The Nicotinic Cholinergic Pathway Contributes to Retinal Neovascularization in a Mouse Model of Retinopathy of Prematurity

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Purpose. To investigate the role of nicotinic acetylcholine receptors (nAChRs) in retinal vascular development and ischemia-induced retinal neovascularization (NV).

Methods. The expression of nAChR subtypes and VEGF signaling pathway components was assessed in mice with and without oxygen-induced ischemic retinopathy by comparing expression levels at postnatal day (P) 14 and P17 in mice exposed to 75% oxygen from P7 to P12 and returned to room air versus mice pups that were exposed to ambient oxygen levels during the same period. The effect of topical or intraocular injection of mecamylamine, a nonspecific nAChR antagonist, or targeted deletion of α7- or α9-nAChRs on ischemia-induced retinal NV was determined by comparing the amount of retinal NV at P17 in these mice versus appropriate controls.

Results. The expression of nAChR subunits and components of the VEGF signaling pathways was increased in ischemic retina. Topical application or intraocular injection of mecamylamine decreased retinal NV in this model. Mecamylamine had no effect on normal retinal vascular development or on revascularization of the central retinal area of nonperfusion in mice with ischemic retinopathy. Targeted deletion of α9, but not α7, nAChR receptor subunits reduced retinal NV in mice with ischemic retinopathy.

Conclusion. These data suggest that nAChR signaling, primarily through the α9 nAChR subunit, contributes to ischemia-induced retinal NV, but not retinal vascular development. Mecamylamine or a specific α9 nAChR antagonist could be considered for treatment of retinopathy of prematurity and other ischemic retinopathies.

Keywords: retinopathy of prematurity, cholinergic pathway, nicotinic acetylcholine receptors, VEGF, retinal neovascularization

There are two major types of ocular neovascularization (NV) that affect the retina: ischemia-induced retinal NV and subretinal NV. Ischemia-induced retinal NV occurs in humans in retinopathy of prematurity (ROP) and diabetic retinopathy and affects the vessels of the inner retina. Subretinal NV occurs in the subretinal space and occurs in diseases of the RPE and Bruch's membrane such as AMD with vessels arising from the choroid. These two types of ocular NV share some characteristics with each other and with NV elsewhere in the body, but they also have unique features. It cannot be assumed that molecular signals implicated in one play a role in the other.

Nicotine stimulates endothelial cell proliferation, migration, and survival, due to the activation of nicotinic acetylcholine receptors (nAChRs).[1,2] Activation of these receptors stimulates angiogenesis in adult animals, in part by increasing VEGF expression as well as phosphorylation of VEGF receptor 2 (VEGFR2).[3–5] However, the function of these receptors in normal vascular development in unclear. Mice deficient in expression of several nAChRs subunits are viable, without reported vascular anomalies, suggesting that these subunits are not indispensable for vascular development.[6–8] In adult animals, both physiological angiogenesis as well as pathologic NV are modulated by nAChRs. For example, activation of nAChRs in the bed of an experimental wound accelerates wound healing,[9,10] and enhances endothelial cell survival.[11] In addition, nAChR may also mediate pathologic angiogenesis including NV of atherosclerotic plaque, tumor angiogenesis, and choroidal NV.[12–15]

Endogenous acetylcholine activates these receptors to induce endothelial cell proliferation, migration, and tube formation in vitro, and angiogenesis in vivo. In the eye specifically, activation of nAChRs by exogenous nicotine contributes to choroidal NV while inhibition of the pathway by mecamylamine suppresses it.[5] Mecamylamine hydrochloride is a secondary amine and a well characterized postganglionic sympathetic system inhibitor that has been shown to block nicotine-induced stimulation of nAChRs and was extensively used as an antihypertensive.[16–19] Studies in mammalian brain and amphibian neuromuscular tissue demonstrated that mecamylamine is a noncompetitive inhibitor that binds to the nAChR ion channel region and decreases the longevity of channel opening rather than by blocking nicotine

binding.[20,21] Mecamylamine is a highly lipophilic small molecule with an excellent biodistribution profile that makes it an excellent candidate for topical administration. In previous studies, where mecamylamine was delivered topically to primate eyes, measurements of mecamylamine in different compartments of the eye indicated that the topical formulation of mecamylamine penetrated into the conjunctiva and sclera to provide substantial levels in the choroid and retina (Hsu HH, et al. *IOVS* 2007;48:ARVO E-Abstract 1775).[5] With a view toward developing a topical therapy for neonates with retinopathy of prematurity, we sought to determine if the nAChR signaling pathway plays a role in a mouse model of ischemia-induced retinal NV and/or in normal retinal vascular development.

Methods

Mice

All experiments were performed in accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research and were reviewed and approved by the Johns Hopkins University Animal Care and Use Committee or the Stanford University Administrative Panel on Laboratory Animal Care. Knock-out mice for the nAChRα7 (B6.129S7-Chrna7tm1-Bay/J) and nAChRα9 (129S-Chrna9tm1Bedv/J) genes were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). C57Bl/6 mice were purchased from Charles River, Harlan Laboratories (both in Frederick, MD, USA), or Jackson Laboratories (Sacramento, CA, USA).

Oxygen-Induced Ischemic Retinopathy in Mice

Retinal neovascularization (rNV) was induced in neonatal mice as previously described.[22] Briefly, at postnatal day 7 (P7), mothers and pups were placed in a hyperoxia chamber (75% O2) to interrupt normal postnatal development of the retinal vasculature in the pup eyes, and returned to room air at P12. For some experiments, immediately upon removal from the hyperoxic chamber, mice were given a 1-μL intravitreal injection of PBS or mecamylamine (Sigma-Aldrich Corp., St. Louis, MO, USA) dissolved in PBS at concentrations ranging from 0.001% to 0.1% in the right eye and PBS alone in the left eye using a Harvard microinjection apparatus (Harvard Apparatus, Holliston, MA, USA) with a pulled glass needle and a dissecting microscope. In other animals, mecamylamine was administered topically by dropping 2 μL to each eye daily from P12 to P16 at concentrations of 0.01%, 0.03%, 0.1%, and 0.3% wt/vol reconstituted in PBS, while control animals received vehicle alone on both eyes. At P17 the mice pups were killed, the eyes were removed and fixed in 10% formalin, and the retinas were dissected out and stained using fluorescein-GSA Isolectin B4 (Invitrogen, Carlsbad, CA, USA) to identify the neovascular tufts. Digital photographs were obtained with a Zeiss Axioskop fluorescence microscope (Zeiss, Oberkochen, Germany) of the flat mounted retinas. ImagePro Plus software (Media Cybernetics, Rockville, MD, USA) was used to highlight and measure the area of retinal NV per retina by an investigator blinded with respect to treatment group.

