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1 **VEGF is an autocrine/paracrine neuroprotective factor for**
2 **injured retinal ganglion neurons**

3
4 Nicolas Froger¹, Frédéric Matonti¹, Christophe Roubeyx¹, Valérie Forster¹,
5 Ivana Ivkovic¹, Nadège Brunel², Christophe Baudouin^{1,3}, José-Alain Sahel^{1,3,4,5}
6 and Serge Picaud^{1,4}

7 ¹Sorbonne Université, INSERM, CNRS, Institut de la Vision, 75012 Paris, France

8 ²UMS 29 INSERM plateforme FluExGen UPMC, 75012 Paris, France

9 ³CHNO des Quinze-Vingts, DHU Sight Restore, INSERM-DGOS CIC 1423, 75012 Paris, France

10 ⁴Fondation Ophtalmologique Adolphe de Rothschild, 75020 Paris, France

11 ⁵Department of Ophthalmology, The University of Pittsburgh School of Medicine, Pittsburgh, PA
12 15213, United States

13
14 **Corresponding Authors:**

15 Dr Nicolas Froger

16 Dr Serge Picaud

17 Institut de la Vision,

18 17 rue Moreau, 75012 Paris, France.

19 Phone: (33) 1 53 46 25 92,

20 E-mail: nifroger@yahoo.fr (NF) serge.picaud@inserm.fr (SP)

21

1

2 **Abstract**

3 Vascular endothelial growth factor-A (VEGF) is the angiogenic factor promoting the
4 pathological neovascularization in age-related macular degeneration (AMD) or diabetic
5 macular edema (DME). Evidences have suggested a neurotrophic and neuroprotective role
6 of VEGF, albeit in retina, cellular mechanisms underlying the VEGF neuroprotection remain
7 elusive. Using purified adult retinal ganglion cells (RGCs) in culture, we demonstrated here
8 that VEGF is released by RGCs themselves to promote their own survival, while VEGF
9 neutralization by specific antibodies or traps drastically reduced the RGC survival. These
10 results indicate an autocrine VEGF neuroprotection on RGCs. In parallel, VEGF produced
11 by mixed retinal cells or by mesenchymal stem cells exerted a paracrine neuroprotection on
12 RGCs. Such neuroprotective effect was obtained using the recombinant VEGF-B,
13 suggesting the involvement of VEGF-R1 pathway in VEGF-elicited RGC survival. Finally,
14 glaucomatous patients injected with VEGF traps (ranibizumab or aflibercept) due to either
15 AMD or DME comorbidity, showed a significant reduction of RGC axon fiber layer thickness,
16 consistent with the plausible reduction of the VEGF autocrine stimulation of RGCs. Our
17 results provide evidence of the autocrine neuroprotective function of VEGF on RGCs is
18 crucially involved to preserve injured RGCs such as in glaucomatous patients.

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30 **Keywords:** VEGF, retinal ganglion cells, autocrine function, neuroprotection, glaucoma

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2 **Introduction**

3 Vascular endothelium growth factor-A (VEGF) is well known for its role in the
4 promotion of angiogenesis. It belongs to a family composed by four distinct glycoproteins:
5 VEGF-A, refers to VEGF as the founding member ¹, while the other main members are
6 VEGF-B, VEGF-C and VEGF-D ²⁻⁴. Their biological actions are mediated by three different
7 VEGF receptors, which belong to the tyrosine-kinase receptor group: VEGF-Receptor1
8 (VEGF-R1); VEGF-Receptor2 (VEGF-R2) and VEGF-Receptor3 (VEGF-R3). While VEGF-B
9 stimulates only VEGF-R1, VEGF-A can activate both VEGF-R1 and -R2. Differently, VEGF-
10 C and VEGF-D bind preferentially to VEGF-R3 ⁴.

11 Except its pro-angiogenic action, VEGF has displayed a protective effect, in particular
12 on neuronal cells as suggested by some studies. Hence, VEGF reduces the glutamate-
13 induced excitotoxicity on hippocampal neurons ⁵ and protects these cells against hypoxia ⁶.
14 In eye, VEGF was described as anti-apoptotic factor for photoreceptors ⁷, as well as for
15 Müller cells ⁸ and retinal pigment epithelium cells ⁹.

