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Abstract: Ischemia-induced angiogenesis is critical for tissue repair, but aberrant neovascularization in the retina causes severe sight impairment. Nitric oxide (NO) has been implicated in neovascular eye disease because of its pro-angiogenic properties in the retina. Nitric oxide production is inhibited endogenously by asymmetric dimethylarginine (ADMA) which is metabolized by dimethylarginine dimethylaminohydrolase (DDAH). The aim of this study was to determine the roles of ADMA and DDAH2 in retinal ischemia-induced angiogenesis. First, vitreous ADMA levels were assessed in patients with proliferative diabetic retinopathy and control subjects. ADMA was found to be significantly elevated in the vitreous of human subjects with retinal ischemia and pathological neovascularization associated with diabetes compared with non-diabetic controls. Next, ADMA and DDAH2 levels were determined in adult C57BL/6J mice and DDAH2 deficient mice were characterized by in vivo fluorescein angiography, immunohistochemistry and retinal function by electroretinogram. The results obtained revealed that retinal ADMA and neurovascular development were unchanged between DDAH2 deficient mice and wildtype control mice under physiological conditions. Finally, DDAH2 deficient mice were studied in the oxygen-induced retinopathy (OIR) model, a model for retinal ischemia and neovascularization, and VEGF and ADMA levels were quantified by ELISA and liquid chromatography tandem mass spectrometry. In the OIR model, DDAH2 deficiency resulted in elevated retinal ADMA which was associated with attenuated aberrant angiogenesis and improved vascular regeneration in a VEGF independent manner. Taken together this study indicates, that in retinal ischemia, DDAH2 deficiency elevates ADMA, promotes vascular regeneration and protects against aberrant angiogenesis. Therapeutic inhibition of DDAH2 may therefore offer a potential therapeutic strategy to protect sight by promoting retinal vascular regeneration and preventing pathological angiogenesis.

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UCL INSTITUTE OF OPHTHALMOLOGY
DEPARTMENT OF GENETICS

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Editor-in-Chief
Experimental Eye Research

Re: "Dimethylarginine dimethylaminohydrolase-2 deficiency promotes vascular regeneration and attenuates pathological angiogenesis"

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Dear Prof Hollyfield, dear ladies and gentlemen.

I should be very grateful if you would consider this manuscript for publication in Experimental Eye Research.

This is an original submission and has not been considered elsewhere.

Kind regards,

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Highlights: Dimethylarginine dimethylaminohydrolase-2 deficiency promotes vascular regeneration and attenuates pathological angiogenesis

Clemens Lange[a, c], Freya Mowat[a], Haroon Sayed[a], Ulrich Luhman[a], M Nandi[d], Peter Kelly[b], Alexander Smith[a], Robin Ali[a], James Leiper[b], James Bainbridge[a]

- Nitric oxide has been implicated in neovascular eye disease.
- Key inhibitor of NO production is ADMA, which is metabolized by DDAH.
- ADMA is elevated in the vitreous of patients with proliferative diabetic retinopathy.
- DDAH2 deficiency results in elevated ADMA and reduced neovascularisation in mice.
- Therapeutic inhibition of ADMA or DDAH2 may offer a potential therapeutic strategy.

Dimethylarginine dimethylaminohydrolase-2 deficiency promotes vascular regeneration and attenuates pathological angiogenesis

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Abstract

Ischemia-induced angiogenesis is critical for tissue repair, but aberrant neovascularization in the retina causes severe sight impairment. Nitric oxide (NO) has been implicated in neovascular eye disease because of its pro-angiogenic properties in the retina. Nitric oxide production is inhibited endogenously by asymmetric dimethylarginine (ADMA) which is metabolized by dimethylarginine dimethylaminohydrolase (DDAH). The aim of this study was to determine the roles of ADMA and DDAH2 in retinal ischemia-induced angiogenesis. First, vitreous ADMA levels were assessed in patients with proliferative diabetic retinopathy and control subjects. ADMA was found to be significantly elevated in the vitreous of human subjects with retinal ischemia and pathological neovascularization associated with diabetes compared with non-diabetic controls. Next, ADMA and DDAH2 levels were determined in adult C57BL/6J mice and DDAH2 deficient mice were characterized by *in vivo* fluorescein angiography, immunohistochemistry and retinal function by electroretinogram. The results obtained revealed that retinal ADMA and neurovascular development were unchanged between DDAH2 deficient mice and wildtype control mice under physiological conditions. Finally, DDAH2 deficient mice were studied in the oxygen-induced retinopathy (OIR) model, a model for retinal ischemia and neovascularization, and VEGF and ADMA levels were quantified by ELISA and liquid chromatography tandem mass spectrometry. In the OIR model, DDAH2 deficiency resulted in elevated retinal ADMA, which was associated with attenuated aberrant angiogenesis and improved vascular regeneration in a VEGF independent manner. Taken together this study indicates, that in retinal ischemia, DDAH2 deficiency elevates ADMA, promotes vascular regeneration and protects against aberrant angiogenesis. Therapeutic inhibition of DDAH2 may therefore offer a potential therapeutic strategy to protect sight by promoting retinal vascular regeneration and preventing pathological angiogenesis.

