An opsin 5–dopamine pathway mediates light-dependent vascular development in the eye

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During mouse postnatal eye development, the embryonic hyaloid vascular network regresses from the vitreous as an adaption for high-acuity vision. This process occurs with precisely controlled timing. Here, we show that opsin 5 (OPN5; also known as neuropsin)-dependent retinal light responses regulate vascular development in the postnatal eye. In Opn5-null mice, hyaloid vessels regress precociously. We demonstrate that 380-nm light stimulation via OPN5 and VGAT (the vesicular GABA/glycine transporter) in retinal ganglion cells enhances the activity of inner retinal DAT (also known as SLC6A3; a dopamine reuptake transporter) and thus suppresses vitreal dopamine. In turn, dopamine acts directly on hyaloid vascular endothelial cells to suppress the activity of vascular endothelial growth factor receptor 2 (VEGFR2) and promote hyaloid vessel regression. With OPN5 loss of function, the vitreous dopamine level is elevated and results in premature hyaloid regression. These investigations identify violet light as a developmental timing cue that, via an OPN5–dopamine pathway, regulates optic axis clearance in preparation for visual function.

Photons from the sun reach our planet at high flux. In response, organisms have evolved detection systems that decode light information for adaptive advantage. Examples from mammals include the visual system, where photons bouncing off an object are detected to decode object identity, and the circadian system, where the 24-h light cycle entrains time-of-day-dependent physiology. Most light detectors in metazoans are opsins, a class of G protein-coupled receptors that convert the energy of a photon into a cellular signalling response. Rhodopsin, the opsin of mammalian rod photoreceptors, is a well-characterized example of a visual opsin, whereas melanopsin (also known as opsin 4 (OPN4)) has a central role in circadian clock phototainment. Neuropsin (also known as OPN5) is another opsin family member. Relatively little is known about OPN5 except that it responds to violet-light wavelengths (λmax of 380 nm) regulates seasonal breeding behaviour in birds and the activity cycle in mice, but also mediates phototainment of the retinal circadian clock. Here, we have investigated OPN5 function in the development of the mouse eye and shown, like the above examples, that it is required for normal biological timing. In this case, OPN5 is required for a light response that regulates vascular regression timing.

Results

OPN5 is expressed in a retinal ganglion cell subset. OPN5 is expressed in retinal ganglion cells (RGCs) in adult mice. To further assess the features of OPN5-expressing cells, we combined an OPN5cre allele (Supplementary Fig. 1) with Ai14, a tdTomato-expressing cre reporter. According to labelling with multiple markers (Supplementary Fig. 2), the overall architecture of the Opn5-null retina is unchanged. In P5 (5 d postnatal) retinal flat mounts, Opn5cre; Ai14 cells were at relatively low density throughout the inner retina (Fig. 1a). In P12 calretinin-labelled cryosections, OPN5-expressing cell bodies were in the ganglion cell layer (Fig. 1c,d). At P5, Ai14-expressing processes were immature (Fig. 1a), but at P12, the processes were prominent and observed as bundles within the nerve fibre layer (NFL) and within several laminations of the inner plexiform layer (IPL; S1–S5 (the sublaminae of the IPL); Fig. 1c,d). These morphological features are consistent with the characteristics of RGCs.

OPN4 antibody labelling in Opn5mice; Ai14 retinas indicated that OPN4 and OPN5 are expressed in distinct RGC subsets. At P5, the density of Opn5mice; Ai14 and OPN4-labelled cells was similar (Fig. 1b). At P12 (Fig. 1e–h), co-labelling again showed that largely, OPN4 and Opn5mice; Ai14 cells were two distinct subsets. Prominent bundles of axons from Opn5 and Opn4 RGCs are cocarcinulated (Fig. 1e–g). Rare co-labelled cells were identified (Fig. 1f,h about 50 cells per retina), but could result from cre lineage marking oversampling. At P24, Opn5mice; Brainbow-labelled retinal cells (Fig. 1i) have the appearance of mature RGCs with extensive dendritic arbores and axons. Brain cryosections from Opn5mice; Ai14 mice showed axons in the optic tracts, lateral geniculate nucleus and superior colliculus as might be expected for RGCs. Labelling of Opn5mice; Ai6 retinas at P8 with the RGC marker RBPMS (RNA-binding protein with multiple splicing) and the RGC/amacrine cell marker calretinin provided evidence that OPN5 is expressed exclusively in RGCs (Supplementary Fig. 3).

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Normal hyaloid vessel regression timing requires **Opn5** and violet light. We have previously shown that light stimulation of **Opn4** regulates hyaloid vessel regression and retinal angiogenesis²³. Prompted by this, we assessed hyaloid regression in the **Opn5**-null mouse. At P1, **Opn5**-null mice showed normal hyaloid vessel numbers (Fig. 2a,b,g,h) and normal vessel cellularity (Fig. 2c,d,

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**Fig. 1 | Opn5 is expressed in a distinct subset of RGCs.** a,b, Flat mount retina from P5, **Opn5**; **Ai14** mice showing the tdTomato cre reporter (a,b), nuclear labelling with Hoechst 33258 (b), and counter labelling for **OPN4** (b). c,d, Retinal cryosections from P12, **Opn5**; **Ai14** mice showing the tdTomato cre reporter (c,d), nuclei with Hoechst 33258 (c,d) and labelling for calretinin (d). Retinal laminae are indicated by the abbreviation between the panels: GCL, ganglion cell layer; S5-S1, sublaminae of the inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer. e-h, As in a and b, except at P12. Magnified regions of f as indicated by the white corner marks are shown (g,h). i,j, Flat mount retinae showing labelling of cell bodies (asterisks), dendritic fields and axons (arrows) for RGCs labelled by the Brainbow3.2 reporter in P24 **Opn5**; **Brainbow** mice. Scale bars, 20 μm. Panels a-j are representative of at least three separate experiments. Additional examples of these images are available on Figshare (https://figshare.com/articles/NCB_Additional_Images_pptx/7450961).
Precocious hyaloid vessel regression is observed (Fig. 2i–k). Thus, OPN5 is required locally within retinal neurons to regulate hyaloid regression.

