiving either Scr- or Si-100-treated GFP-labeled MSCs intravitreally, 2 days post I/R injury (20X magnification).

Figure 3.4.

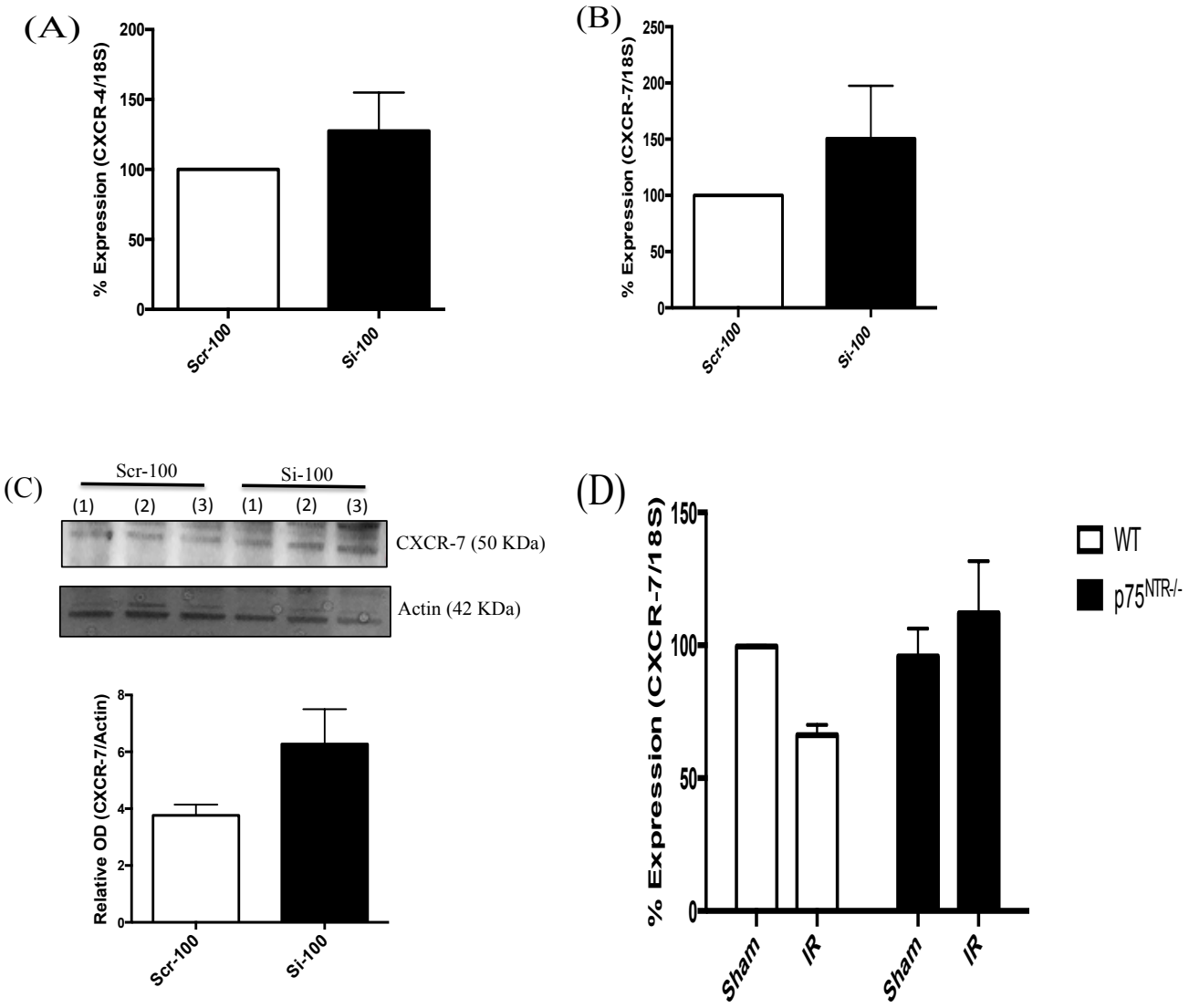

(A)
% Expression (CXCR-4/18S)
200
150
100
50
0
Scr-100
Si-100
(B)
% Expression (CXCR-7/18S)
250
200
150
100
50
0
Scr-100
Si-100
(C)
Scr-100
Si-100
(1) (2) (3) (1) (2) (3)
CXCR-7 (50 KDa)
Actin (42 KDa)
Relative OD (CXCR-7/Actin)
8
6
4
2
0
Scr-100
Si-100
(D)
% Expression (CXCR-7/18S)
150
100
50
0
Sham
IR
Sham
IR
WT
p75NTR-/-

Figure 3.4. Genetic deletion of $p75^{NTR}$ enhanced SDF-1α/CXCR-4 & -7 signaling axis.