In other experiments, mouse pups were killed at P12 and P17 following 5-day exposure to hyperoxia and the retinas were fixed, isolated, and stained as above. Flat-mounted retinas were measured as above to delineate the central area of nonperfusion (ANP) that results from vessel regression during the hyperoxia phase.

Effect of Mecamylamine on Normal Vascular Development

On the first day after birth (P1), mouse pups were given a periorbital injection of 0.03% mecamylamine in PBS or PBS alone using a Harvard microinjection apparatus with a pulled glass needle inserted just below the closed eyelid so as not to puncture the eye or enter the venous plexus. The mice were killed at P7, P10, or P18. The retinas were fixed, dissected out, stained, and flat mounted as above. For P7 retinas, the length of vascular development of the superficial retinal vessels was measured from the optic nerve head (ONH) to the edge of the peripheral vascular bed in all four quadrants per retina and the results were averaged to yield one measurement per retina. At P10 and P18, measurements were performed in the same manner focusing on the deeper capillaries that grow into the retina from the superficial vessels in a pattern starting from the central vessels to the periphery.

Platelet Endothelial Cell Adhesion Molecule (PECAM) Selection of Retinal Cells and Quantitative PCR

Wild-type C57Bl/6 mice were exposed to 75% oxygen for 5 days beginning at P7 and then returned to room air. Some mouse pups were euthanized at P14 while the remaining pups were euthanized at P17, both with age-matched room air controls. Retinas were dissected out immediately and placed in Hanks balanced salt solution (Gibco, Gaithersburg, MD, USA) and rinsed once. The retinas from three to five mice were pooled together. Pooled retinas were digested with 1-mg/mL collagenase D (Roche Scientific, Indianapolis, IN, USA) in serum-free Debulcco's modified Eagle's medium (DMEM; Invitrogen) for 40 minutes at 37°C, pipetting gently seven to eight times at the beginning and at 10-minute intervals to break up the retinas into single cell suspensions. The suspensions were filtered through a 70-μm nylon mesh (ThermoFisher, Waltham, MA, USA), which was rinsed with DMEM to make a final volume of 10 mL. The single cell suspensions were pelleted at 400 × g for 10 minutes and rinsed twice in DMEM. The final rinse was removed and the cells were resuspended in 1.5-mL DMEM and incubated with sheep anti-rat IgG-coated Dynabeads (Life Technologies, Carlsbad, CA, USA) that had been preincubated with rat anti-mouse PECAM (BD Biosciences, San Diego, CA, USA) antibodies to separate the retinal endothelial cells from the neuronal cells. After incubating at 4°C for 1 hour with gentle agitation, the beads were isolated using the proprietary magnetic rack. The PECAM (–) cell suspension was rinsed 3X with PBS as were the PECAM (+) cells adhering to the beads. Total RNA was isolated from both PECAM (+) and (–) fractions using Trizol reagent (Invitrogen) according to the manufacturer's instructions. After isolation, the RNA was used to prepare cDNA with iScript DNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the instructions. Real-time quantitative PCR was performed using a Rotor Gene Q instrument (Qiagen, Hilden, Germany) and Rotor-Gene SYBR Green PCR Kit (Qiagen) to investigate the expression levels of angiogenesis-related genes and nicotinic acid receptor alpha subunits in the retinas of normal and hypoxic mice. Cyclophilin A expression was used to standardize expression levels of the test genes. Fold changes in genes expression were calculated using ΔΔCt values.[23] Primers used in these experiments are listed in the Table.

In other experiments, mouse pups from nAChR α9 (+/–) or nAChR α7 (+/–) mice were exposed to hyperoxia as above, returned to room air, and killed at P17. This mating paradigm produces experimental mice as well as controls in the same litters. Retinas were isolated and cDNA was prepared from total

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Table. Real Time PCR Primers

Gene	Forward	Reverse	Note[24,46]
Cyclophillin A	CAGACGCCACTGTCGCTTT	TGTCTTTGGAACTTTGTCTGCAA	*
nAChRα2	GTGCCCAACACTTCCGATG	TGTAGTCATTCCATTCCTGCTTT	†
nAChRα3	CCAGTTTGAGGTGTCTATGTC	TCGGCGTTGTTGTAAAGC	†
nAChRα4	CTCAGATGTGGTCCTTGTC	GAGTTCAGATGGGATGCG	†
nAChRα5	CATCGTTTTGTTTGATAATGC	TGCGTCCAAGTGACAGTG	†
nAChRα6	TGTCTCCGATCCCGTCAC	TTGTTATACAGAACGATGTCAGG	†
nAChRα7	GGTCATTTGCCCACTCTG	GACAGCCTATCGGGTGAG	†
nAChRα9 exon 4	GGTGGATGTCACCTATTTCC	TGAAGTCAGAGAGGTCACCA	
nAChRα10	TCTGACCTCACAACCCACAA	TCCTGTCTCAGCCTCCATGT	†
PECAM	CCTCAGTCGGCAGACAAGATG	GCATAGAGCACCAGCGTGAGT	
PEDF	CACCCGACTTCAGCAAGATTACT	TCGAAAGCAGCCCTGTGTT	
VEGF A	GGCTGCTGTAACGATGAAG	CTCTCTATGTGCTGGCTTTG	*
VEGF R2	GCCCTGCCTGTGGTCTCACTAC	CAAAGCATTGCCCATTCGAT	

* Shih SC, Ju M, Liu N, Smith LE. Selective stimulation of VEGFR-1 prevents oxygen-induced retinal vascular degeneration in retinopathy of prematurity. *J Clin Invest*. 2003;112:50–57.

† Smith ML, Souza FG, Bruce KS, Strang CE, Morley BJ, Keyser KT. Acetylcholine receptors in the retinas of the α7 nicotinic acetylcholine receptor knockout mouse. *Mol Vis*. 2014;20:1328–1356.

retinal RNA as above. Real-time quantitative PCR was performed to investigate differences in gene expression between knockout animals and littermate controls.