To mimic Opn5 loss of function using lighting conditions, we raised mice from birth in the absence of the 380-nm wavelengths that maximally stimulate OPN5 (refs. 11–14). Control mice were raised in a light–dark cycle of 'VBGR' (violet (380 nm), blue (480 nm), green (520 nm) and red (630 nm)) lighting and showed a typical hyaloid vessel number at P8 (Fig. 2l,n, grey bar). By contrast, mice raised in 'BGR' lighting that omitted violet light showed a typical hyaloid vessel number at P8 (Fig. 2l,n, grey bar). By contrast, mice raised in 'BGR' lighting that omitted violet light showed a typical hyaloid vessel number at P8 (Fig. 2l,n, grey bar).

Control: 13.3 ± 1.2, Opn5 null: 13.2 ± 1.6 nuclei per 100-µm length, (P = 0.92). At P8, Opn5-null mice had fewer hyaloid vessels (Fig. 2e–h), indicating precocious regression. This phenotype is unique as all hyaloid phenotypes described so far, including the Opn4-null mouse, show hyaloid persistence. Precocious hyaloid regression in the Opn5-null mouse is best illustrated when vessel numbers are quantified over a P1–P8 time course (Fig. 2g,h, grey line) and compared with the control (Fig. 2g,h, blue line) and the Opn4-null mouse (Fig. 2h, green line). When Opn5 is conditionally deleted in the retina using Chx10-cre or Rx-cre, precocious hyaloid regression is observed (Fig. 2i–k). Thus, OPN5 is required locally within retinal neurons to regulate hyaloid regression.

![Fig. 2](image_url)
the Opn5-null mice and is consistent with a model in which 380-nm photons stimulate OPN5 to suppress hyaloid vessel regression.

A light–OPN5 pathway regulates dopamine levels in the eye. In Opn4-null mice, elevated levels of vascular endothelial growth factor A (VEGFA) explain hyaloid vessel persistence\(^1\). Assessment of the levels of VEGFA and its inhibitor FLT1 (ref. \(^2\)) in the vitreous of Opn5-null mice indicated no change (Supplementary Fig. 4d,e). This suggested that the OPN5–light response pathway regulated hyaloid regression by a distinct mechanism. In the Opn5-null retina,
we noted an unusual labelling pattern for tyrosine hydroxylase (TH). In wild-type (WT) retina at P8, TH immunoreactivity was faint and largely restricted to the perinuclear region of a subset of amacrine cells (Fig. 3a,b). In Opn5-null mice, TH labelling was stronger and prominent in cell processes (Fig. 3c,d). Elevated intensity of TH labelling in the Opn5-null retina was also evident at P15 when dopaminergic amacrine cells are more fully developed (Fig. 3e,f). TH is the rate-limiting enzyme that mediates the first step in the biosynthesis of dopamine. As TH levels are under feed-back regulation, these data indicated that dopamine levels might be modulated in the Opn5-null eye. Interestingly, dopamine is known to have in vitro anti-vascular activity via suppression of VEGF receptor 2 (VEGFR2) signalling. This meant that Opn5-dependent regulation of dopamine could be an explanation for Opn5-dependent regulation of hyaloid regression. Thus, we hypothesized that OPN5-dependent release of dopamine from the retina promoted hyaloid vessel regression by direct signalling.

Dopaminergic amacrine cells that express TH develop in the first few days after birth in the mouse. This also means that, normally, dopamine levels in the retina climb rapidly after birth. Using enzyme-linked immunosorbent assay (ELISA) quantification, we confirmed that retinal dopamine levels rise over a P2–P8 time course (Fig. 3g). Dopamine in solubilized retinal tissue is primarily enzyme-linked immunosorbent assay (ELISA) quantification, we confirmed that retinal dopamine levels rise over a P2–P8 time course (Fig. 3g). Dopamine in solubilized retinal tissue is primarily

A light–OPN5 pathway suppresses dopamine release to the vitreous by enhancing DAT activity. The biological effects of dopamine are regulated by its release, signalling and reuptake. A key regulator of uptake, and thus a good candidate for an OPN5-dependent activity, is the dopamine transporter DAT (also known as SLC6A3). Threonine 53 of DAT is phosphorylated and enhances the rate of dopamine uptake. This activation marker can be detected with a phospho-specific antibody. Basal phosphorylation stoichiometry of TS3-DAT is typically 50% but is increased by stimuli that elevate dopamine uptake. To determine whether phospho-TS3-DAT levels were regulated by light and were OPN5-dependent, we labelled retinal cryosections from cohorts of P8 littermate Opn5+/+ and Opn5−/− mice after they were dark adapted followed by 30 min of 380-nm light at 1 × 1012 photons cm−2 s−1. Labelling for phospho-TS3-DAT was found throughout the IPL andNFL of all samples (Fig. 4a–d). The phospho-TS3-DAT signal was quantified by averaging pixel intensity across horizontal pixel rows of images aligned like those shown (Fig. 4a–d), generating intensity profiles (Fig. 4e, grey and blue profiles) and calculating the area under the peaks (indicated by the grey vertical dashed lines in Fig. 4e,f). This revealed that, for the IPL (Fig. 4g, grey and blue bars), but not for

![Image](https://www.nature.com/naturecellbiology)
the NFL (Fig. 4h, grey and blue bars), the phospho-T53-DAT signal was significantly increased in response to the 380-nm light.