(A) Quantitative real-time PCR of mRNA expression of CXCR-4 in GFP-labeled MSCs treated with Scr- or Si-100 against $p75^{NTR}$ receptor (n=3). (B) Quantitative real-time PCR of mRNA expression of CXCR-7 in GFP-labeled MSCs treated with Scr- or Si-100 against $p75^{NTR}$ receptor (n=3). (C) Western blot representative and bar graph of CXCR-7 expression in GFP-labeled MSCs lysates treated with Scr- or Si-100 against $p75^{NTR}$ receptor (n=3). (D) Quantitative real-time PCR of mRNA expression of CXCR-7 in WT and $p75^{NTR-/-}$ retinas subjected to I/R (n=4-5).

Figure 3.5 (A, B)

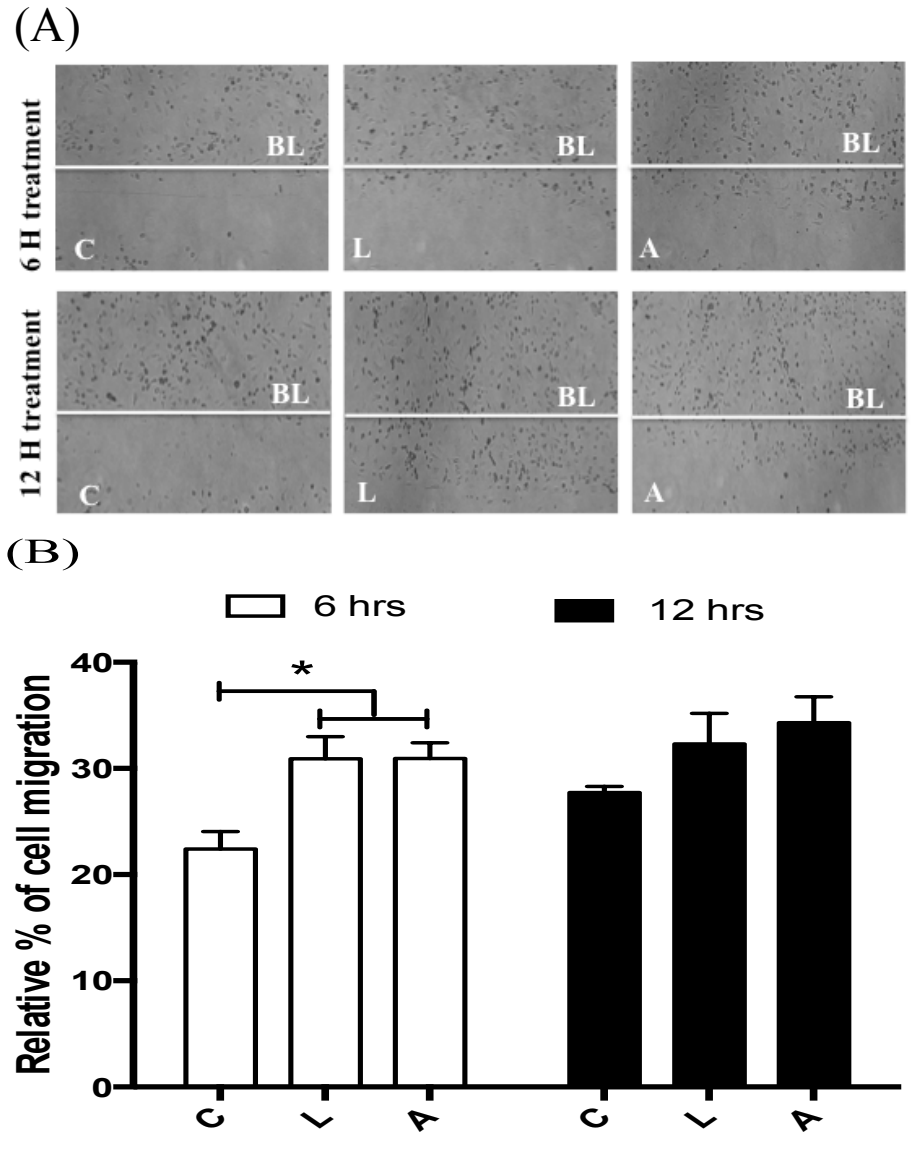
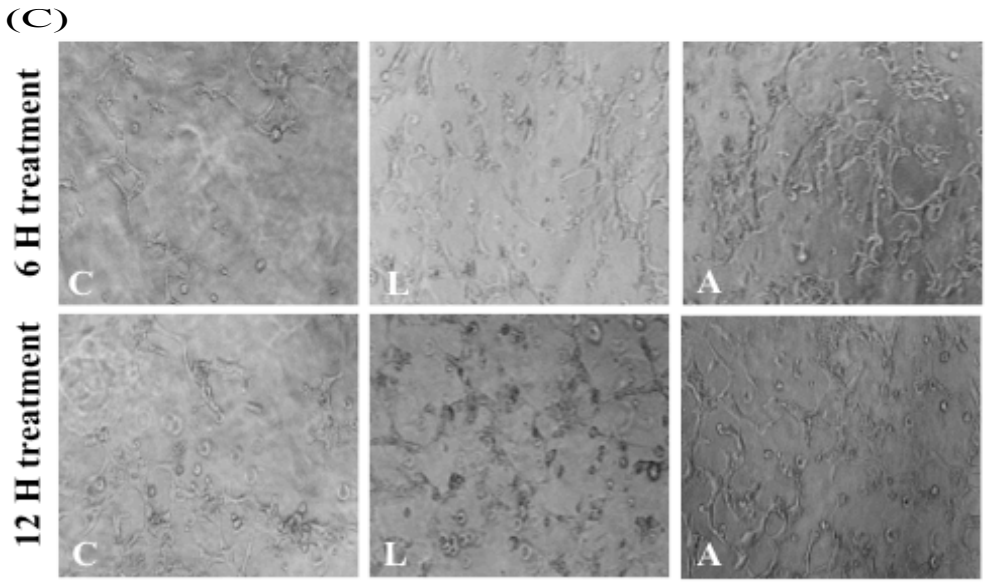