Statistical Analysis

Data are expressed as means ± SEM. Statistical analysis was performed using Student's *t*-test with *P* less than 0.05 considered significant, or Dunnett's test for multiple comparisons.

Results

Mecamylamine Suppresses Ischemia-Induced Retinal NV

Oxygen-induced ischemic retinopathy (OIR) is a model of rNV. Mouse pups with their mothers were placed in a hyperoxic chamber with oxygen maintained at a concentration of 75% from P7 to P12 when they were returned to room air. Immediately after removing them from the chamber, the pups received a 1-μL intraocular injection of mecamylamine in PBS at a concentration ranging from 0.001% to 0.1% in the right eye and PBS alone in the left eye. In a dose-dependent manner, mecamylamine reduced ischemia-induced NV significantly compared with PBS alone (Fig. 1a). Similar results were seen in mice that received daily topical administration of mecamylamine (Supplementary Fig. S1).

To investigate whether mecamylamine, which readily diffuses through ocular tissue, also inhibited normal vascular development, mouse pups were given a 1-μL periorbital injection of 0.03% mecamylamine or PBS at P1 and the extent of retinal vessel formation was measured at three time-points during development. At P7, the extent of superficial vessel network coverage toward the peripheral retina was the same in retinas from mice treated with mecamylamine compared with those of mice treated with PBS (Figs. 1b, 1c). The deeper capillary network forms from branches of the superficial vessels that grow into the retina starting in the central retina and progressing peripherally. The extent of coverage of the deeper capillary network was measured at P10 and P18 and was the same in mice treated with mecamylamine compared with mice treated with PBS at both time-points. Thus, mecamylamine reduces pathologic NV but has no effect on normal vascular development.

Expression of Components of the VEGF Pathway and nAChR Alpha Subtypes in Retinal Endothelial Cells

Because nAChRs are expressed on both neuronal cells as well as vascular cells, and because ischemic retinopathy can alter VEGFA and VEGFR2 levels in both cell populations, it was desirable to isolate these populations and investigate any changes that may occur, especially with regard to nAChR subtypes. To investigate the expression of VEGFA, VEGFR2, and the nAChR alpha subunits in retinal neuronal and endothelial cell populations, retinal endothelial cells were isolated from dissociated total retinal cell suspensions from mice with OIR at P14 and P17 using anti-PECAM-1–coated magnetic beads. The average enrichment factor of PECAM expression in the PECAM (+) cell fraction generated by the purification technique was 27.4+/− 4.7 compared with the PECAM (−) cell fraction (Fig. 2a). The change in VEGFA and VEGFR2 expression levels was investigated in PECAM (−) and (+) cell populations from the retinas of OIR mice. Two days after the onset of ischemia at P12, VEGFA expression levels were elevated in both PECAM (−) neuronal and PECAM (+) endothelial cell populations and the elevation was sustained at P17 (Fig. 2b). Vascular endothelial growth factor receptor 2 is expressed on several cell types in the retina, including ganglion cells, rod photoreceptors, and endothelial cells, but its expression was only significantly increased at P14 or P17 in the PECAM (+) fraction of retinal cells, indicating that it is only upregulated by OIR in endothelial cells.

We next investigated the expression levels of the nAChRα subtypes in control and OIR retinas. A previous study indicated that eight subtypes are expressed in the retina.[24,25] Our study demonstrates that the eight subtypes are expressed in both the PECAM (+) endothelial cell fraction as well as in the PECAM (−) neuronal fraction of retinal cells and that they are modulated by hypoxia (Figs. 2c, 2d). The nAChR subtypes α2, α9, and α10 were transiently upregulated at P14 in the PECAM (−) fraction but returned to control levels at P17 in retinas from OIR mice compared with controls. The nAChRα5 subtype was increased at P17, while the α3 subtype was increased at both P14 and P17 in the PECAM (−) fraction. In the PECAM (+) cell fraction, the α9 and α10 subtypes were significantly increased at P14 but not at P17 compared with controls while the α3 and α5 subtypes were significantly increased at P17. Interestingly, the α2, α4, and α6 subtypes were all significantly suppressed at