Keywords: DDAH2, ADMA, Nitric oxide, angiogenesis, retinal neovascularization, diabetic retinopathy

1. Introduction

Adaptive tissue responses to ischemia promote blood flow and angiogenesis that are critical for normal development, tissue repair and regeneration. In the mature retina, however, ischemia-induced angiogenesis is typically misdirected into the vitreous gel where it not only fails to redress retinal ischemia but also exacerbates impairment of sight with haemorrhage into the vitreous gel and tractional retinal detachment (Foster and Resnikoff, 2005). Pathological angiogenesis is the result of a complex interplay of molecular mediators, cellular interactions and extracellular matrix modulation, and is the target of novel therapeutic approaches (for a review see (de Oliveira Dias et al., 2011)). Local therapeutic inhibition of vascular endothelial growth factor (VEGF) attenuates pathological neovascularization (Avery et al., 2006) but this strategy fails to promote effective revascularization of ischemic retina.

The ubiquitous biological messenger nitric oxide (NO) promotes vascular dilatation by cGMP-induced smooth muscle relaxation(Archer et al., 1994). In the eye, NO promotes angiogenesis in experimental models of pathological neovascularization (Ando et al., 2002) and is elevated in the vitreous of human subjects with proliferative diabetic retinopathy (Hernandez et al., 2002). NO synthesis from L-arginine is catalyzed by 3 isoforms of nitric oxide synthase (NOS) with distinct tissue distributions: endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). Asymmetric dimethylarginine (ADMA) and other asymmetrically methylated arginine analogs (L-NMMA) are key regulators of NO synthesis as they competitively inhibit the binding of L-arginine for the active site of NOS (Vallance et al., 1992). Asymmetric methyl-arginines are endogenously produced on degradation of proteins containing asymmetrically methylated L-arginine residues, and are metabolized to citrulline and dimethylamine by dimethylarginine dimethylaminohydrolases-1 (DDAH1) and -2 (DDAH2) (Fig. 1) (Leiper et al., 1999)(Ogawa et al., 1987). These two DDAH isoforms have distinct tissue distributions (Leiper et al., 1999) suggesting isoform-specific regulation of NOS. DDAH1 is predominantly found in tissues that

express nNOS whereas DDAH2 is found in high levels in tissues expressing eNOS, which has a role in promoting angiogenesis in the retina (Fukumura et al., 2001)(Brooks et al., 2001).

Here we show that in retinal ischemia, DDAH2 deficiency elevates ADMA, promotes retinal vascular regeneration and protects against aberrant neovascularization.

2. Material and Methods

***2.1 Human study population and sample collection*:** Twenty-four human subjects having surgery for advanced proliferative diabetic retinopathy (PDR) and 10 non-diabetic subjects having surgery for idiopathic full-thickness macular hole or epiretinal membranes were enrolled after informed consent. Samples of undiluted vitreous were obtained from the mid-vitreous using a 20-gauge vitreous cutter. After centrifugation, supernatants were stored at -80 °C.

***2.2 Generation and identification of DDAH2 knockout mice*:** Heterozygous *DDAH2*$^{+/-}$ genetic knockout mice were obtained from the Texas Institute for Genomic Medicine (http://www.tigm.org/) and bred to generate homozygous *DDAH2*$^{-/-}$, heterozygous *DDAH2*$^{+/-}$ and wildtype *DDAH2*$^{+/+}$ mice. All animals were managed in accordance with the guidelines of the Association for Research in Vision and Ophthalmology. In all experiments, weight-matched homozygous, heterozygous and wildtype littermates were compared to circumvent inter-litter variability.

***2.3 Oxygen-induced retinopathy mouse model (OIR)*:** Nursing dams and their pups were kept at 75 ±3% O_2 in an oxygen supply chamber from postnatal day (p) 7 to p12, returned to room air on p12 and culled at p12 or p17 as described elsewhere (Lange et al., 2009). The area of ischemia and neovascularization was studied as previously described (X. Wang et al., 2013).

2.4 Laser-induced choroidal neovascularization (CNV): Laser-CNV induction and *in vivo* fundus fluorescein angiography 14 days after laser was performed as previously described (X. Wang et al., 2013).

2.5 Electroretinography**:** Standard photopic and scotopic Ganzfeld ERG's were recorded bilaterally in dark-adapted mice using the electrophysiological system Espion 2000 (Espion E^2, Diagnosys LLC, Cambridge, UK) as previously described (Mowat et al., 2012).

2.6 Chemical analysis**:** Methylarginines were quantified using liquid chromatography tandem mass spectrometry as previously described (Caplin et al., 2012). Western blotting protein analysis was performed for DDAH2 in retinal and choroidal tissue as previously described (Mowat et al., 2010). VEGF protein levels were determined using a commercially available ELISA kit (mouse VEGF DuoSet ELISA kit, R&D, Systems Europe, Abingdon, UK) and were corrected for total protein levels.

2.7 Immunohistochemistry**:** Eyes of anaesthetized animals were fixed by intracardiac perfusion using 1% paraformaldehyde. Haematoxylin and eosin staining histology and immunohistochemistry were performed as previously described (Lange et al., 2012).

2.8 Statistical analysis**:** Data from knockout animals were normalized to littermate controls. Data were compared using the non-parametric Mann-Whitney U test. Mean variables of more than two groups were compared by ANOVA with Bonferroni corrections for multiple comparisons. P-values less than 0.05 were considered statistically significant.