To determine whether light induction of the phospho-T53-DAT signal was OPN5 dependent, we performed the same analysis in Opn5-null mice (Fig. 4c,d,f–h). This revealed that, in the Opn5-null retina, the phospho-T53-DAT signal for the IPL did not elevate in response to light exposure (Fig. 4g, orange and red bars), indicating OPN5 dependence. Interestingly, in the NFL, Opn5-null mice have a lower level of phospho-T53-DAT independent of light exposure (Fig. 4h, compare grey bar and orange bar). However, this low level of phospho-T53-DAT can be rescued by light exposure (Fig. 4h, compare orange and red bars). This indicates that NFL phospho-T53-DAT levels are regulated both by OPN5 (negatively, light independent) and by a distinct light response pathway (positively). Although we do not currently understand the pathway for the positive regulation of phospho-T53-DAT in the NFL (other opsins are obvious candidates), it serves as a useful internal control to show that the Opn5-null retina can be light responsive. An immunoblot detecting phospho-T53-DAT in total solubilized retina from the light phase (Fig. 4i) revealed that, overall, the level is lower in the Opn5-null mice, suggesting that NFL phospho-T53-DAT is the smaller proportion of the total. Collectively, analysis of T53-DAT indicates that OPN5 is required for a light-dependent upregulation within the IPL. As T53-phosphorylated DAT sequesters dopamine in the vitreous, this finding is consistent with a model in which loss of OPN5 function results in diminished dopamine uptake by DAT, and thus elevated levels of vitreal dopamine.

Inhibition of DAT promotes hyaloid regression in an Opn5-dependent manner. To determine whether DAT had a functional role in the regulation of hyaloid regression in vivo, we took advantage of the DAT inhibitor GBR12909 (ref. 38). Based on data showing that dopamine signalling can suppress VEGFR2 activation via the phosphatase SHP2 (ref. 10), we would predict that suppressing DAT activity would elevate dopamine levels in the eye and thus should counter the consequences of dark rearing because the latter elevates levels of VEGFA in the vitreous. In the absence of OPN5, or the violet light that stimulates OPN5, the vitreous dopamine level is precociously elevated. This results in premature activation of the dopamine receptor DRD2 in hyaloid VECs, suppression of VEGFR2 survival signalling and precocious regression. These data indicate that both 480-nm blue light via OPN4 and 380-nm violet light via OPN5 function as developmental timing cues.
at P8 showed that WT mice did not respond significantly (Fig. 4k), but that in heterozygote mice, GBR12909 produced a precocious hyaloid regression equivalent to the Opn5 homozygote phenotype (Fig. 4k). GBR12909 produced no change in homozygote mice (Fig. 4k). Inhibitor activity may be buffered by the intact feedback regulation of the WT mouse[45]. However, in Opn5 heterozygote mice, in which dopamine levels are elevated (Fig. 3k) and feedback regulation may be compromised, the DAT inhibitor was able to convert the hyaloid phenotype from normal to precocious regression. It is likely that the inhibitor produces no effect in the Opn5 homozygote because the endogenous vitreal dopamine level is already high (Fig. 3k) and signaling activity may be close to maximal. These data indicate a finely balanced interaction between Opn5 and DAT that is consistent with OPN5-dependent regulation of DAT activity via phosphorylation.

VGAT in OPN5 RGCs is required for regulation of phospho-T53-DAT and hyaloid regression. Glutamate, γ-aminobutyric acid (GABA) and glycine are neurotransmitters important for visual function. In the adult mouse, glutamate is used as an excitatory neurotransmitter by canonical photoreceptors[41,42] and OPN4 RGCs[43]. GABA and glycine are inhibitory neurotransmitters and their receptors are detected in various retinal neurons, including amacrine cells and RGCs[44]. Glutamate and GABA/glycine are loaded into presynaptic vesicles by the VGLUT (vesicular glutamate transporter) and VGAT (vesicular GABA transporter) family of transporters, respectively. During mouse retinal development, VGAT is expressed soon after birth and precedes the expression of VGLUT2 (ref. [45]). Loss of function of these transporters eliminates neurotransmitter activity[46]. To determine whether OPN5 RGCs might use one of these neurotransmitters for the vascular response pathway, we quantified hyaloid vessels in Opn5−/− conditional deletion mutants of Vglut2 and Vgat. Although deletion of Vglut2[46] had no consequence (Fig. 5a), homozygous deletion of Vgat[46] phenocopied the Opn5 germline null precocious hyaloid regression (Fig. 5b–f). Furthermore, the Opn5−/−; Vglut2−/−; Vgat[46]−/− mice showed a significant precocious regression (Fig. 5d,f). This transheterozygote phenotype is genetic evidence that Opn5 and Vgat function in the same pathway and in the same cell type. If this is true, then we would predict that the Vgat conditional deletion would, like Opn5 loss of function, result in diminished levels of phospho-T53-DAT under lighted conditions. This was confirmed using immunofluorescence labeling of the retina from Opn5−/−; Vgat[46]−/−; Vgat−/− mice (Fig. 5i,j). These data indicate that OPN5 RGCs use VGAT to signal within the vascular regression pathway.