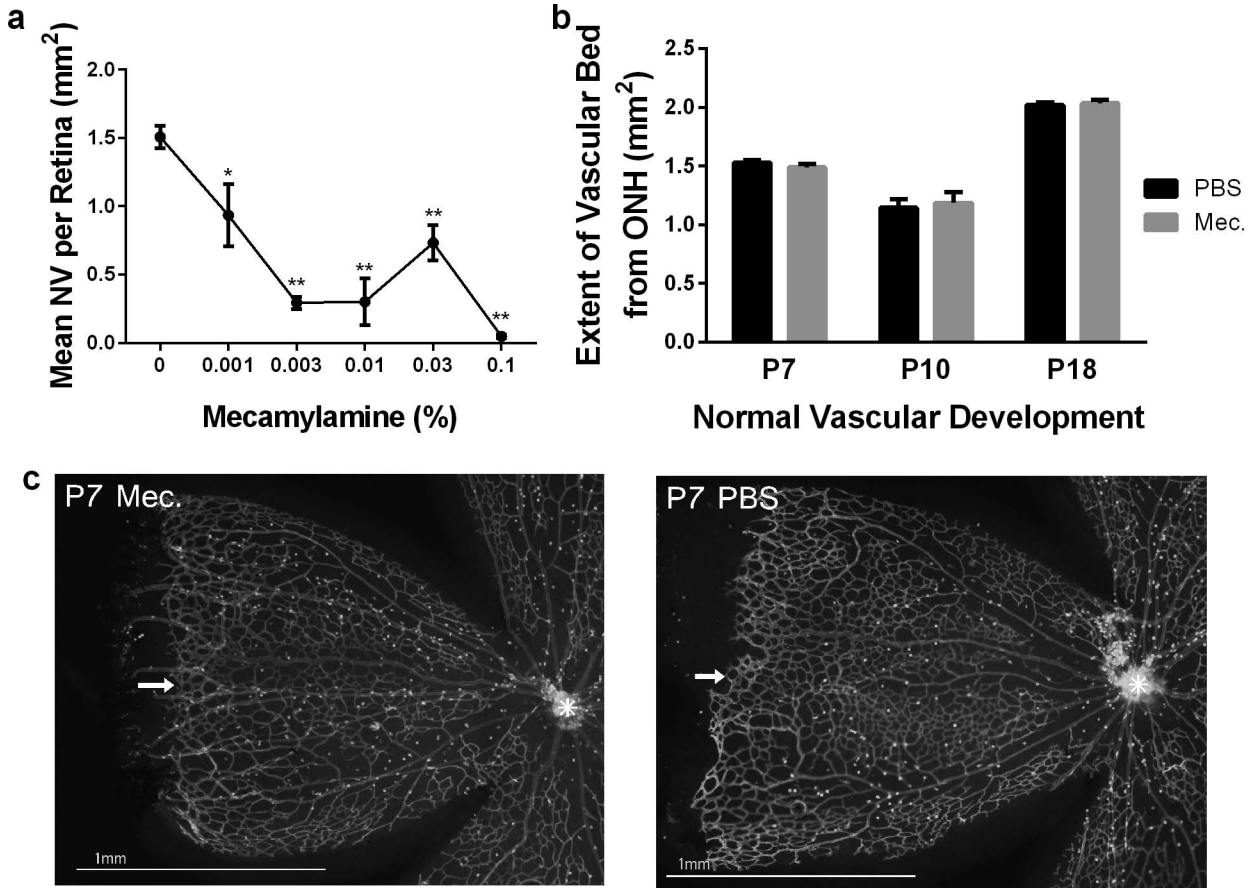

Figure 1. Mecamylamine reduces rNV in OIR but does not affect normal vascular development. (**a**) Mouse pup litters ($n = 4$–9 pups per litter) were treated by intraocular injection of mecamylamine or PBS at the onset of hypoxia (P12). $^{*}P < 0.05$, $^{**}P < 0.001$ for control versus treated rNV. (**b**) Distance from the optic nerve head (*asterisk*) to the peripheral edge of vascularization (*arrow*) was measured at P7 in the superficial vascular bed and at P10 and P18 for the deep capillary vascular bed. The distance was measured in each of four quadrants in the flat-mounted retinas and averaged to generate one value per eye. $n = 4$ to 10 per group. (**c**) Images of representative retinal quadrants for superficial vessels at P7 of mecamylamine- and PBS-treated eyes.

P17 in the OIR mice. And surprisingly the α7 subtype, which has been shown repeatedly to play a role in proliferation of endothelial cells from other circulations, was unmodulated at either time-point for either cell fraction.

Effect of Targeted Deletion of nAChRα7 or nAChRα9 on Ischemia-Induced Retinal NV and Gene Expression

Previous studies in vitro using siRNA to knockdown expression of specific nAChRα subtypes demonstrated that suppression of α7 decreased endothelial cell proliferation and increased apoptosis while suppression of α9 increased proliferation without affecting apoptosis.[4] Therefore, in order to discern whether these two subtypes played similar roles in vivo, we investigated the effect of systemic ablation of α7 or α9 in mice on the development of rNV in the OIR model using commercially available knockout mice. Mice hemizygous for either deletion were mated to produce litters that contained (+/+), (+/–), and (–/–) littermate controls to correct for any interlitter variations. Compared with wild-type mice, there was no difference in the amount of rNV in OIR retinas from either hemizygous or homozygous nAChRα7 knockout mice (Fig. 3a). In the nAChRα9 knockout mice, hemizygous deletion resulted in a 14% decrease on the amount of rNV compared with controls while homozygous ablation resulted in a 34% decrease in the amount of rNV that developed (Figs. 3a, 3b). Because the amount of rNV that develops in the OIR model depends upon the amount of vessel ablation that occurs in the hyperoxic phase, we measured the central ANP in nAChRα9 (+/+) and (–/–) mice at P12 immediately after removal from hyperoxia. Both populations of mice showed identically sized ANP. Additionally, when the central ANP remaining at P17 was measured in a separate cohort of mice, there was no difference compared with controls. Thus the regrowth of normal vasculature to fill in the ablated zone occurred at the same rate in nAChRα9 (–/–) mice compared with (+/+) mice suggesting that nAChRα9 is not necessary for normal vessel formation.

Previous work has demonstrated that activation of nAChr receptors can alter the ratio of the proangiogenic VEGF and the antiangiogenic pigment epithelial derived factor (PEDF) in favor of VEGF.[26] To investigate whether a change in the VEGF:PEDF ratio could account for the decreased rNV in nAChRα9 (–/–) mice compared with controls, mRNA levels were compared for VEGF, PEDF, and VEGFR2 in P17 OIR knockout mice versus OIR wild-type controls (Fig. 4). Gene expression was not significantly different for any of these. However, when expression levels for the different nAChRα subtypes were measured, we found significant increases in the expression levels for α2, α4, and α5 in the retinas of knockout mice compared with controls. This suggests that there may be some compensatory upregulation of these subtypes in the absence of nAChRα9.