3. Results

3.1 Vitreous ADMA is raised in human eyes with proliferative diabetic retinopathy

To investigate the role of ADMA in retinal ischemia in human eye disease we measured ADMA in the vitreous of human subjects. In proliferative diabetic retinopathy vitreous ADMA was elevated by 58% compared to non-diabetic control subjects (0.03 ±0.002 vs. 0.019 ±0.001 μM, p= 0.0004, Fig.2).

3.2 Methylarginines and DDAH isoforms are differentially distributed in the murine eye

Next, we investigated the distributions of ADMA, L-NMMA and their catabolizing enzyme DDAH2 in the retina and choroid/RPE of normal adult C57BL/6J mice by liquid chromatography tandem mass spectrometry, Western blotting and immunohistochemistry. ADMA was evenly distributed throughout the murine retina and choroid; L-NMMA was also evenly distributed in the retina and choroid, at concentrations substantially higher than ADMA (Fig. 3A). DDAH2 protein was present in the retina at a higher level than in the choroid and retinal pigment epithelium (Fig. 3B). Immunohistochemistry demonstrated localization of DDAH2 to the ganglion cell layer and photoreceptor layers (Fig. 3C-D).

3.3 DDAH2 is not essential for normal retinal development and function

We next explored the role of *DDAH2* in normal retinal development and function. We identified no abnormality of retinal development or retinal vasculature in adult homozygous *DDAH2*$^{-/-}$ or heterozygous *DDAH2*$^{+/-}$ mice on fundus imaging, *in vivo* fluorescein angiography, immunohistochemistry or electroretinography (Fig. 3E-H, Fig. S1A-E).

3.4 DDAH2 promotes pathogenic retinal neovascularization

Having established that loss of DDAH2 has no effect on retinal vascular development in *DDAH2*$^{-/-}$ mice, we next investigated the role of DDAH2 in murine oxygen-induced retinopathy (OIR), a model of retinal ischemia-induced neovascularization. In OIR, exposure of young mice to hyperoxia (75% inhaled oxygen) from postnatal day 7 (p7) results in ablation of immature retinal vasculature. On return to room air at p12 the ischemic retina becomes hypoxic, leading to upregulation of adaptive angiogenic processes. Neovascularization, however, fails to revascularize ischemic retina appropriately and instead is misdirected into the vitreous, in a pattern that recapitulates key features of proliferative diabetic retinopathy. We investigated the role of DDAH2 in retinal vascular regeneration by characterizing the response to OIR in *DDAH2*$^{+/-}$ knockout mice. At p12, following 5 days exposure to hyperoxia, heterozygous *DDAH2*$^{+/-}$ knockout mice were similarly susceptible to oxygen-induced retinal vascular ablation as their littermate (*DDAH2*$^{+/+}$) controls. At p17 however, heterozygous *DDAH2*$^{+/-}$ knockout mice developed greater revascularization of the area of retinal vascular ablation, resulting in less extensive ischemia, and less extensive aberrant pre-retinal neovascularization. Having identified an effect of *DDAH2* haploinsufficiency we then determined that in *DDAH2* null (*DDAH2*$^{-/-}$) mice the magnitude of this response to OIR was greater still (Fig. 4A-I). These data indicate that dose dependent reduction of *DDAH2* promotes appropriate revascularization and reduces aberrant angiogenesis in retinal ischemia.

3.5 DDAH2 deficiency does not alter retinal VEGF levels in the OIR model

Since DDAH2 can induce expression of vascular endothelial growth factor (VEGF), which is well recognized for its pro-angiogenic role in OIR, we next investigated retinal VEGF protein levels in *DDAH2*-deficient mice during OIR. The concentration of VEGF protein was significantly raised in the retina during the hypoxic phase of OIR. However, the concentration of VEGF was unaffected by *DDAH2* deficiency (Fig. 4J) indicating that the observed attenuated neovascular response is independent of local VEGF.

3.6 Retinal ADMA is increased by DDAH2-deficiency in retinal ischemia

Next, we determined the impact of DDAH2 on retinal ADMA and L-NMMA in OIR by liquid chromatography tandem mass spectrometry. During the hypoxic phase of OIR at p17, retinal ADMA was significantly increased in *DDAH2*$^{-/-}$ mice (Fig. 4K) suggesting that increased ADMA attenuates the development of retinal neovascularization. Although L-NMMA is present in the normal retina at higher levels than ADMA, we identified no measurable impact of OIR or *DDAH2*-deficiency on local L-NMMA (Fig. 4L).

3.7 DDAH2 does not influence pathogenic choroidal neovascularization

To investigate the role of DDAH2 in angiogenesis in choroidal neovascularization (CNV), a feature of age-related macular degeneration, we measured the extent of CNV induced by laser-rupture of Bruch's membrane in DDAH2-deficient mice. We identified no significant difference in the extent of CNV (Supplementary Fig. 2) suggesting that, in contrast to its role in ischemia-induced retinal neovascularization, DDAH2 deficiency does not affect the development of CNV.