Dopamine has a direct action on hyaloid vascular endothelial cells to promote hyaloid regression. We have hypothesized that dopamine release from the neonatal retina is light and OPN5 dependent and that dopamine then signals directly to hyaloid vascular endothelial cells (VECs) to promote regression. To assess whether a retinal source of dopamine regulated hyaloid regression, we conditionally deleted a Thβ allele. Chx10-cre[47], although effective for studies of TH function in adult mice[48], did not delete Thβ efficiently during the postnatal period. However, Rxs-cre[47] was effective (Fig. 6a,b) and resulted in a hyaloid vessel persistence (Fig. 6c–e), indicating that the active dopamine is produced locally in the retina. To assess the involvement of dopamine receptors in hyaloid regression, we first took advantage of SK38393, a receptor agonist[49]. SK38393 was injected daily into Opn5−/−, Opn5−/− and Opn5−/− mice from P1–P8. Although SK38393 had no significant effect on WT mice (Supplementary Fig. 4f), it produced precocious hyaloid regression in heterozygous mice (Supplementary Fig. 4f). SK38393 did not produce a significant reduction in hyaloid vessel numbers in Opn5-null mice (Supplementary Fig. 4f). This pattern of response is very similar to that observed with the DAT inhibitor (Fig. 4k). Again, this pattern of modulation is probably explained by the resilience of an intact dopamine feedback pathway in WT animals, the sensitized background of the heterozygote and the already saturated level of dopamine signalling in the homozygote. Injection of two different dopamine receptor antagonists from P1–P8 in WT mice produced elevated numbers of hyaloid vessels (Supplementary Fig. 4g). Thus, pharmacological manipulations indicate that hyaloid vessel regression can be regulated both positively and negatively by dopamine receptor modulators.

One prediction of the hypothesis that retinal dopamine regulates hyaloid regression was that dopamine receptors would be expressed within the hyaloid vessels. As dopamine receptor D2 (DRD2) was implicated in the suppression of VEGFR2 signalling[49], we focused on this member of the family. Vascular cells, but not hyaloid-associated myeloid cells, showed Drd2-GFP reporter expression (Fig. 6g,i). Furthermore, labelling with an anti-DRD2 antibody detected cells within the hyaloid vessels (Fig. 6b,h) and this was eliminated in the Drd2−/−; Pdgfb-icreERT2 conditional deletion that targets VECs (Fig. 6i). These data show that DRD2 is expressed in hyaloid VECs. In Drd2−/−; Pdgfb-icreERT2 mice, the hyaloid vessels are persistent (Fig. 6j), but there are no quantifiable consequences for the development of the superficial retinal vasculature (Supplementary Fig. 5). This identifies hyaloid VECs as a dopamine-responsive cell and indicates that dopamine signalling promotes hyaloid regression. A further prediction of the hypothesis is that, in hyaloid VECs, dopamine signalling would suppress the activation of VEGFR2 (ref. [49]). To test this, we performed immunoblotting for VEGFR2 and the activated, phosphotyrosine-1173 form of VEGFR2 (pY1173-VEGFR2), from both Drd2−/− control and Drd2−/−; Pdgfb-icreERT2 hyaloid vessels at P5. Pooling dissected hyaloids from six animals of each genotype allowed threshold detection of the pY1173-VEGFR2 in the control (Fig. 6k, left lane). To assess the reliability of comparative immunoblotting, we performed a three step, twofold loading dilution and quantified immunoblot band intensities. Presented graphically, band intensities for VEGFR2, pY1173-VEGFR2 and β-tubulin showed high Pearson coefficients, indicating a linear relationship between lysate quantity and band intensity (Supplementary Fig. 6). When pY1173-VEGFR2 values were normalized to VEGFR2 (Fig. 6l), Drd2−/−; Pdgfb-icreERT2 genotype values were much higher (Fig. 6l), consistent with the observed band intensities on the immunoblot (Fig. 6k). These data show that deletion of Drd2 in hyaloid VECs permits elevated activation of VEGFR2 and indicates that, normally, dopamine signalling suppresses VEGFR2 activity. In an additional test of this model, we assessed pY1173-VEGFR2 and pS473-AKT levels in the Opn5-null mice. pY1173-VEGFR2 levels were lower in the hyaloid vessels of the Opn5-null mice (Fig. 6m). In addition, across an allelic series, pS473-AKT levels were lower only in the Opn5 homozygote, consistent with precocious hyaloid regression only in this genotype (Fig. 6n). As dopamine levels are high in the Opn5-null mice, these data are consistent with a model in which dopamine promotes hyaloid vessel regression by suppressing VEGFR2 activity and the downstream survival signalling mediated by AKT.

As a genetic test of the relationship between OPN5 and vascular signalling, we determined whether deletion of Drd2 in VECs would reverse precocious hyaloid regression in the Opn5-null mice. We compared P8 hyaloid vessel numbers in mice of genotype Opn5−/−, with Opn5−/−; Pdgfb-icreERT2; Drd2−/− and with Opn5−/−; Pdgfb-icreERT2; Drd2−/−. This experiment confirmed the precocious regression of hyaloid vessels due to Opn5 loss of function (Fig. 6o,p,r, light blue bar), but showed that deletion of Drd2 in VECs could switch the hyaloid phenotype to persistence (Fig. 6q,p, dark blue bar). This outcome establishes that Opn5 and Drd2 function in the same developmental pathway and have opposing influences on hyaloid regression.
Fig. 6 | Retinal dopamine promotes hyaloid vessel regression via DRD2-dependent suppression of VEGFR2 activity. a,b, TH labelling (green) in four regions of P8 flat mount retinas from Th\(^{+/+}\) (a) and Rx-cre; Th\(^{+/+}\) (b) mice. c,d, Hyaloids from P8 control Th\(^{+/+}\) (c) and Rx-cre; Th\(^{+/+}\) (d) mice. e, P8 hyaloid vessel numbers in control (Th\(^{+/+}\) or Th\(^{+/+}\)), Rx-cre; Th\(^{+/+}\) and Rx-cre; Th\(^{+/+}\) mice. f,g, Hyaloid vessels from Drd2-GFP mice showing reporter expression (green) in vessels but not macrophages (circles). GFP, green fluorescent protein. h, Immunoblotting for DRD2 in P8 hyaloids from tamoxifen-treated Drd2\(^{+/+}\) (h) and Drd2\(^{+/+}\); Pdgfb-icreERT2 (i) mice. j, P8 hyaloid vessel numbers in Drd2\(^{+/+}\) and Drd2\(^{+/+}\); Pdgfb-icreERT2 mice. k, Immunoblots for VEGFR2, pY1173-VEGFR2, pS473-AKT levels are lower in Opn5\(^{+/+}\) and Opn5\(^{+/+}\); Pdgfb-icreERT2 mice. l, Band intensity normalized to VEGFR2. Numbers at the base of each chart bar is \(n\). Error bars are s.e.m. Images are representative of at least three separate experiments.