(A)
6 H treatment
12 H treatment
BL
C
L
A
(B)
6 hrs
12 hrs
Relative % of cell migration
40
30
20
10
0
*
C
L
A
C
L
A

Figure 3.5 (C, D)


(C)
6 H treatment
12 H treatment
C
L
A
C
L
A

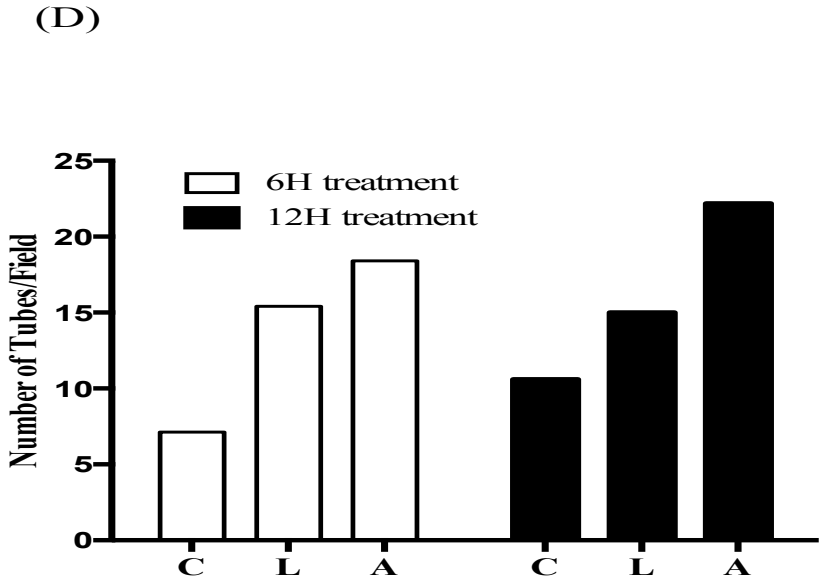

(D)
Number of Tubes/Field
25
20
15
10
5
0
6H treatment
12H treatment
C
L
A
C
L
A

Figure 3.5. Pharmacological inhibition of MSCs-p75^{NTR} increased angiogenic behavior of their conditional media on endothelial cells.