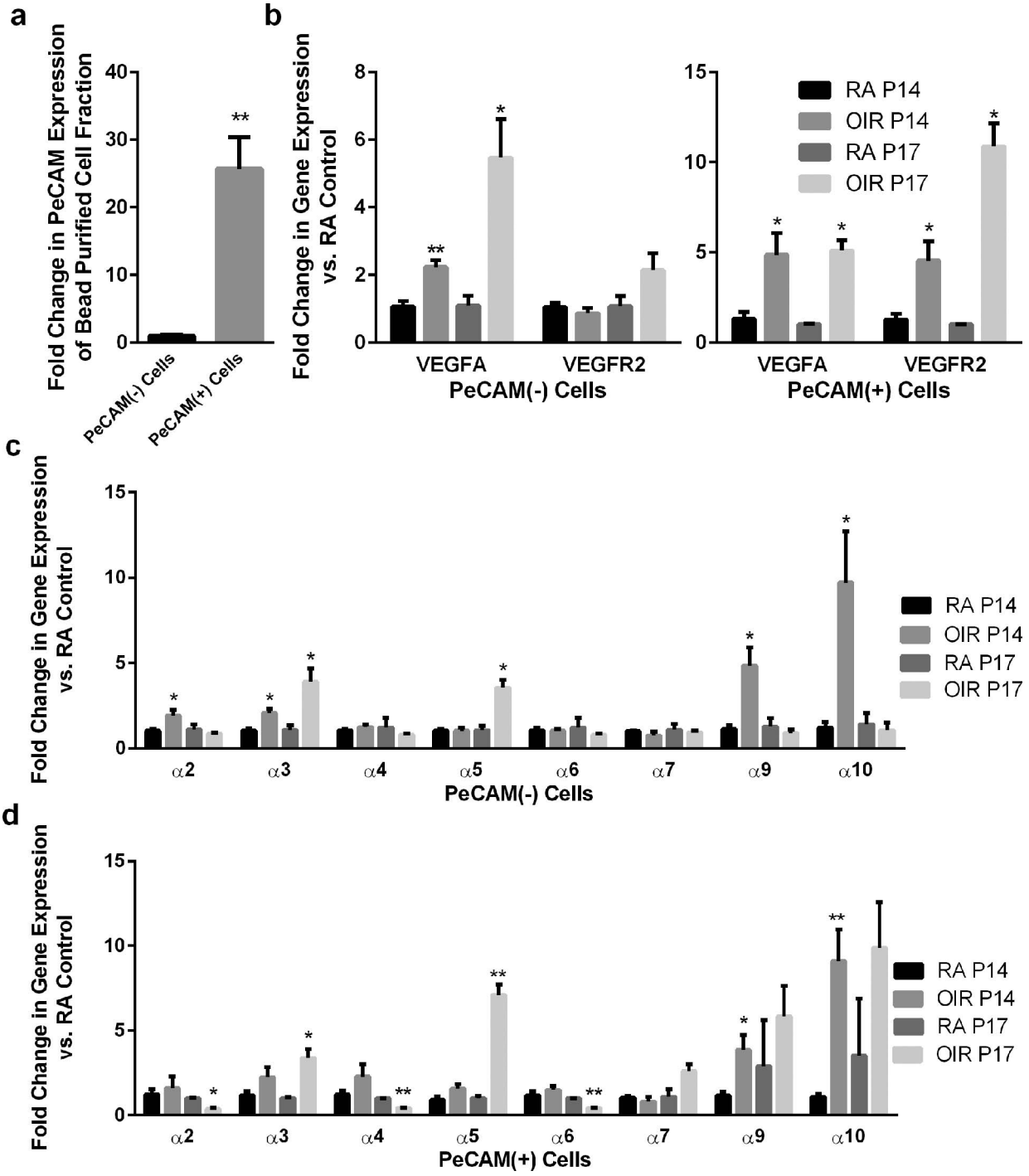

Figure 2. Gene expression in PECAM (+) and PECAM (–) fractions of retinal homogenates from RA control versus OIR mice at P14 and P17 was compared. (**a**) The enrichment factor of PECAM expression in bead-selected fractions versus nonselected fraction was measured. $n = 12$, $^{**}P = 2.65 \times 10^{-5}$. (**b**) Vascular endothelial growth factor A and VEGFR2 expression levels were measured at P14 ($n = 6$) and P17 ($n = 3$) in both PECAM (+) and PECAM (–) fractions from OIR retinas and RA control retinas. $^{*}P < 0.05$, $^{**}P < 0.01$. (**c**) Expression levels of the eight nAChRα subtypes were measured at P14 ($n = 6$) and at P17 ($n = 3$) in the PECAM (–) fraction of retinal homogenate samples from OIR mice and RA controls. $^{*}P < 0.05$. (**d**) Expression of the nAChR α subtypes were measured at P14 ($n = 6$) and P17 ($n = 3$) in the PECAM (+) fraction of retinal homogenate samples from OIR mice and RA controls. $^{*}P < 0.05$, $^{**}P < 0.01$.

Discussion

Overview

The salient findings of this study are that in the murine OIR model of rNV, the expression of nAChR subunits and components of the VEGF signaling pathways are increased in the ischemic retina. Furthermore, topical or intraocular administration of the nAChR antagonist mecamylamine decreased retinal NV in this model. Additionally, targeted deletion of α9, but not α7, nAChR receptor subunits reduced retinal NV in OIR. These data suggest that nAChR signaling, primarily through the α9 nAChRs, contributes to ischemia-induced

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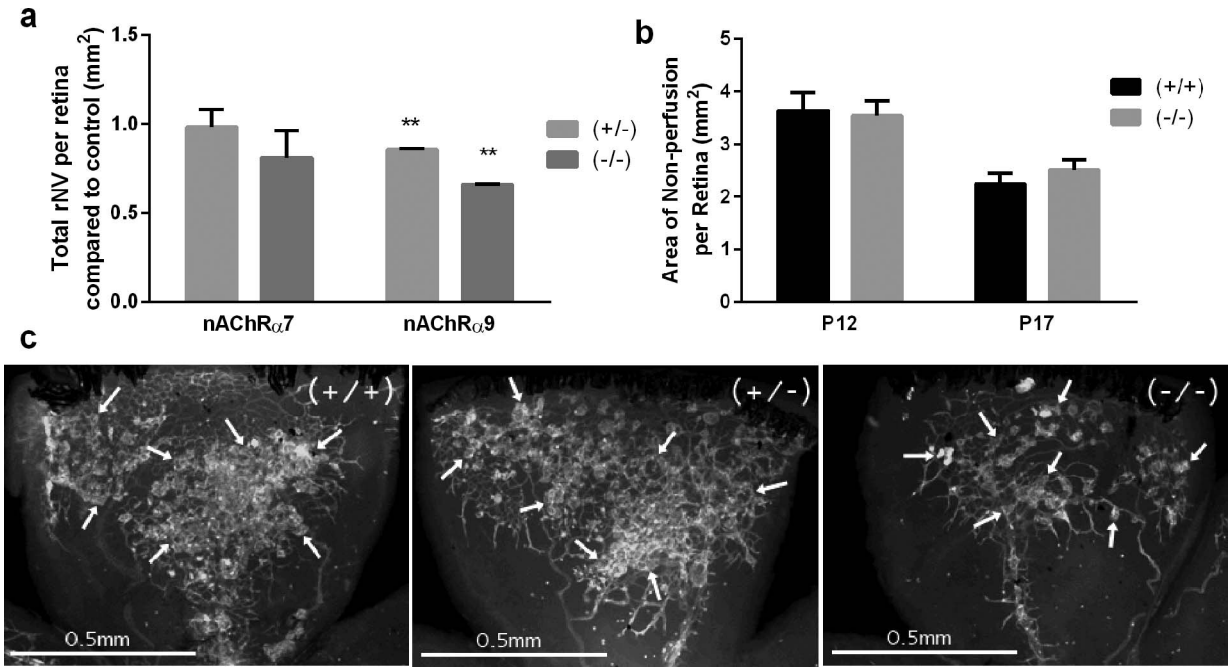

Figure 3. Pathologic rNV is reduced in nAChRα9 KO mice but not in nAChRα7 KO mice. (**a**) Retinal NV was quantified in retinas of P17 OIR that were from nAChR α7 (+/–) or (–/–), nAChR, α9 (+/–) or (–/–), and wild-type control mice. $n = 6$ to 15. $P < 0.01$. (**b**) The central ANP that results from exposure to hyperoxia was measured in nAChR α9 (+/+) and (–/–) mice at P12 and also at P17 as normal vessels grow to revascularize the central retina. $n = 3$ to 7. (**c**) Representative images of one quadrant of flat mounted retinas from (+/+), (+/–), and (–/–) nAChR α9 KO mice display the amount of rNV for each group (*arrows*).

retinal NV, but not retinal vascular development. Mecamylamine or a specific α9 nAChR antagonist could be considered for treatment of ROP and other ischemic retinopathies.