One implication of immunoblotting data for pY1173-VEGFR2 (Fig. 6k) and the genetic analysis (Fig. 6a–r) is that a balance of VEGFA and dopamine signalling determines the fate of the hyaloid vessels. To determine whether we could demonstrate this balance at the level of receptor ligands, we designed a rescue experiment. We generated an elevated level of VEGFA activity, and thus hyaloid vessel persistence, by conditionally deleting the gene encoding the naturally occurring VEGFA inhibitor FLT1 in the retina. Chx10-cre deletion of Flt1\(^{+/+}\) produces hyaloid persistence (Supplementary Fig. 4a–c), but in this case, we used Rx-cre (Fig. 6s–v, light blue bar). To determine whether dopamine receptor signalling could reverse the hyaloid persistence, we injected (each day, from P1 to P8) a littermate cohort of Rx-cre; Flt1\(^{+/+}\) mice with the dopamine receptor agonist SKF38393. This resulted in a reversal of the hyaloid persistence (Fig. 6s–v), an outcome that illustrates the balance of VEGFA and dopamine signalling that regulates hyaloid regression.
Discussion

We have identified an unanticipated vascular development pathway in the eye. OPN5, an atypical opsin known to respond to near-UV photons\textsuperscript{11–14}, initiates the pathway response and functions postnata-

ally (Fig. 5l). Dopamine, a broadly functional neurotransmitter and neuromodulator, is a signalling intermediate that is regulated by OPN5 and elicits a direct response in hyaloid VECs to limit VEGFR2 signalling via DRD2 (Fig. 5l). Based on OPN5-dependent and light-dependent phosphorylation at TS3, and its pharmacologi-
cal inhibition, our data also indicate that the dopamine transporter DAT is a key component of this pathway that normally suppresses the levels of dopamine in the vitreous (Fig. 5l). Therefore, the GABA transporter VGAT is implicated in OPN5 RGC signalling as its conditional deletion in OPN5 RGCs phenocopies the Opn5-null precocious hyaloid regression and low phospho-TS3-DAT level. Thus, the light–OPN5–VGAT–dopamine–DRD2–VEGFR2–hya-
loid pathway is characterized by two suppressive steps: light–OPN5 suppresses the levels of dopamine in the vitreous, whereas dopa-
mine suppresses VEGFR2 signalling in the hyaloid vessels (Fig. 5l).

Light-dependent regulation of vitreal dopamine occurs against a backdrop of generally rising levels of dopamine in the postnatal eye. This means that, whereas the function of dopamine is to pro-

mote hyaloid vessel regression, the effect of 380-nm photons and OPN5 is to suppress regression of the hyaloid vessels. It is likely that this has evolved as a mechanism to optimize the timing of hyaloid vessel regression and ensure that they remain functional postna-
tally until the superficial retinal vascular plexus is complete. We have previously shown that OPN4 also mediates light-dependent vascular development in the eye\textsuperscript{26} and suppresses VEGFA levels because it keeps retinal cellularity in check, and thus limits the oxygen

demand that can elevate levels of VEGFA (Fig. 5l). The crucial window for activation of the OPN4 response is in late gestation and requires a direct light stimulation of the mouse fetus\textsuperscript{26}. Thus, the OPN4 and OPN5 response pathways use distinct mediators to regulate vascular development and, as they function at different stages of development, can be thought of as developmental timing cues (Fig. 5l). Notably, the spontaneous waves of neuronal activity that arise in the neonatal mouse retina\textsuperscript{52,53} are partly dependent on OPN4 modulation of gap junctions that are, in turn, regulated by dopamine\textsuperscript{53}. In the future, it will be interesting to assess the relationship of retinal wave activity to vascular development.

OPN5 is highly conserved and we might anticipate that the path-
way that we describe (Fig. 5l) will be relevant to human biology. The latter steps in the pathway involving DRD2-dependent suppression of VEGF2 activity may be an explanation for the observation that premature infants treated with dopamine (for hypotension) have a higher risk of retinopathy of prematurity\textsuperscript{55,56}, a vascular overgrowth disease. We suggest that therapeutic dopamine promotes regression of the hyaloid vessels and thus exacerbates the hypoxia that leads to rebound vascular overgrowth. Furthermore, we have previously shown that the risk of retinopathy of prematurity in premature infants is partly dependent on their season of gestation, with short days and lower light exposure associated with higher risk\textsuperscript{57}. It is pos-

sible that the OPN5–dopamine pathway is a component of this risk equation because insufficient light would be expected to result in elevated levels of vitreal dopamine, precocious hyaloid regression and thus a more profound hypoxia in the premature eye. An understand-
ing of the relationship between OPN4-dependent and OPN5-depen-
dent regulation of vascular development in the eye (Fig. 5l) raises the interesting possibility that premature infants at risk for retinopathy of prematurity might be treated with a light therapy that differentially targets each pathway response. Finally, it has been proposed that both violet light in the 360–400 nm range\textsuperscript{58} and dopamine\textsuperscript{59} are key regulators of refractive development and that each can suppress progression to myopia. The current observations suggest that the OPN5–dopamine pathway is likely to be involved.
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Methods