(A, B) Representative and bar graph analysis of % migration of HRECs in response to treatment with MSCs conditional media receiving (LM11A-31; L, 200 nM) or (Compound A, 20 uM) (*, significant using one-way ANOVA, n=5-6). (C, D) Representative and bar graph analysis of tube formation assay of HRECs in response to treatment with MSCs conditional media receiving (LM11A-31; L, 200 nM) or (Compound A, 20 uM) (n=3).

CHAPTER 5

DISCUSSION

Angiogenesis can be defined as expansion of microvasculature from pre-existing capillaries [1]. It can be beneficial during wound healing and post-ischemic repair, or detrimental in some pathological diseases including; ischemic retinopathy diseases such as diabetic retinopathy (DR) and retinopathy of prematurity (ROP) as well as arthritis and tumor growth (reviewed in [2,3]). In ischemic retinopathy diseases, pro-oxidative stress and pro-inflammatory milieu stimulate apoptosis of retinal vascular endothelial cells leading to ischemia [4]. Normally to counteract the ischemic condition and salvage injured ischemic tissue, growth of collateral arteries from preexisting arterioles (reparative angiogenesis) is initiated [5]. This reparative mechanism appears to be impaired in the ischemic retina. Alternatively, in an effort to meet the high metabolic demand of the retina, vertical sprouting of mal-functional capillaries is triggered towards the vitreous, eventually leading to (pathological angiogenesis/neovascularization) [6] [7]. Current therapeutic approaches for ischemic retinopathy diseases fail to completely overcome mal-functional pathological neovascularization occurring in late stages of the disease, moreover, they do not approach initial ischemic insult.

Since the retina is a typical neurovascular unit, i.e. composed of neurons, vasculature and glia; neuron-mediated angiogenesis has received great interest recently. Nerve growth factor (NGF) and its precursor proNGF as well as their receptors: the high affinity survival TrkA receptor and the low affinity $p75^{NTR}$ have been well studied in the diabetic retina, however their contribution to ischemic retinopathy is not well characterized. Our project aimed at elucidating the specific role of targeting proNGF/$p75^{NTR}$ axis and how this can prevent ischemic insult and enhance vascular repair in models of ischemic retinopathy. Our project addressed three specific

aims. The first aim was to examine role of proNGF and its mature form; NGF in vascular alterations associating ischemic retinopathy diseases both, clinically as well as in experimental models (first aim complied in manuscript 1 (chapter 2) and manuscript 2 (chapter 3)). Our second aim was to evaluate possible vascular repair induced by deletion of $p75^{NTR}$ receptor in an experimental model of ischemic retinopathy and assess underlying mechanism (second aim complied in manuscript 2 (chapter 3)). The third aim was to examine whether mesenchymal stem cells (MSCs) constitute part of the underlying mechanism in vascular protection mediated by deletion of $p75^{NTR}$ receptor in an experimental model of ischemic retinopathy (third aim complied in manuscript 3 (chapter 4)).

The main findings of the project can be summarized as follows: 1) proNGF contributes to angiogenic response observed in PDR **(Figures 1.1-1.3)** in a Trk-A dependent manner **(Figures 1.5-1.7)**. 2) Inhibition of $p75^{NTR}$ receptor did not impact proNGF-induced angiogenic behavior in PDR, rather, it activated TrkA receptor **(Figure 1.4)**. 3) Deletion of $p75^{NTR}$ prevented vascular cell death **(Figures 2.1, 3.2)** enhanced vascular repair and ameliorated pathological angiogenesis in a mouse model of ischemic retinopathy **(Figures 2.1, 2.2)**. 4) The underlying mechanism involves, at least in part, increased expression/activation of TrkA receptor **(Figure 2.6)** as well as preserved ratio of mature to immature neurotrophins **(Figures 2.4, 2.5)** and VEGF signaling **(Figure 2.8)**. 4) Enhanced MSCs homing to ischemic vasculature after deletion of $p75^{NTR}$ receptor is a contributor to vascular repair observed in ischemic retinopathy **(Figure 3.3)**, at least in part, through SDF-1/CXCR-4& -7 axis **(Figure 3.4)**.