ROP Versus OIR

Retinopathy of prematurity is a vasoproliferative retinal disease affecting premature infants (for reviews see Refs. 27–31). Normally, retinal vascular development occurs prenatally and is complete around gestational age 37 weeks. In infants born prematurely, normal vascular development is interrupted due to the loss of maternally derived factors such as insulin-like growth factor 1 (IGF1), IGF binding protein 3 (IGFBP3), and ω-3 polyunsaturated fatty acids (PUFAs), as well as the down regulation of proangiogenic factors such as VEGF due to increased oxygen tension relative to that in utero. This interruption of normal vascular development is Phase 1 of ROP. Phase 2 occurs as the neural retina develops and oxygen requirements increase leading to hypoxia of the retina, and upregulation of VEGF and other angiocytokines. This leads to dysregulated vascular cell proliferation into the epiretinal vitreous space forming membranes that can cause retinal detachment and/or neuronal defects that can result in poor visual acuity for life. Interventions to decrease the severity and progression of the disease include careful regulation of oxygen to prevent ROP, and the use of cryotherapy or laser ablation. More recently, anti-VEGF therapies have been assessed in ROP, but concerns remain about possible systemic effects of this approach.[32]

To model the human disease, we used the well-characterized mouse OIR model.[22,33] As in ROP, the mouse OIR model has a biphasic pathology whose initial phase includes loss of proangiogenic stimuli due to increased oxygen tension resulting in a cessation of retinal vascular development, which phase is followed by a hypoxia-induced upregulation of proangiogenic factors leading to aberrant NV. However, there are some key differences.[30,34,35] First, retinal vascular development in humans occurs by a combination of vasculogenesis

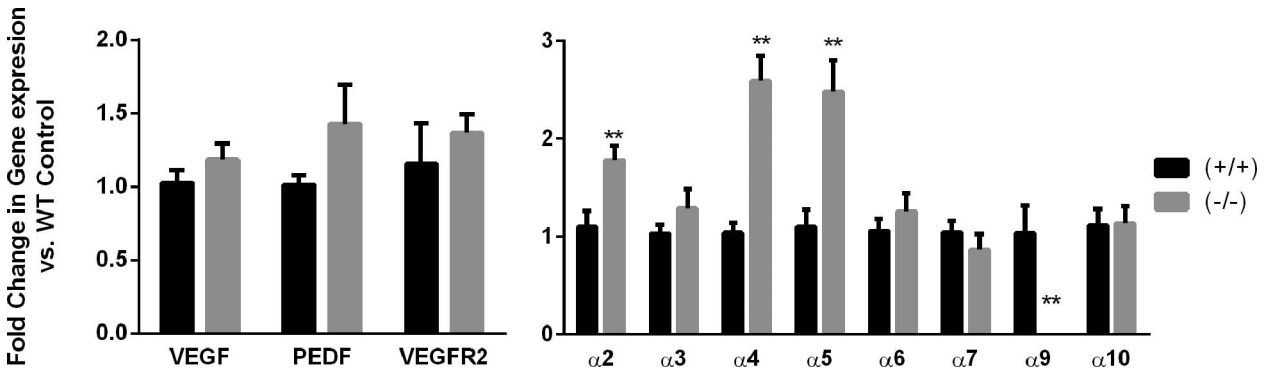

Figure 4. The expression levels of VEGF, PEDF, VEGFR2, and the nAChRα subtypes were compared in P17 OIR retinas from nAChR α9 (+/+) and (–/–) mice. (+/+) $n = 8$, (–/–) $n = 13$. $^{**}P < 0.01$.

and angiogenesis and is normally complete by gestational age of 37 weeks (i.e., prenatal) while that in mice is entirely postnatal and is now believed to involve only angiogenesis. As a result of this difference, while human ROP is exacerbated by the loss of maternally derived factors such as IGF1 and ω-3 PUFAs, murine OIR does not involve alterations in maternal trophic support. Additionally, human ROP involves a disruption of vessel formation in the peripheral retina during phase 1, while mouse OIR in addition includes ablation of central retina vessel. Lastly, murine OIR does not cause retinal detachment, resolves without intervention, and is not reported to cause neuronal cell death. Thus, the OIR model has been helpful to increase our understanding of the mechanisms of ROP and to identify potential therapeutic interventions, but may not be entirely predictive of the benefit of such new therapeutic approaches.

The Angiogenic Effect of Endothelial nAChRs

We previously discovered that endothelial cell (EC) nicotinic acetylcholine receptors (nAChRs) induce EC survival, proliferation, and function.[2] Notably, endothelial cells have the capacity to synthesize and degrade acetylcholine, the endogenous ligand for nAChRs.[15,36–40] In vivo, activation of the nAChRs may enhance physiological angiogenesis or contribute to pathologic NV.[2–5,9,10]

We have shown that there are positive, reinforcing interactions between the cholinergic and VEGF pathways. Stimulation of EC nAChRs increases VEGF expression and metalloproteinase expression[41,42]; and induces phosphorylation of the VEGFR.[43] Vascular endothelial growth factor–induced EC migration is inhibited by nAChR antagonists. Reciprocally, VEGF stimulation increases the expression of EC nAChR α7.[44] These findings indicate that the cholinergic and VEGF system are synergistic proangiogenic pathways.