Mice. Animals were housed in a pathogen-free vivarium and all pharmacological treatments were in accordance with protocols approved by the Institutional Animal Care and Use Committee at Cincinnati Children’s Hospital Medical Center. This study is compliant with all relevant ethical regulations regarding animal research. Day of birth is defined as P1. Genetically modified mice used in this study were: C57BL/6-Tg(129PgdIbcreERT2)2Flk1 (jax source 010515), Pdgfb-icreERT2(T2)J-, R2-cre, Ai4 (jax stock 007914), Brainbow (jax stock 021227; Brainbow 3.2), Dn2(+/–) (TgDn2-EGFP-311St8a1), Dn2(–/–) (jax stock 020651), Flt1(loxp) (loxP camKIIα-CreERT2) (Jax stock 003927), Vegaf2 (jax stock 012898), Vegaf2 (jax stock 012897) and Opn5 (ref. 21) and Opn5m62.1 (generated from C57BL/6 embryonic stem cells obtained from KOMP (embryonic stem clone ID: KOMP-(HTGRS6008_A_B12-Opn5-ampicillin). The embryonic stem cells harbour a genetic modification in which a loxp cassette is flanked by an FRT site located between exons 3 and 4, and a FRT site separates Lacz from the neomycin cDNA coding region (Supplementary Fig. 1). Loxp sites also flank exon 4 of Opn5, allowing multiple mouse lines that can be used for reporter nulls, conditional floxed and null mice. The Opn5m5 allele was created by crossing the Opn5m5 allele to FLP mice (jax stock 003946) to remove the LacZ cassette. The Opn5m5 allele line was created by crossing the Opn5m5 mice to E2a-cre (jax stock 003724). Littermate control animals were used for all experiments with the exception of C57BL/6 mice, which were reared under different lighting conditions.

The genotyping primers and protocol for alleles except Opn5 are described in the cited publication or on the Jackson Labs website. Primer sequences for genotyping the Opn5m5 or Opn5mf5 alleles are: F1: GAGGCATCTGCCACTCT; R1: CACAGTATGTGTGACAACCT; R2: GTGGACAGATTAACTGAAGC; R3: GTGAAAGAGATGCATTTGTGAG; R4: TGGAGTCCTACTCGCGGACG. Sanger sequencing was performed to validate the knock-in sequence of founder mice. The genotyping primers and protocol for alleles except Opn5 are described in the cited publication or on the Jackson Labs website.

Lighting conditions. Animals were housed in standard fluorescent lighting (photon flux: 1.62 × 1011 photons cm−2 s−1) in a 12 light/12 dark cycle except where noted. For full-spectrum lighting (VBGR), LEDs were used to yield a comparable total photon flux of 1.68 × 1011 photons cm−2 s−1. Spectral and photon flux information for LED lighting: violet (λmax = 380 nm, 4.23 × 1011 photons cm−2 s−1 in 370–400 nm range), blue (λmax = 480 nm, 5.36 × 1011 photons cm−2 s−1 in 430–530 nm range), green (λmax = 530 nm, 5.82 × 1011 photons cm−2 s−1 in 480–600 nm range) and red (λmax = 630 nm, 1.93 × 1011 photons cm−2 s−1 in 590–660 nm range). For wavelength-restricted hyaloid assessment, C57BL/6 animals were housed in a 12 light/12 dark cycle starting at late gestation (embryonic day 18 (E18)) either in full spectrum (VBGR) or without violet (BGR) lighting. For dark-reared experiments, pregnant dams were moved to the dark at gestation age E16. For light induction experiments, on P7 at lights off, nursing females and pups were moved to the dark for 24 h dark adaptation. Opn5m5 and Opn5m5 pups were subjected to ≤30 min of 380-nm light at 1 × 1011 photons cm−2 s−1 (approximately 1% of clear sky summer day sunlight) at this wavelength) at 2 h after subjective lights off.

Immunohistochemistry and imaging. Animals were anesthetized under isoflurane and killed by cervical dislocation or decapitation for early post-natal pups. Preparation and immunofluorescence staining of retinae and hyaloid vessels were as described previously20. For phospho-T53-DAT quantification, the retina of each genotype and light condition were collected in dim red light (dark adapted) or normal light from at least three different induction experiments and mounted in the same OCT plate at matching temporal indices. Retinal sections were processed, stained and imaged together to compensate for batch differences. Alexa-conjugated secondary antibodies were purchased from Jackson Immunoresearch. Images were captured using Zeiss ApoTome AX10 or Zeiss LSM700 confocal microscopes and processed by ImageJ (NIH) and Adobe Photoshop (Adobe Systems). The primary antibodies and lectins used in this study are listed in Supplementary Table 1.

ELISA. Vitreous and retina from pups were collected and rapidly frozen on dry ice. To detect dopamine levels, vitreous samples were pooled from three to six pups depending on age and six retinae for each n. Dopamine extraction and ELISA were performed according to the manufacturer’s protocol using BA E-5300 (Rocky Mountain Diagnostics). For dark-adapted experiments, the vitreous and retina were collected under dim red light. To detect VEGFA and FLI1 levels, samples from P5 pups were pooled from six eyes for each n. The mouse VEGFA kit Quantikine (MV900) and the mouse VEGFR1 (FL1T1) kit Quantikine (MV1R00) from R&D systems were used. ELISA was read by using the EnVision Multimode Plate Reader (Perkin Elmer).