4. Discussion

Therapeutic strategies that promote new vessel growth into the ischemic retina and away from the vitreous body would be extremely beneficial for patients with ischemic retinopathy, such as proliferative diabetic retinopathy and retinal vein occlusion. In this study we aimed to explore the role of ADMA and its catabolizing enzyme DDAH2, which are potent regulators of NO synthesis, on vascular regeneration and pathological neovascularization. To do this we investigated the expression of ADMA in the vitreous of patients with PDR and in the ischemic murine retina and characterized DDAH2 knockout mice in health and in an established model for retinal ischemia and neovascularization.

We found that ADMA levels were significantly increased in the vitreous of patients with proliferative diabetic retinopathy. This finding is consistent with a previous report describing elevated aqueous ADMA in diabetic retinopathy (Sugai et al., 2007). Since ADMA readily traverses cell membranes (Closs et al., 1997), it is likely that its concentration in the vitreous reflects that in the retina. The extent to which the elevated vitreous ADMA is the consequence of increased local production, as opposed to delivery from the systemic circulation, is uncertain. Both diabetic nephropathy and retinopathy are associated with elevated plasma ADMA which is a potent inhibitor of NO synthetases (Malecki et al., 2007)(Ueda et al., 2007). Exacerbated NO, on the other hand, is associated with the development of diabetic nephropathy, cardiovascular disease, cancer and the development of retinal neovascularization (Palmer et al., 1987)(Bazzaz et al., 2010)(Ando et al., 2002). Increased endogenous ADMA in the diabetic eye may therefore help to protect against NO-induced ischemia and aberrant neovascularization at least in part by inhibiting VEGF-induced chemotaxis and angiogenesis (Fiedler et al., 2009). This hypothesis is in line with previous findings demonstrating that ADMA protects against apoptosis of neural cells (X.-Y. Wang et al., 2013) and acts as a potent endogenous inhibitor of angiogenesis (Konishi et al., 2007)(Jang et al., 2000). In the eye, intervention to elevate ADMA via inhibition of DDAH activity may therefore offer a potential novel therapeutic approach for conditions including diabetic retinopathy in which excessive NO production is implicated (Hernandez et al., 2002).

To determine the effect of DDAH2 on ADMA levels and retinal ischemia and neovascularization we investigated DDAH2 deficient mice in health and in the OIR mouse model. Under normal conditions DDAH2 is predominately expressed in the ganglion cell layer, photoreceptor layers and to a lesser extent in the inner nuclear layer. DDAH2 deficiency caused no abnormality of retinal development or retinal vasculature in adult mice on fundus imaging, *in vivo* fluorescein angiography, immunohistochemistry or electroretinography indicating that DDAH2 does not affect normal neuroretinal development or function. These findings are consistent with previous

reports demonstrating that neither iNOS nor eNOS are required for normal retinal vascular development or normal retinal function (Al-Shabrawey et al., 2003). Under ischemic conditions, however, DDAH2 deficiency was associated with increased ADMA levels, reduced aberrant angiogenesis and improved vascular regeneration. These data indicate that DDAH2 deficiency and increased ADMA promotes appropriate revascularization and reduces aberrant angiogenesis in retinal ischemia most likely via an inhibition of NO synthase. These data are consistent with previous studies demonstrating that deficiency of endothelial- or inducible-NOS suppresses retinal neovascularization and improves vascular repair in the OIR model (Sennlaub et al., 2001)(Brooks et al., 2001). *iNOS*-deficient mice develop a substantial reduction of the area of ischemia by about 70% and a reduction of preretinal neovascularization by about 85% at p17 (Sennlaub et al., 2001). *eNOS*-deficient mice exhibit a 46% reduction of the area of retinal ischemia and a reduction of retinal neovascularisation by about 66% (Brooks et al., 2001) similar to our own findings in *DDAH2*-deficient mice. In addition to their roles in the regulation of NO production, DDAH enzymes are also involved in NOS-independent pathways. Since DDAH2 can induce expression of vascular endothelial growth factor (VEGF) (Hasegawa et al., 2006), which is well recognized for its pro-angiogenic role in OIR (Aiello et al., 1995), we investigated retinal VEGF protein levels in *DDAH2*-deficient mice during OIR. The concentration of VEGF protein was significantly raised in the retina during the hypoxic phase of OIR, a finding that is consistent with previous reports (Pierce et al., 1996). However, the concentration of VEGF was unaffected by *DDAH2* deficiency indicating that *DDAH2* deficiency and locally increased levels of the NOS inhibitor ADMA promotes retinal vascular regeneration and attenuates aberrant neovascularization independently of local VEGF concentration. These data are consistent with previous studies demonstrating that deficiency of endothelial- or inducible-NOS suppresses retinal neovascularization and improves vascular regeneration in retinal ischemia independent of VEGF (Ando et al., 2002)(Brooks et al., 2001)(Sennlaub et al., 2001).

5. Conclusions

In summary, our results demonstrate that DDAH2 prevents ADMA upregulation in retinal ischemia, impairing retinal vascular regeneration and promoting aberrant neovascularization. Deficiency of DDAH2 does not affect normal neuroretinal development or function but, in the context of ischemia, strongly promotes vascular regeneration and protects against pathological neovascularization. This mechanism is gene dose-dependent, tissue-selective and independent of VEGF. Local endogenous ADMA is modestly elevated in human eyes with advanced diabetic retinopathy but appears either insufficient or too late to prevent aberrant retinal neovascularization. Therapeutic intervention to reduce NO at an earlier stage in the disease, for example by small molecules inhibition of DDAH2 (Leiper and Nandi, 2011), may offer the means to protect against blindness in common conditions associated with retinal ischemia by promoting vascular regeneration and preventing retinal neovascularization.