16 In recent years, anti-VEGF therapies have become important treatments to limit
17 tumor growth and suppress neovascularization in retinal diseases such as age-related
18 macular degeneration (AMD) and diabetic macular edema (DME) ¹⁰. Anti-VEGF therapies
19 for eye diseases have clear-cut medical benefits, resulting in the regression of neovessels
20 and improvement of visual acuity ^{11,12}. However, recent data suggest that anti-VEGF
21 therapies may alter the thickness of the nerve fiber layer, which is composed of retinal
22 ganglion cell (RGC) axons on their way to the optic nerve ¹³. This negative effect of anti-
23 VEGF therapies on RGCs is consistent with recent reports on the role of VEGF in the
24 neuroprotection of RGCs in animal models of retinal ischemia ¹⁴, optic nerve transection ¹⁵,
25 and glaucoma ¹⁶. However, the cellular mechanisms of VEGF-mediated neuroprotection on
26 RGCs are still unclear, including whether it acts directly or indirectly on RGCs. Here, we

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- 1 provide evidence that VEGF can directly affect RGC survival by activating their VEGF
- 2 receptors in an autocrine/paracrine mode.

1 **Results**

2 ***VEGF is a paracrine neuroprotective factor for RGC***

3 We previously showed that conditioned medium isolated from mixed retinal cell
4 cultures (CM-M) can promote the survival of enriched adult rat RGCs in cultures¹⁷. This
5 effect occurred either in the presence or in the absence of B27 supplement and persisted
6 until 21 days *in vitro* (DIV) (Fig. S1). We identified the neuroprotective factors contained in
7 this CM-M using the Luminex assay for rat trophic factors. All samples (n=4) of the CM-M
8 contained a large amount of VEGF-A₁₆₄, but no other factors known to promote RGC
9 survival (*i.e.* BDNF, G-CSF, and GM-CSF; Table 1). The presence of VEGF was confirmed
10 and quantified by ELISA technique (106.1 ± 22.6 pg/ml, n=9).

11 Mixed retinal cultures are composed of a monolayer of macroglial and microglial
12 cells, upon which are found retinal neurons (mainly cone photoreceptors and bipolar cells;
13 Fig. S2). We attributed the origin of VEGF synthesis to microglial cells because these
14 purified cells can produce substantial levels of VEGF in most cultures after only 48h ($17.8 \pm$
15 21.7 pg/ml; n=22; Fig. S3). Purified macroglial cells (including Müller glial cells) did not
16 produce VEGF (Fig. S3), in contrast to results reported in previous studies on isolated
17 Müller cells¹⁸ or a human Müller cell line (MIO-M1)⁹.

18 In parallel, we found that conditioned medium from mesenchymal stem cells (CM-
19 MSC), which contained VEGF, can also promote RGC survival (Fig.1A-E). We assessed
20 whether the VEGF contained in these two different conditioned media (CM-MSC and CM-M)
21 contributed to RGC survival. First, purified adult rat RGCs were incubated with the CM-
22 MSC, in the presence of a specific antibody against VEGF-A₁₆₄ (rabbit polyclonal IgG, 0.5
23 μ g/ml). This anti-VEGF antibody completely suppressed the RGC survival effect produced
24 by the CM-MSC ($p < 0.01$, Fig. 1C-D). Indeed, there was a linear correlation ($r^2 = 0.78$)
25 between the VEGF concentration in each CM-MSC and its efficiency in promoting RGC

1 survival (Fig. 1E). Secondly, we obtained a significant reduction of the CM-M-induced
2 neuroprotection ($p < 0.01$) when the anti-VEGF antibody was added to retinal CM-M (Fig.
3 1F). However, the neuroprotective effect of CM-M was not completely suppressed and
4 remains significant ($p < 0.01$ as compared to control) in presence of the anti-VEGF. This
5 observation may result from the presence of other neurotrophins in CM-M (e.g. NGF or
6 GDNF, other than BDNF which were undetectable in CM-M), because the detection of
7 growth factors via our Luminex assay was not exhaustive. Finally, we tested a control rabbit
8 polyclonal IgG targeting another protein (anti-NF200), and we found that the CM-M-
9 enhanced RGC survival was not significantly affected (Fig. 1G). This result indicates that
10 the VEGF present in the two different conditioned media is partially responsible for the
11 trophic effects on RGCs.