NGF is secreted as precursor form (proNGF) that gets cleaved to the mature NGF [8]. So far, researchers have focused on studying angiogenic response of NGF which was extensively demonstrated in retinal endothelial cells [9,10], as well as retinal Muller cells [11]. NGF was

also shown to induce reparative angiogenesis in a VEGF-A-dependent mechanism in diabetic ischemic limbs[12]. Regarding ischemic retinopathy diseases, prior studies showed a positive correlation of NGF with progression of retinal pathological angiogenesis accompanying PDR in humans [13] [14] or experimental retinal neovascularization models [15], which rationalized the previous trials to inhibit NGF effect in an attempt to attenuate pathological neovascularization in ischemic retinopathy [15]. Nevertheless, these studies detected NGF at mRNA level or utilized ELISA assays, both of which cannot distinguish NGF from its precursor, proNGF.

Our previous analyses showed that diabetes-induced oxidative stress disturbs the homeostasis of NGF by hampering the cleavage of proNGF resulting in accumulation of proNGF and reducing NGF levels in experimental [16] and ocular fluids from PDR patients [17]. Therefore, it is conceivable that the previously reported angiogenic behavior associating NGF in ischemic retina is due to increases in mixed proNGF/NGF rather than NGF alone.

To our knowledge, our project is the first identifying the individual contribution of proNGF or its mature form; NGF in different retinal angiogenic patterns associating ischemic retinopathy diseases including PDR and ROP. Using cleavage-resistant form of proNGF, we have shown that proNGF contributed to angiogenic behavior of aqueous humor samples isolated from patients diagnosed with PDR **(Figures 1.1-1.3)**, an effect mediated by activation of TrkA receptor **(Figures 1.5-1.7)**. We claim this angiogenic behavior to be "pathological pattern" of angiogenesis associating ischemic retinopathy diseases since the samples were isolated from patients characterized with "proliferative" stage of DR. Our results lend further support to another study showing that the angiogenic effect of proNGF in cancer cells is exerted mainly via TrkA [18]. Our results also show that p38 MAPK is involved downstream of TrkA activation induced by proNGF to induce angiogenesis in endothelial cells **(Figure 1.4)**. This notion lend

further support to other studies implicating the role of TrkA/p38 MAPK signal in promoting cell growth, migration and invasion of cancer cells [19,20]. In addition, another study in smooth muscle cells showed also that activation of p38 MAPK and ERK was necessary for TrkA-mediated cell proliferation [20].

On the other hand, using antibodies that can separately distinguish mature NGF from its precursor form; proNGF, we have seen imbalance in the level of both of them during the two distinct vascular stages associating ischemic retinopathy diseases. In oxygen-induced retinopathy (OIR) mouse model which is one of the most widely used experimental model clinically resembles ischemic retinopathy diseases including; ROP [21] and PDR [22], this imbalance was observed during vaso-obliteration stage which clinically resembles "ischemic/non-proliferative" stage of ischemic retinopathy diseases **(Figure 2.4)**. Moreover, the imbalance was also observed during neovascularization stage **(Figure 2.5 A-D)**, which clinically resembles "proliferative" stage of ischemic retinopathy diseases. Accumulated amounts of proNGF, as well as decreases in NGF levels were associated with significant retinal ischemia **(Figure 2.1 B)** as well as pathological retinal neovascularization **(Figure 2.2 E)**. This notion lends further support to our previous conclusion that proNGF is the one involved in "pathological" pattern of angiogenesis **(Figures 1.1-1.3)** and interventions are required to decrease its level and shifting the balance towards mature NGF to preserve retinal neuronal and vascular function [23].

Interestingly, our results showed that inhibition of $p75^{NTR}$ did not significantly alter proNGF-induced angiogenic response in retinal endothelial cells **(Figures 1.5-1.7)**, most importantly, deletion of $p75^{NTR}$ modestly activated TrkA receptor **(Figure 1.4)** which is known to induce cell survival/proliferation signal [24,25]. This gave us the rationale for the second part

of the project; studying impact of $p75^{NTR}$ deletion in ischemic retinopathy and examine whether enhanced TrkA signaling plays a role.