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Disclosures

The authors declare no conflict of interests.

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FIGURES

Fig. 1: Regulation of Nitric Oxide synthesis by Methylarginines (ADMA and L-NMMA). L-arginine is the substrate for nitric oxide synthase (NOS) enzymes. Arginine residues in proteins are methylated by protein arginine methyl transferases. Following proteolysis of arginine-methylated proteins, methylarginines (ADMA and L-NMMA) accumulate in the cytosol where they can inhibit NOS activity by competing with arginine at the NOS active site. Inhibitory methylarginines are metabolized by the action of dimethylarginine dimethylaminohydrolase (DDAH1 and DDAH2).

Fig. 2: Vitreous ADMA levels are elevated in subjects with proliferative diabetic retinopathy (PDR, n=24) compared to control subjects having surgery for idiopathic macular hole or epiretinal membranes (n=10). Bars represent mean (± SEM). *** = p= 0.0004 (Mann-Whitney U-test).

Fig. 3: DDAH2 deficiency does not alter retinal development or function. A) ADMA and L-NMMA concentration in the retina and choroid/RPE in adult C57BL/6J mice (n= 5 per group). **B)** DDAH2 protein levels in the retina and choroid/RPE in adult C57BL/6J mice quantified by Western blotting (n= 4 per group). **C, D)** DDAH2 immunohistochemistry (with and without primary DDAH2 antibody) in adult C57BL/6J mice. **E, F)** H&E histology and collagen 4 (Col4) immunohistochemistry in one month old *DDAH2*$^{-/-}$, *DDAH2*$^{+/-}$ and *DDAH2*$^{+/+}$ littermate control mice (n= 3-4 per group). **G, H)** Representative scotopic electroretinogram recordings (G) and quantification of the a- and b-wave amplitude at 1 Cds/m^2 intensity (H) in adult *DDAH2*$^{+/+}$ control

mice (n= 6), *DDAH2$^{+/-}$ (n=6) and DDAH2$^{-/-}$ (n=6)* littermates; GCL= ganglion cell layer; IPL= inner plexiform layer; INL inner nuclear layer; OPL= outer plexiform layer; ONL = outer nuclear layer; Cho= choroid; RPE = retinal pigment epithelium. Bars represent mean (± SEM).

Fig. 4: In retinal ischemia DDAH2-deficiency increases ADMA levels, promotes revascularization and attenuates aberrant neovascularization. A-F) Representative vessel-stained retinal flatmounts of *DDAH2$^{+/+}$ (control)*, *DDAH2$^{+/-}$* and *DDAH2$^{-/-}$* littermates at p12 (A-C) and p17 (D-F) in oxygen-induced retinopathy (OIR). The ischemic area is outlined in white; the area of aberrant neovascularization is highlighted in yellow. **G-H)** Mean area of neovascularization (G) and ischemic fraction (H) in *DDAH2$^{+/+}$* (wildtype controls, n=15), *DDAH2$^{+/-}$* (n=21) and *DDAH2$^{-/-}$* littermates (n=10) at P17 after OIR induction (data is presented as percentage of total retinal area relative to wildtype littermate controls). **I)** Timecourse of mean ischemic fraction of total retinal area in *DDAH2$^{+/+}$*, *DDAH2$^{+/-}$* and *DDAH2$^{-/-}$* littermates at p12 and p17 in oxygen induced retinopathy (range n=5-8 per group). **J-L)** Mean retinal VEGF protein (J), ADMA (K) and L-NMMA (L) levels in *DDAH2$^{+/+}$ controls, DDAH2$^{+/-}$* and *DDAH2$^{-/-}$* littermates (range n=5-8 per group) at p17 under normoxic condition and after OIR induction (data is presented relative to normoxic wildtype littermate controls). Bars represent mean (± SEM). Ctr. = *DDAH2$^{+/+}$* controls. NV = neovascularization. **** = p<0.0001, *** = p<0.001, ** = p<0.01 (ANOVA with the Bonferroni correction for multiple significance tests).

Supplementary Fig. 1: DDAH2 is not required for normal retinal vascular development. A-E) Representative infrared fundus images (A), fluorescein angiography (B) and vessel-stained retinal flatmounts of control (*DDAH2*$^{+/+}$, C-E), *DDAH2*$^{+/-}$ (´) and *DDAH2*$^{-/-}$ littermates (´´) at one month of age. **D, E)** higher magnification images of the superficial (D) and deep vascular layer (E). Scale bar: 1 mm (C), 250 µm (D and E)

Supplementary Fig. 2: DDAH2 does not contribute to laser induced CNV. **A-C)** Early and late (‘) fluorescein angiographies of representative CNV lesions of adult *DDAH2*$^{+/+}$ *(control), DDAH2*$^{+/-}$ *and DDAH2*$^{-/-}$ littermates 2 weeks after laser CNV induction. **D, E:** Mean area of CNV in *DDAH2*$^{+/+}$ (control, n=6), *Ddah-2*$^{+/-}$ (n=7) and *DDAH2*$^{-/-}$ littermates (n=6) 2 weeks after laser CNV induction (data is presented relative to wildtype littermate controls). Bars represent mean (± SEM).