Antagonism of nAChR Blocks Pathologic NV

Kiuchi and colleagues[5] showed that pretreating mice with topical administration of mecamylamine, a nonselective nAChR antagonist, effectively reduced choroidal NV in the mouse model of ruptured Bruch's membrane. Our present results demonstrate that mecamylamine can also mitigate the prolific vascularization seen in OIR. Notably, mecamylamine did not inhibit the revascularization of the central vaso-obliterated region, indicative of the restoration of normal physiological vascularization in this animal model. Likewise, administration of mecamylamine during normal development did not produce any changes in normal vascular development, as assessed by analyzing the rate of radial outgrowth from the ONH. These studies indicate that normal vascular development can proceed in the presence of a nAChR antagonist, whereas pathologic NV is inhibited.

Previous observations of human retinal endothelial cells in vitro using nAChR-specific siRNAs suggested that nAChRα7 activation induces a proangiogenic response while α9 activation decreases endothelial cell proliferation.[4] Therefore, it was predicted that nAChRα7 (–/–) mice would have less rNV than wild-type (+/+) mice, whereas nAChRα9 (–/–) mice would have greater NV. However, the reverse is true in the mouse eye. In the pathologic NV associated with murine OIR, it appears that α9 nAChRs play a prominent role in the development of rNV. Indeed, mice that lack the α9 nAChR subtype developed less rNV than control mice. We found no difference in the extent of neovasculariz between wild-type and α7 (–/–) mice, which suggests that this subtype plays a less important role in the development of rNV than the in vitro experiments would lead one to believe. The roles played by the different subtypes thus appear to be context dependent. Our data also suggest that multiple subtypes play a role in the neovascular response because inhibition of all of the nAChR subtypes by mecamylamine reduced rNV by greater than 95% at the highest concentration while nAChRα9 ablation resulted in only a 34% reduction. Other nAChR subtypes may compensate for the loss of the α9 nAChR subtype. Indeed, there was a 2-fold increase in the mRNA expression of nAChRα2 in the α9 (–/–) mice, and a 2.5-fold increase for nAChRα4 and α5 mRNA. Further studies will need to be done to determine how much each subtype contributes to the neovascular response.

Conclusions

In summary, we have shown the existence of nAChRs in the murine neural retina and the retinal vasculature, and their upregulation in the murine OIR model of retinal NV. Furthermore, pharmacologic or genetic suppression of nicotinic acetylcholinergic receptors inhibited the development of retinal NV in this model. Our work justifies a more comprehensive analysis of the role of different nAChR subunits in rNV by genetic manipulations or through use of specific subtype inhibitors to further define the specific receptors that may be antagonistic and agonistic to the pathologic NV process in the retina. Finally, nAChR antagonists such as mecamylamine may represent a novel therapeutic approach to alleviate ischemic retinopathies that develop in diseases such as ROP and diabetic macular edema. Partial evidence for this was suggested by a small phase I/II trial investigating the efficacy of topical 1% mecamylamine drops twice per day in alleviating macular edema and improving visual acuity (VA).[45] The mixed results demonstrated that a subset of patients showed improvements while others showed no improvement or even worsening of VA. Much like our results, the authors suggest that the heterogeneity of effect may be a reflection of the differing and possibly opposing roles the nAChR subtypes are reported to play and a better understanding of these differing roles is necessary to develop therapies that can effectively target this angiomodulatory system.

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References

1. Villablanca AC. Nicotine stimulates DNA synthesis and proliferation in vascular endothelial cells in vitro. *J Appl Physiol*. 1998;84:2089–2098.
2. Heeschen C, Jang JJ, Weis M, et al. Nicotine stimulates angiogenesis and promotes tumor growth and atherosclerosis. *Nat Med*. 2001;7:833–839.
3. Heeschen C, Weis M, Aicher A, Dimmeler S, Cooke JP. A novel angiogenic pathway mediated by non-neuronal nicotinic acetylcholine receptors. *J Clin Invest*. 2002;110:527–536.
4. Wu JC, Chruscinski A, De Jesus Perez VA, et al. Cholinergic modulation of angiogenesis: role of the 7 nicotinic acetylcholine receptor. *J Cell Biochem*. 2009;108:433–446.
5. Kiuchi K, Matsuoka M, Wu JC, et al. Mecamylamine suppresses basal and nicotine-stimulated choroidal neovascularization. *Invest Ophthalmol Vis Sci*. 2008;49:1705–1711.
6. Caffery PM, Krishnaswamy A, Sanders T, et al. Engineering neuronal nicotinic acetylcholine receptors with functional

sensitivity to alpha-bungarotoxin: a novel alpha3-knock-in mouse. *Eur J Neurosci*. 2009;30:2064–2076.