12

13 ***VEGF is an autocrine factor produced by RGCs to promote their own survival***

14 Surprisingly, the antibody directed against VEGF-A₁₆₄ has also reduced *per se* the
15 survival of purified RGCs by 34.8% when they were cultured in a commercial VEGF-free
16 culture medium ($p < 0.05$, see Fig. 1F). This result suggests that RGCs themselves released
17 VEGF into culture medium. Indeed, when RGCs were initially seeded at 8,000 cells per well,
18 measurable concentrations of VEGF-A₁₆₄ were detected in the culture medium at 6 and 12
19 DIV by ELISA, whereas VEGF was non-detectable at 1 DIV (not shown). From 70 to 75% of
20 harvested supernatants contained detectable VEGF-A₁₆₄ after 6 to 12 DIV, with
21 concentrations reaching 81.3 ± 12.7 pg/ml at 6 DIV.

22 At 6 DIV, the increase of seeding density from 8×10^3 to 50×10^3 cells/well improved
23 the RGC survival (Fig. 2A-B) and increased the percentage of VEGF-positive supernatants
24 from 12% to 100%, respectively (Fig 2C). VEGF concentrations also substantially and
25 significantly increased with higher seeding density ($p < 0.001$, Kruskal-Wallis test; Fig 2C).

1 More importantly, the VEGF-A₁₆₄ concentrations highly correlated with the actual number of
2 surviving RGCs ($p < 0.001$, Spearman test), the curve being best fitted with an exponential
3 regression ($r^2 = 0.65$) (Fig. 2D). These measures are therefore consistent with a correlation
4 between the level of free VEGF (0-600pg/ml) and RGC survival (0-6000 RGCs per well).
5 The expression of VEGF in healthy RGCs as in cultured RGCs was confirmed by anti-VEGF
6 immunolabelling of purified RGC cultures and retinal sections (Fig. S4) in agreement with
7 previous reports^{9,19}. These measures are also consistent with the detection of VEGF-A
8 mRNA in embryonic RGCs²⁰ and our adult rat RGCs (data not shown). Altogether, these
9 results indicate that VEGF is an autocrine factor for RGCs.

10 The importance of VEGF for RGC survival was finally verified using various VEGF
11 trapping molecules: (i) a mouse monoclonal anti-human VEGF (anti-pan, non-selective for
12 VEGF-A isoforms), (ii) a rabbit polyclonal antibody against murine VEGF-A₁₆₄ (*i.e.* selective
13 for isoform 164), and (iii) ranibizumab (the Fab fragment from a humanized mouse
14 monoclonal anti-VEGF-A, non-selective for isoforms). These anti-VEGF proteins all induced
15 a statistically significant dose-dependent reduction of RGC survival after 6 DIV (Fig. 2E).
16 The efficacy of ranibizumab became statistically significant at relatively high concentrations
17 (above 250 $\mu\text{g/ml}$; Fig. 2E). Both the specific rabbit polyclonal antibody against murine
18 VEGF-A₁₆₄ (2 $\mu\text{g/ml}$), as well as ranibizumab (at least above 250 $\mu\text{g/ml}$), prevented the
19 detection of VEGF produced by RGCs since 100% of samples displayed undetectable
20 VEGF levels (Fig. 2F). In contrast, the pan antibody (non-selective for VEGF-A isoforms)
21 reduced the proportion of samples with a detectable level of VEGF but did not significantly
22 affect the mean VEGF concentrations in the medium, although it affected its neuroprotective
23 effectiveness, probably due to the reduction of free VEGF (not bound to an antibody). The
24 mean values of VEGF concentrations were calculated, including undetectable values
25 defined as zero. We found that if anti-pan did not significantly decrease the VEGF