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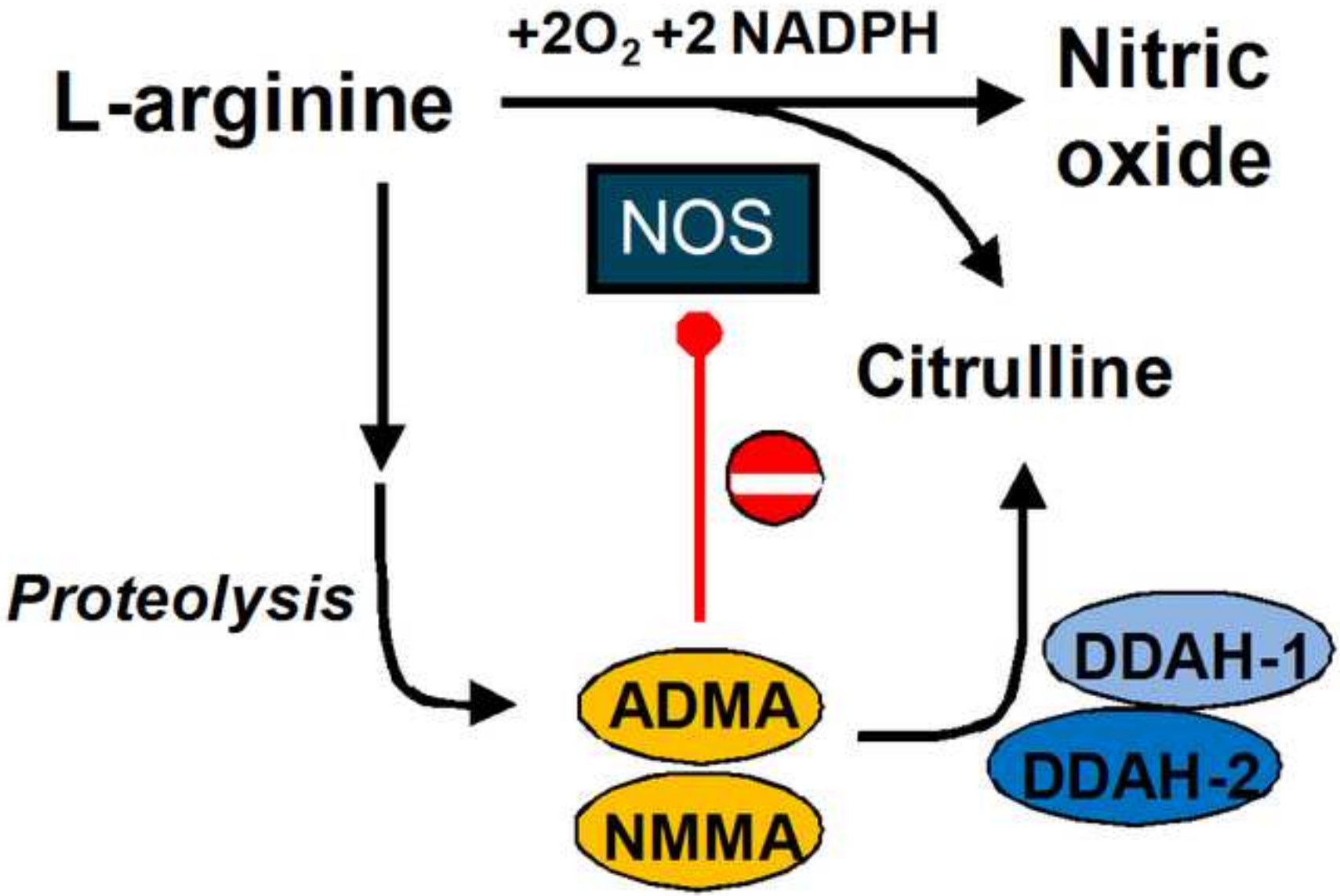