$p75^{NTR}$ is a member of TNF-α receptor superfamily that can bind all neurotrophins with approximately equal affinity in most cells [26]. The main action of $p75^{NTR}$ receptor is to mediate apoptosis via forming a co-receptor with sortilin; a member of Vps10p-domain receptor family [27]. $p75^{NTR}$ implication in regulation of cell death as well as its role is ischemic vascular diseases, including retinal diseases, is extensively demonstrated in literature (as reviewed in [28]). ProNGF has great affinity to bind and activate $p75^{NTR}$-sortilin complex to mediate cell death [29]. proNGF was shown to promote microvascular damage following myocardial infarction [30] and ischemic stroke [31] in a $p75^{NTR}$-dependent manner. proNGF-$p75^{NTR}$ axis was also shown to be involved in neuronal damage associating Alzheimer disease [32]. We, and others, have shown that upregulation of proNGF induces $p75^{NTR}$-mediated retinal neurodegeneration [16] [17,33]; inflammation [33] as well as endothelial cell death [34]. Using OIR model, we have seen increased expression of $p75^{NTR}$ receptor during vaso-obliteration phase that was accompanied by marked retinal ischemia **(Figure 2.1)** and enhanced cell death signal **(Figure 2.3)**. The increased $p75^{NTR}$ expression reported in our studies came in agreement with multiple reports showing increased expression of $p75^{NTR}$ in ischemic retina models including ischemia induced by elevated intra-ocular pressure [35], diabetes-induced metabolic ischemia [36] and in an inherited retinal degeneration model, Royal College of Surgeons rats, that was associated with progressive capillary dropout and subretinal neovascularization [37]. Our results came in agreement also with a study that showed that hypoxia stimulates shedding of $p75^{NTR}$ rather than modulate its expression [38]. In contrast, a recent study using similar OIR model showed that expression of $p75^{NTR}$ receptor did not change in response to hyperoxia (p8, p10 and

p12), yet it showed significant increase in $p75^{NTR-/-}$ expression in response to hypoxia (p14 and p17) [39]. This further strengthened our rationale to delete $p75^{NTR}$ receptor and study the vascular implications in ischemic retina.

In OIR model, we have seen that genetic deletion of $p75^{NTR}$ receptor exerted vascular protection in ischemic retina as illustrated by ameliorated central vascular retinal ischemia **(Figure 2.1)** as well as mal-functional retinal neovascularization **(Figure 2.2)**. Our results came in accordance with Barcelona PF et al., 2016 where pharmacological inhibition of $p75^{NTR}$ receptor exerted vascular protection in two different models of ischemic retinopathy diseases [39]. Le Moan N et al. reported more similar results where genetic deletion of $p75^{NTR}$ receptor protected against retinal neovascularization in OIR model [38].

Multiple underlying mechanisms could be contributing to enhanced vascular repair seen in OIR in response to genetic deletion of $p75^{NTR}$ receptor. Deletion of $p75^{NTR}$ attenuated vascular cell death coincided with preserved activation of the survival pAkt pathway as well as attenuated expression of apoptotic markers, cleaved and total-PARP **(Figure 2.3)**. This came in accordance with previous reports by our group showing that vaso obliteration of OIR was associated with increased expression of apoptotic markers including; cleaved caspase-3 and PARP as well as decreased activation of Akt [40,41].

As discussed previously, retinal ischemic diseases were shown to be associated with accumulated amounts of proNGF at the expense of NGF **(Figures 2.4, 2.5)**, an imbalance that coincided with pathological form of angiogenesis in vitro **(Figures 1.1-1.3)** and in vivo **(Figure 2.2)**. In agreement, several mature neurotrophins; NGF, BDNF, NT-3 and GDNF were shown to down regulate in an ischemic rat retina model [42]. Clinically, preterm infants experienced decreased serum levels of mature BDNF compared to full-term [43,44]. Genetic deletion of

$p75^{NTR}$ preserved the ratio of mature neurotrophins; NGF and BDNF during both vaso-obliteration **(Figure 2.4)** and neo-vascularization phases of OIR **(Figure 2.5)** which could further explain vascular protection underlying genetic deletion of $p75^{NTR}$ receptor observed in our experimental model of ischemic retinopathy. NGF and BDNF are members of the neurotrophin family that can exert neuroprotective, vascular protective and angiogenic effects as reviewed in [23]).

Based on our previous observation that inhibition of $p75^{NTR}$ receptor was accompanied by increased activation of TrkA receptor **(Figure 1.4)**, we hypothesized that TrkA signaling could be a potential player in vascular repair regulated by deletion of $p75^{NTR}$ receptor. Genetic deletion of $p75^{NTR}$ resulted in increases in TrkA expression and activation during vaso-obliteration phase of OIR **(Figure 2.6)**. As discussed previously, preserved levels of mature neurotrophins were also observed throughout both vaso obliteration and neovascularization phases of OIR **(Figures 2.4, 2.5)**. NGF/TrkA signaling is known to enhance survival/reparative angiogenic signal [12,23,24]. Thus, we came to the notion that preserved NGF/TrkA signal could be another potential player underlying vascular protection observed by genetic deletion of $p75^{NTR}$ in models of ischemic retinopathy.