7. Orr-Urtreger A, Göldner FM, Saeki M, et al. Mice deficient in the α7 neuronal nicotinic acetylcholine receptor lack α-bungarotoxin binding sites and hippocampal fast nicotinic currents. *J Neurosci*. 1997;17:9165–9171.
8. Vetter DE, Liberman MC, Mann J, et al. Role of alpha9 nicotinic ACh receptor subunits in the development and function of cochlear efferent innervation. *Neuron*. 1999;23: 93–103.
9. Jacobi J, Jang J, Sundram U, Dayoub H, Fajardo LF, Cooke JP. Nicotine accelerates angiogenesis and wound healing in genetically diabetic mice. *Am J Pathol*. 2002;161:97–104.
10. Morimoto N, Takemoto S, Kawazoe T, Suzuki S. Nicotine at a low concentration promotes wound healing. *J Surg Res*. 2008;145:199–204.
11. Smedlund K, Tano JY, Margiotta J, Vazquez, G. Evidence for operation of nicotinic and muscarinic acetylcholine receptor-dependent survival pathways in human coronary artery endothelial cells. *J Cell Biochem*. 2011;112:1978–1984.
12. Santanam N, Thornhill BA, Lau JK, et al. Nicotinic acetylcholine receptor signaling in atherogenesis. *Atherosclerosis*. 2012;225:264–273.
13. Schaal C, Chellappan SP. Nicotine-mediated cell proliferation and tumor progression in smoking-related cancers. *Mol Cancer Res*. 2014;12:14–23.
14. Tang J, Li Z, Lu L, Cho CH. β-Adrenergic system, a backstage manipulator regulating tumour progression and drug target in cancer therapy. *Semin Cancer Biol*. 2013;23:533–542.
15. Lee J, Cooke JP. Nicotine and pathological angiogenesis. *Life Sci*. 2012;91:1058–1064.
16. Ascher P, Large WA, Rang HP. Studies on the mechanism of action of acetylcholine antagonists on rat parasympathetic ganglion cells. *J Physiol*. 1979;295:139–170.
17. Fieber LA, Adams DJ. Acetylcholine-evoked currents in cultured neurones dissociated from rat parasympathetic cardiac ganglia. *J Physiol*. 1991;434:215–237.
18. Nooney JM, Peters JA, Lambert JJ. A patch clamp study of the nicotinic acetylcholine receptor of bovine adrenomedullary chromaffin cells in culture. *J Physiol*. 1992;455:503–527.
19. Gyermek L. Methods for the examination of ganglion-blocking activity. In: Kharkevich DA, ed. *Pharmacology of Ganglionic Transmission*. New York, New York: Springer; 1980:63–121.
20. Varanda WA, Aracava Y, Sherby SM, VanMeter WG, Eldefrawi ME, Albuquerque EX. The acetylcholine receptor of the neuromuscular junction recognizes mecamylamine as a non-competitive antagonist. *Mol Pharmacol*. 1985;28:128–137.
21. Williams M, Robinson JL. Nicotinic receptors in mammalian brain. *Prog Neuropsychopharmacol Biol Psychiatry*. 1984;8: 769–772.
22. Smith LE, Wesolowski E, McLellan A, et al. Oxygen-induced retinopathy in the mouse. *Invest Ophthalmol Vis Sci*. 1994; 35:101–111.
23. Yuan JS, Wang D, Stewart CN Jr. Statistical methods for efficiency adjusted real-time PCR quantification. *Biotechnol J*. 2008;3:112–123.
24. Smith ML, Souza FG, Bruce KS, Strang CE, Morley BJ, Keyser KT. Acetylcholine receptors in the retinas of the α7 nicotinic acetylcholine receptor knockout mouse. *Mol Vis*. 2014;20: 1328–1356.
25. Dmitrieva NA, Strang CE, Keyser KT. Expression of alpha 7 nicotinic acetylcholine receptors by bipolar, amacrine, and ganglion cells of the rabbit retina. *J Histochem Cytochem*. 2007;55:461–476.
26. Pons M, Marin-Castaño ME. Nicotine increases the VEGF/PEDF ratio in retinal pigment epithelium: a possible mechanism for CNV in passive smokers with AMD. *Invest Ophthalmol Vis Sci*. 2011;52:3842–3853.
27. Lutty GA, Chan-Ling T, Phelps DL, et al. Proceedings of the Third International Symposium on Retinopathy of Prematurity: an update on ROP from the lab to the nursery. (November 2003, Anaheim, California). *Mol Vis*. 2006;12:532–580.
28. Heidary G, Vanderveen D, Smith LE. Retinopathy of prematurity: current concepts in molecular pathogenesis. *Semin Ophthalmol*. 2009;24:77–81.
29. Rivera JC, Sapieha P, Joyal JS, et al. Understanding retinopathy of prematurity: update on pathogenesis. *Neonatology*. 2011; 100:343–353.
30. Hartnett ME, Penn JS. Mechanisms and management of retinopathy of prematurity. *N Engl J Med*. 2012;367:2515–2526.
31. Hellstrom A, Smith LE, Dammann O. Retinopathy of prematurity. *Lancet*. 2013;382:1445–1457.
32. Klufas MA, Chan RV. Intravitreal anti-VEGF therapy as a treatment for retinopathy of prematurity: what we know after 7 years. *J Pediatr Ophthalmol Strabismus*. 2015;52:77–84.
33. Connor KM, Krah NM, Dennison RJ, et al. Quantification of oxygen-induced retinopathy in the mouse: a model of vessel loss, vessel regrowth and pathological angiogenesis. *Nat Protoc*. 2009;4:1565–1573.
34. Kim CB, D'Amore PA, Connor KM. Revisiting the mouse model of oxygen-induced retinopathy. *Eye Brain*. 2016;8:67–79.
35. Grossniklaus HE, Kang SJ, Berglin L. Animal models of choroidal and retinal neovascularization. *Prog Retin Eye Res*. 2010;29:500–519.
36. Kirkpatrick CJ, Bittinger F, Unger RE, Kriegsmann J, Kilbinger H, Wessler I. The non-neuronal cholinergic system in the endothelium: evidence and possible pathobiological significance. *Jpn J Pharmacol*. 2001;85:24–28.
37. Lips KS, Pfeil U, Reiners K, et al. Expression of the high-affinity choline transporter CHT1 in rat and human arteries. *J Histochem Cytochem*. 2003;51:1645–1654.
38. Mosinger JL, Olney JW. Photothrombosis-induced ischemic neuronal degeneration in the rat retina. *Exp Neurol*. 1989; 105:110–113.
39. Parnavelas JG, Kelly W, Burnstock G. Ultrastructural localization of choline acetyltransferase in vascular endothelial cells in rat brain. *Nature*. 1985;316:724–725.
40. Wessler I, Kirkpatrick CJ, Racke K. Non-neuronal acetylcholine, a locally acting molecule, widely distributed in biological systems: expression and function in humans. *Pharmacol Ther*. 1998;77:59–79.
41. Carty CS, Soloway PD, Kayastha S, et al. Nicotine and cotinine stimulate secretion of basic fibroblast growth factor and affect expression of matrix metalloproteinases in cultured human smooth muscle cells. *J Vasc Surg*. 1996;24:927–935.
42. Dom AM, Buckley AW, Brown KC, et al. The α7-nicotinic acetylcholine receptor and MMP-2/-9 pathway mediate the proangiogenic effect of nicotine in human retinal endothelial cells. *Invest Ophthalmol Vis Sci*. 2011;52:4428–4438.
43. Jho D, Mehta D, Ahmmed G, et al. Angiopoietin-1 opposes VEGF-induced increase in endothelial permeability by inhibiting TRPC1-dependent Ca2 influx. *Circ Res*. 2005;96:1282–1290.
44. Ng MK, Wu J, Chang E, et al. A central role for nicotinic cholinergic regulation of growth factor-induced endothelial cell migration. *Arterioscler Thromb Vasc Biol*. 2007;27:106–112.
45. Campochiaro PA, Shah SM, Hafiz G, et al. Topical mecamylamine for diabetic macular edema. *Am J Ophthalmol*. 2010; 149:839–851.
46. Shih SC, Ju M, Liu N, Smith LE. Selective stimulation of VEGFR-1 prevents oxygen-induced retinal vascular degeneration in retinopathy of prematurity. *J Clin Invest*. 2003;112:50–57.

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