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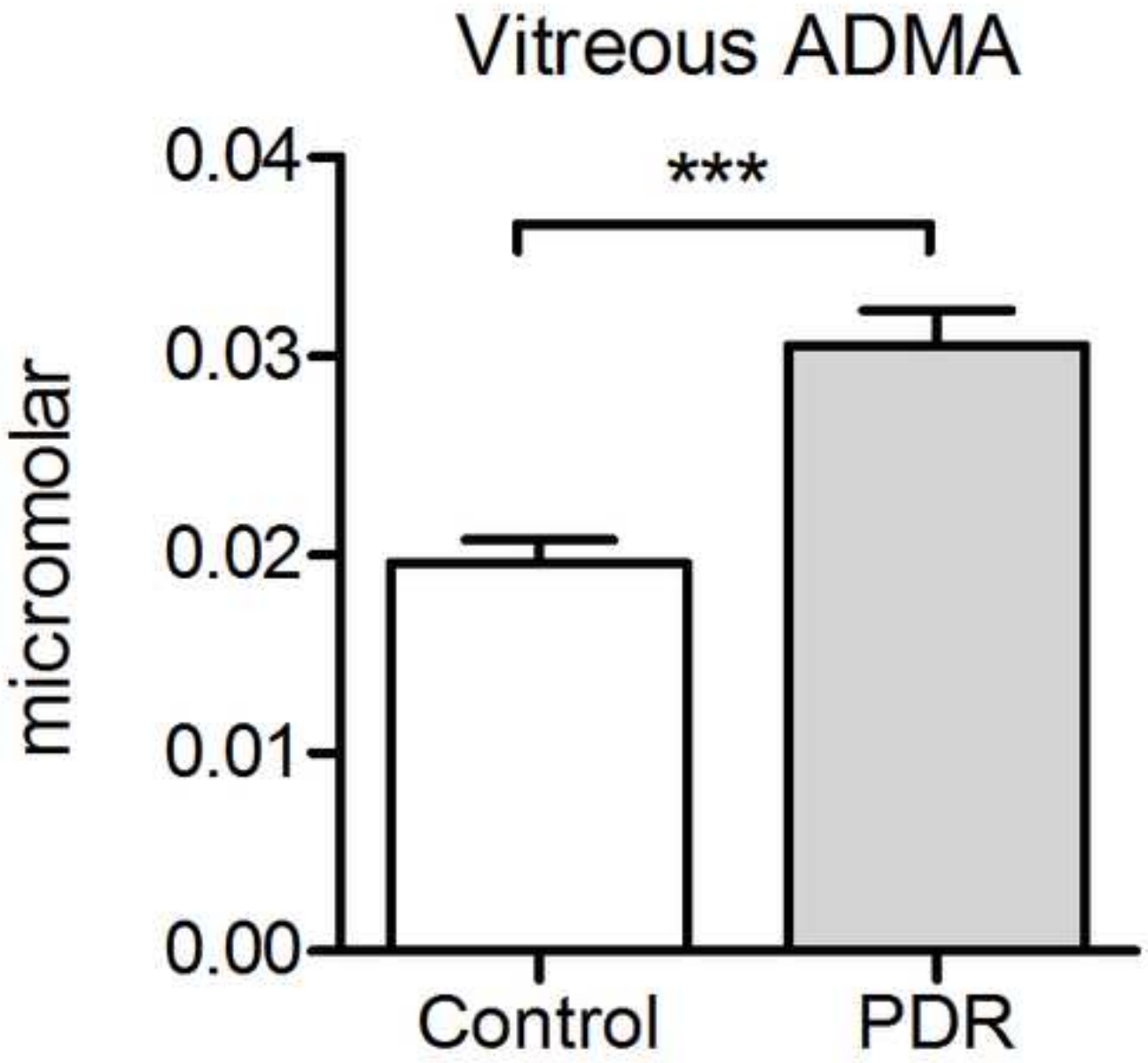

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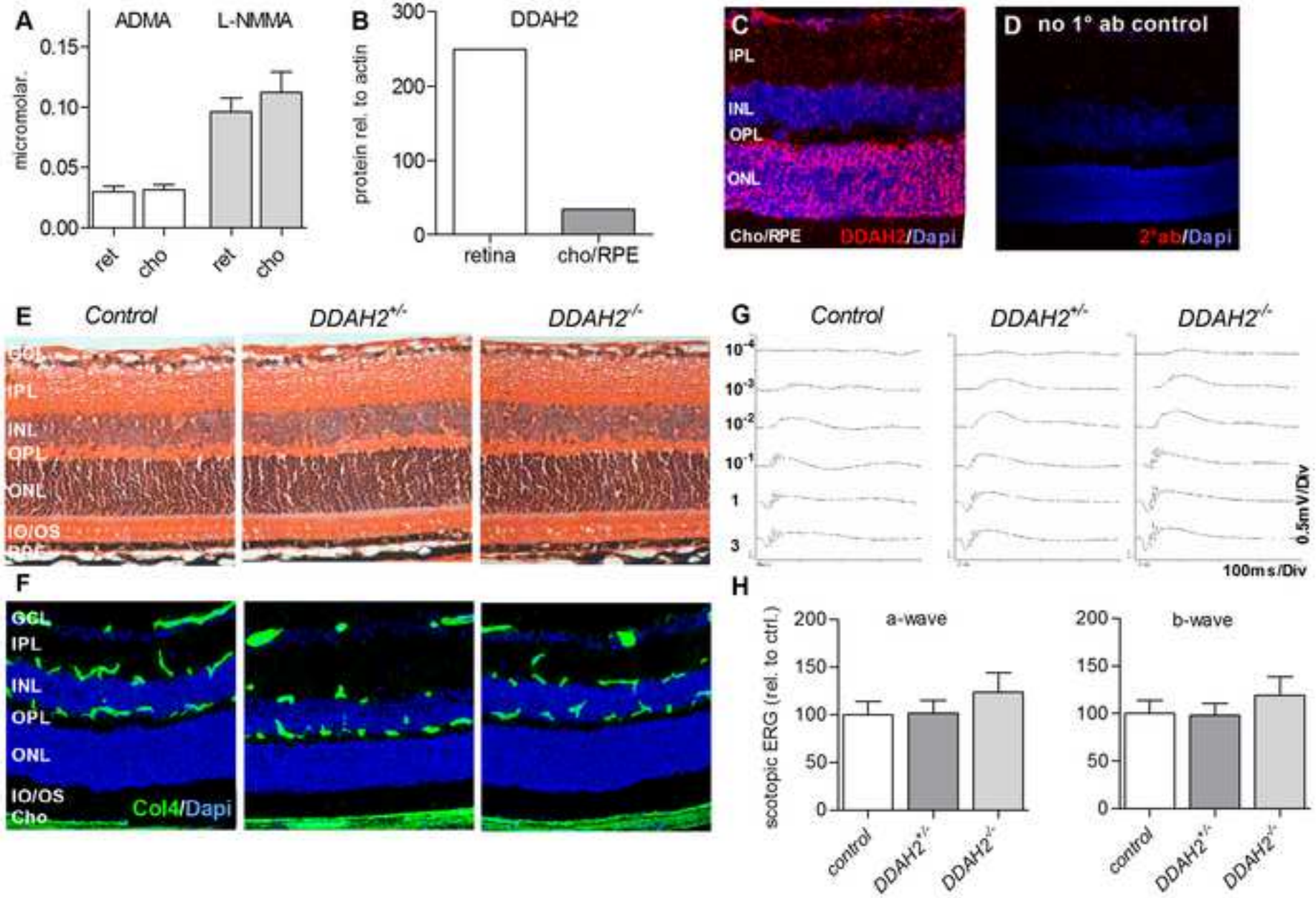