To further support our hypothesis that Trk(s) survival signal is involved in vascular repair observed by genetic deletion of $p75^{NTR}$ receptor, we used pharmacological approach to interfere with Trk(s) signal and assessed how this would implicate vascular protection induced by genetic deletion of $p75^{NTR}$ receptor in ischemic retinopathy diseases. K-252a is an alkaloid and a staurosporine analog that is potent inhibitor of various protein kinases including protein kinase A, protein kinase C and protein kinase G [45]. K-252a can also inhibit Trk(s) receptors and was shown to selectively block the effects of NGF on PC12 cells [46] [47]. Intra-vitreal injection of

K-252a in $p75^{NTR-/-}$ pups impaired central intra-retinal reparative angiogenesis and increased pathological neovascularization, i.e. reversed vascular protection associating genetic deletion of $p75^{NTR}$ receptor in our model of ischemic retinopathy **(Figure 2.7)**. This implicates the importance of TrkA survival signal in vascular protection mediated by deletion of $p75^{NTR}$ in terms of maintaining healthy retinal vasculature.

VEGF could be a potential contributor to vascular protection observed in $p75^{NTR-/-}$ mice pups. During the hypoxic stage of OIR, we have seen significant increases in gene and protein expression of VEGF **(Figure 2.8 A-C)**, which came in corroboration with previous findings [38,48]. Deletion of $p75^{NTR}$ sustained hypoxia-induced VEGF and VEGFR2 activation **(Figure 2.8 B, D)**. These results support prior findings that gene delivery of $p75^{NTR}$ impaired neo-vascularization and blood flow recovery in diabetic mouse with hind limb ischemia through depression of VEGF [34], however our results came in contrast to results by Le Moan et al. where genetic deletion of $p75^{NTR}$ receptor resulted in decreased stabilization of HIF-1α and VEGF expression at p17 and subsequent decrease in retinal neovascularization in OIR model [38].

To further focus on initial ischemic insult in ischemic retinopathy diseases apart from pathological neovascularization, we employed retinal ischemia/reperfusion (I/R) model as previously described by [49]. The surrogate marker for retinal ischemia after I/R is degenerated capillaries, i.e. acellular capillaries. Morphologically, degenerated capillaries in retinal I/R seem comparable to acellular capillaries found in diabetic retinopathy. Degenerated, acellular capillaries are not perfused, and are believed to represent a discrete event that progressively contributes to the development of retinal ischemia, and ultimately, to neovascularization [50,51].

Our previous results implicating that genetic deletion of $p75^{NTR}$ receptor protected against retinal ischemia during vaso obliteration phase of OIR **(Figure 2.1)** was further supported in I/R model where decreased number of acellular capillaries, the surrogate marker for retinal ischemia, was observed upon genetic deletion of $p75^{NTR}$ **(Figure 3.2)**. Our results confirm that $p75^{NTR}$ is a main regulator of vascular cell death, contributing to retinal ischemia that came in accordance with a previous report by Wei Y. et al [35]. They reported that $p75^{NTR}$ was upregulated (60 KD fragment (mature non-glycosylated form) and 50 KD fragment (ectodomain fragment), in addition to its co-receptor sortilin (90 KD, mature form) at 3, 5 and 7 days after retinal ischemia [35]. In addition to previously discussed mechanisms that contributes to vascular protection induced by genetic deletion of $p75^{NTR}$, preserved SDF-1 signaling axis could be another potential contributor. SDF-1 is known to regulate physiological retinal angiogenesis as described by Unoki N. et al. [52]. Our results indicated preserved gene expression of CXCR-7, one of SDF-1α receptors upon genetic deletion of $p75^{NTR}$ receptor in ischemic retina **(Figure 3.4)**. Further studies are warranted to assess expression of SDF-1 and any of its other receptors that can be potentially involved in inducing vascular repair in ischemic WT and $p75^{NTR-/-}$ retinas.