Pharmacological reagents and experiments. All dopamine pharmacological modulators, except for the antagonist 2-CMDO (2-chloro-11-(4-methylpiperazino) dibenz(Z)[f]loxepine maleate), were injected at 1 mg per kg body weight intraperitoneally into nursing dams on the day of birth and at P2, then directly into pups until P8. 2-CMDO was injected into pups at P5–P8 at 2 mg per kg body weight. Injection was done in dim red light 1 h before the lights were turned on. Dopamine agonist SKF38393 hydrobromide, the high-affinity D2 antagonist L-741626, the dopamine transporter inhibitor GBR12909 dihydrochloride and 2-CMDO were all purchased from Tocris Biosciences. For experiments with Pdgfb-icreERT2 mouse lines, 2 mg tamoxifen was injected into nursing dams on the day of birth and on P2 to activate tamoxifen-dependent cre.

Western blotting. Western blot experiments were performed using standard protocols. Immediately after dissection, 12 hyaloid vascular tissues or retinae of P6 (6 pups) were pooled in 100 μl of 1× Laemml sample buffer and sonicated. After 20 μl supernatant was loaded on 4–20% gradient protein gel (THERMO Fisher Scientific). Separated protein bands were transferred to a PVDF (polyvinylidene difluoride) membrane, and bands were visualized by chemiluminescence (THERMO Fisher Scientific). Unprocessed immunoblots are available in Supplementary Fig. 6. Band intensity was measured by ImageJ (NIH). The following antibodies were used for western blotting: VEGFR2 (9698, Cell Signalin Technology), phospho-VEGFR2 (2478, Cell Signaling Technology), β-actin (ab6046, Abcam), DAT (NB300-254, Novus), phospho-DAT (PAS-53414, Thermo Fisher Scientific), AKT (4061, Cell Signaling Technology) and phospho-AKT S473 (4060, Cell Signaling Technology). All antibodies were used at 1:1,000 dilution.

Statistics and reproducibility. Samples for immunoblots were pooled from multiple animals (six pups for hyaloid vasculature and six retinae) and each experiment was repeated at least twice with independent samples. Unprocessed western blots are available in Supplementary Fig. 6. ELISA assessment was performed at least twice with independent biological samples. Each n in this analysis is a separate animal with the exception of immunoblots and ELISA, for which pooled samples from animals of the same genotype represent one n. Retinal images with immunofluorescence labelling represent n = 3 independent biological samples from separate litters. Data gathered for hyaloid vessel quantification represent samples from multiple litters to reach n indicated on charts for each genotype and condition. Data are presented as mean ± s.e.m. (standard error of the mean) in aligned dot plots overlaid with a bar or line graph. Statistical analyses were performed using GraphPad Prism version 4.00 (GraphPad Software) and Microsoft Excel for two-tailed Student’s t-test, one-way or two-way analysis of variance (ANOVA) as indicated. Two-tailed distribution, two-sample unequal variance t-test was used to determine the statistical significance between two independent groups except for Fig. 6m, which is one-tailed. Sidak’s or Tukey’s multiple comparison test were performed post-hoc when significance differences were found in ANOVA.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Source data for all figures have been provided as Supplementary Table 2. Additional experimental repeats for key retinal labelling experiments have been deposited on Figshare (https://doi.org/10.6084/m9.figshare.7450961). All other data supporting the findings of this study are available from the corresponding author on reasonable request.

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Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
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- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection
Perkin Elmer Envision Plate Reader: Wallac Envision Manager version 1.12. Zeiss Zen Software for image acquisition

Data analysis
Fiji by ImageJ (NIH) Photoshop CS3 (Adobe) Excel 2016 (Microsoft) GraphPad Prism v4.00 (GraphPad Software, Inc.)

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Source data for all figures have been provided as Supplementary Table 2. Additional experimental repeats for key retinal labelling experiments have been deposited on Figshare (doi:10.6084/m9.figshare.7450961). All other data supporting the findings of this study are available from the corresponding author on reasonable request.
Field-specific reporting

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Life sciences disclose on these points even when the disclosure is negative.

Sample size
Sample sizes were chosen based on previous publications of hyaloid analysis and by using Power and Sample Size Calculator (http://www.statisticalsolutions.net/): Rao, S et al. A direct and melanopsin-dependent fetal light response regulates mouse eye development. Nature 494, 243-6 (2013)

Data exclusions
No data was excluded

Replication
For all genetic models and pharmacological treatments, multiple litter cohorts were measured to eliminate the effect of litter, and demonstrate reproducibility. For all immunohistchemistry, tissues from multiple animals (n >= 3) across different litters were collected and analyzed. Drd2-eGF hyaloid images were from 2 Drd2-eGFP+ eyes (A generous gift from Dr. D. Copenhagen). For Western blot experiments, hyaloids were pooled from different litters (n = 8 eyes) for each genotype and experiments were repeated three times. For VEGFA and FLT1 ELISA, vitreal for each genotype was pooled from 6 individual eyes for each n, n = 3. For Dopamine extraction and ELISA, vitreal fluid for each genotype was pooled from >= 6 eyes for each n, n = 3 except for Opn5+/+ (n=2). Retinal tissue from six individual eyes were pooled for each n of dopamine extraction and ELISA. 2-3 independent ELISA were performed, depending the genotype and tissues. For pDAT quantification, retinae of each genotype and light condition were collected from at least 3 different induction experiments and mounted in the same OCT blocks. Retinal sections were processed, stained, and imaged together to compensate for batch differences. For C57BL/6J LD and DD dopamine time course, pups from each litter were split randomly to different time points.