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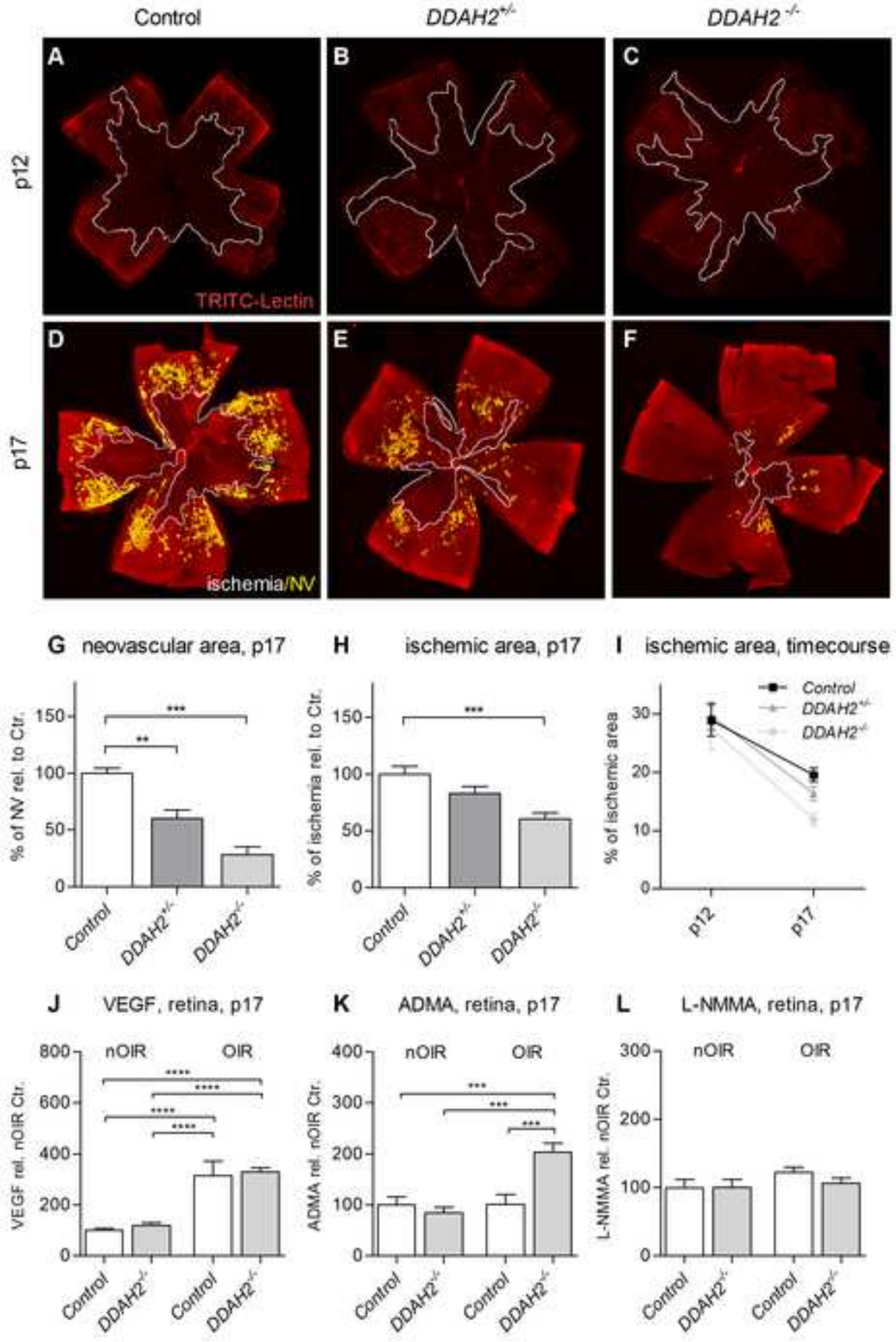

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