Stem cells have been shown to be an attractive candidate for retinal regeneration [53]. Among which mesenchymal stem cells (MSCs) gained big interest because they can be obtained from the patients' bone marrow in quantities appropriate for clinical application. MSCs are multipotent stem cells present in adult marrow and have the potential to differentiate into lineages of mesenchymal tissues, including bone, cartilage, fat, tendon, muscle and marrow stroma [54]. Several studies have shown that MSCs differentiate into retinal neurons in vivo and in vitro [55]. Animal studies have also demonstrated that subretinal transplantation of MSCs delays retinal degeneration and preserves retinal function [56]. Nevertheless, vascular protection

of MSCs transplantation in ischemic retina has not been fully elucidated. $p75^{NTR}$ receptor, also known as CD271, enriches several progenitor/stem cells subtypes [57]. MSCs were stained positive for $p75^{NTR}$ as early as 1988 in a study done by Thomson et al [58], nonetheless, its role in regulating MSCs vascular biology is still not fully addressed. $p75^{NTR}$ was shown to directly inhibit differentiation of MSCS into multiple cell types through inhibition of transcription factors including; Runx2 and OSX, which are essential for osteoblast differentiation and for expression of chondrogenesis marker; Sox9 and the myogenic marker; Myf5 as reported by Mikami et al [59]. In our project, we tried to evaluate the role of MSCs-expressed $p75^{NTR}$ in regulating their vascular homing and repair in I/R mouse model.

Intravitreal injection of MSCs in ischemic retinas showed maximum vascular homing and integration one-week post injection **(Figure 3.1)** and exerted additional vascular protection against the surrogate marker of retinal ischemia; acellular capillaries, in both WT and $p75^{NTR-/-}$ mice **(Figure 3.2)**. To our knowledge, our project is the first to examine vascular protection of MSCs in ischemic retina, meanwhile, neuroprotection has been described elsewhere [60,61]. Li et al., reported preserved number of RGCs after transplantation of MSCs in I/R rat retinas. MSCs were shown to express markers of neurons; neurone specific enolase, neurofilament and various neurotrophic factors [60]. Additionally, Arnhold S. et al reported the rescue of photoreceptor cells upon transplantation of bone marrow-derived MSCs in an experimental model of retinitis pigmentosa [61].

To specifically elaborate on role of MSCs-expressed $p75^{NTR}$ in regulating their vascular homing, we transiently knocked down $p75^{NTR}$ gene expression in MSCs. Our results implicated increased homing of MSCs into ischemic retinal vasculature upon knocking down $p75^{NTR}$ expression **(Figure 3.3)**. Knocking down MSCs-$p75^{NTR}$ potentiated their vascular protective

effect, decreased number of acellular capillaries and preserved retinal structure. In vitro, we have seen similar results where, pharmacological inhibition of MSCs-p75^{NTR} increased the paracrine angiogenic behavior of their conditional media on endothelial cells **(Figure 3.5)**. The underlying mechanism behind increased MSCs homing to ischemic retinal vasculature upon genetic deletion of p75^{NTR} involves, at least in part, activation of SDF-1α/CXCR-4/ -7 axis. In support of our notion, increased gene expression of SDF-1α receptors; CXCR-4 & -7 on MSCs lysates was observed **(Figure 3.4)**. SDF-1/CXCR-4 is a known signaling axis regulating physiological retinal angiogenesis [52]. Moreover, SDF-1/CXCR-7 was reported to enhance migration of MSCs in a transient cerebral I/R rat hippocampus model [62]. Further studies are warranted to examine expression of SDF-1α in MSCs conditional media, upon genetic deletion of p75^{NTR} expression.

In summary, the project illustrates the importance of proNGF/p75^{NTR} axis in pathophysiology of ischemic retinopathy diseases. proNGF is implicated in pathological neovascularization whereas, p75^{NTR} regulates retinal vascular ischemia, a prerequisite for pathological neovascularization. Our results highlight pre-clinical evidence and support therapeutic utility of anti-proNGF or pharmacological inhibitors against p75^{NTR} to combat ischemic retinal diseases characterized by aberrant angiogenesis. We demonstrate a number of unraveled novel pathways by which interfering with proNGF/p75^{NTR} axis contributes to vascular protection in ischemic retinopathy diseases. These explored pathways involve preserving levels of mature neurotrophin, TrkA survival signal, sustaining VEGF/VEGFR2 activation as well as increased MSCs vascular homing in SDF-1-dependent manner, all together maintain healthy retinal vasculature.

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