Randomization
The studies conducted in this manuscript compared wildtype (control) and mutant (experimental) animals, which were allocated into groups based on genotype. There was randomization while assigning litters from genetic models to different experiments. Pups from C57BL/6J litters were randomly selected and designated for pharmacological or vehicle treatment. Animals were randomized into different experiments from a cohort of litters, where one control and experimental animal from each litter was designated for a particular experiment while the littermates were assigned to another purpose. For light induction experiments, pups of different genotype were randomly assigned to dark-adapted only or dark-adapted plus light-induced from different litters to achieve enough sample size.

Blinding
For all pharmacological treatments, the investigators were blinded to the genotype during the course of treatment except for C57BL/6J. For Western blot and ELISA investigators were not blinded to the genotypes of the animals since samples were pooled. Investigators were not blinded for experiments with differential lighting conditions (hyaloid vessel and retinal labelling). Investigators were blinded to the genotype for other hyaloid vessel and retinal vessel quantification, and genotype was assigned to data at the end point.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
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| ☑   | Eukaryotic cell lines |
| ☑   | Palaeontology         |
| ☑   | Animals and other organisms |
| ☑   | Human research participants |
| ☑   | Clinical data         |

Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☑   | ChIP-seq              |
| ☑   | Flow cytometry        |
| ☑   | MRI-based neuroimaging |

Antibodies

Antibodies used

Primary antibody for IF Source Calretinin (1:100) MAB1568 (Millipore) Drd2 (1:200) ADR-002 (Alomone) ChAT (1:200) AB144 (Millipore) DAT/SLC6A3 (1:200) MAB369 (Millipore) RBPMS (1:200) AB194213 (Abcam) Melanopsin (1:1000) AB-N38 (Advance Targeting Systems) pDAT (1:500) PAS-35414 (Thermo Fisher Scientific) Tyrosine Hydroxylase (1:1000) AB1542 (Millipore) Primary antibody for Western Source AKT (1:1000) #4691 (Cell Signaling Technology) pAKT-Ser473 (1:1000) #4060 (Cell Signaling Technology) DAT (1:1000) NB300-254 (Novus) pDAT (1:1000) PAS-35414 (Thermo Fisher Scientific) VEGFR2 (1:1000) #9698 (Cell Signaling Technology) ˜-Tubulin (1:1000) ab6046 (Abcam)
Validation

All antibodies were validated by both the manufacturer and using negative controls in this study.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)
MK4 cells were used within the CCHMC Gene Targeting Core Facility to test the efficacy of guide RNAs for CRISPR

Authentication
MK4 cells were generated at CCHMC (from mouse metanephric mesenchyme) and so are the original source material

Mycoplasma contamination
We do not have information on whether this cell line was tested for mycoplasma contamination.

Commonly misidentified lines
(See ICLAC register)
MK4 are an original, in house isolate

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals
Animals were housed in a pathogen-free vivarium and all pharmacological treatments were in accordance with CCHMC institutional policies. Afternoon on day when pups were seen in the morning is defined as P1. Genetically modified mice used in this study were: Chx10cre1 (Jax stock #00515), PdgfbicreER(T2)2, Rxcre3, A144 (Jax stock #007914), Brainbow5 (Jax stock #021227 Brainbow 3.2), Drd2EGFP [ref 6] (Tg[Drd2-EGFP];S118Gsat), Drd2loxp (Jax stock #020631), Flt1lox (ref 8) (Jax stock #02809 Vegfr-1lox), Tnflox (ref 9) Opn410 and Opn5tm1a(KOMP)Wtsi that were generated from C57BL/6N ES cells obtained from KOMP (ES clone ID:KOMP-(HTGRS6008_A_B12-Opn5-amplicillin). The ES cells harbour a genetic modification wherein a LacZ-Neomycin cassette is flanked by FRT sites, between exon 3 and exon 4 and a loxp site separates LacZ from the neomycin coding region. Loxp sites also flank exon 4 of Opn5 allowing multiple mouse lines that can serve as reporter nulls, conditional floxed and null mice. The Opn5Sfl allele was created by crossing the Opn5tm1a(KOMP)Wtsi mice to FLPeR11 (Jax stock #003946) to remove the LacZ cassette. The Opn5Sfl mice were used for all experiments with the exception of C57BL/6J mice reared under different lighting conditions. The Opn5cre was generated in-house using CRISPR-Cas9 technology. Four gRNAs that target exon 1 of Opn5 were selected to knock in the Cre cassette. Plasmids containing the gRNA sequence were transfected into MK4 cells (an in-house mouse cell line representing induced metanephric mesenchyme undergoing epithelial conversion). The editing efficiency of gRNA was determined by T7E1 assay of PCR products of the target region amplified from genomic DNA of transfected MK4 cells. The sequence of the gRNA that was subsequently used for the transfection is TGGAGTCCTACTCGCGGACG. Sanger sequencing was performed to validate the knock-in sequence of founder mice. Mice were placed on normal chow diet (NCD: 29% Protein, 13% Fat and 58% Carbohydrate kcal; LAB Diet #5010) ad libitum with free access to water. With the neonatal stage for this analysis, animals were not matched for sex.

Wild animals
Study did not involve wild animals

Field-collected samples
Study did not involve samples collected in the field

Ethics oversight
IACUC and CCHMC institutional policies

Note that full information on the approval of the study protocol must also be provided in the manuscript.