1 concentrations whatever the doses used (0.5, 1. or 2.0 µg/ml), the selective anti-VEGF-A₁₆₄
2 significantly induced a significant decrease in VEGF concentrations in the culture medium at
3 the two doses tested (0.5 and 2.0 µg/ml, p<0.05 as compared to control condition).
4 Similarly, ranibizumab induced a dose-dependent decrease of VEGF concentrations in
5 culture medium, which appeared statistically significant at the dose of 250, 500 and 1000
6 µg/ml, respectively (p<0.05 as compared to control condition).

7

8 ***VEGF-elicited RGC survival involves VEGF-R1 pathway***

9 Using the recombinant VEGF-A₁₆₄ protein (from rat), we consolidated that VEGF *per*
10 se promotes the RGC survival. At the 10 ng/ml concentration the recombinant protein
11 significantly increased the RGC survival (+41.8%; p<0.01), whereas incubation at 1 ng/ml
12 produced a slight increase (+14.3%; Fig 3A), but not statistically significant. To further
13 assess the pharmacological pathway of the VEGF-elicited RGC survival, we investigated
14 the involvement of VEGF receptors in VEGF-elicited RGC survival. Because VEGF-A binds
15 at two distinct receptors named VEGF-R1 and VEGF-R2²¹, we used pharmacological
16 agents in order to discriminate which of these receptors are activated by VEGF to promote
17 the RGC survival. The VEGF-R2 receptor antagonist, ZM 323881 (ZM) allows discerning
18 the activity of VEGF-R1 versus -R2 receptors. Co application of ZM (5nM) in the culture
19 medium with the recombinant VEGF-A (10 ng/ml) did not affect the VEGF-mediated
20 increase in RGC survival (ZM+VEGF-A: +31.4%, VEGF-A: +38.5%; Fig. 3B), while
21 incubation of ZM alone did not show any significant changes on RGC survival (+8.0%).
22 These data suggested that the RGC survival is rather mediated through the VEGF-R1
23 activation. To verify this hypothesis, we applied a recombinant VEGF-B protein, which
24 stimulates selectively the VEGF-R1. Addition of VEGF-B (1 ng/ml) into the culture medium
25 for 6 DIV significantly increased RGC survival (+30.5%; Fig. 3C). In order to verify the

1 absence of VEGF-R3 implication in this neuroprotective efficacy, we finally applied the
2 recombinant VEGF-D protein, which activates VEGF-R3 and in some proportion VEGF-R2
3 (4). However, VEGF-D (0.5 ng/ml) failed to increase the RGC survival (+1.7%; Fig. 3C).
4 These results are consistent with the involvement of VEGF-R1 activation by VEGF, to
5 promote RGC survival by VEGF-A. We could show expression of both VEGF-R1 and
6 VEGF-R2 – at the mRNA level – in freshly purified RGCs (n=3), as shown by RT-PCR
7 experiments (Fig. 3D-E). We concluded that RGC survival relies on the VEGF-R1 activation.

8

9 ***Anti-VEGF treatments alter the retinal nerve fiber layer (RNFL) thickness in***
10 ***glaucomatous patients***

11 One report has already suggested that anti-VEGF therapy could negatively affect
12 RGC survival¹³. Such negative effects could be more important in patients with already
13 compromised RGCs. Thus, we focused our investigations by measuring the RNFL thickness
14 of glaucomatous patients, with either AMD or DME comorbidity, as compared to non-
15 glaucomatous patients, all receiving anti-VEGF therapy. There was a statistically significant
16 decrease of RNFL thickness in the treated eyes (injected eyes) from the glaucomatous
17 group, which appeared during the third month of treatment (Fig. 4D). We can observe a
18 quick decrease of the RNFL thickness during the first 3 months corresponding to the
19 induction phase (one intravitreal injection [IVI] each month) whereas during the next phase
20 the decrease is continuing, but less intensely (but remaining significant), probably due to a
21 less intensive treatment (3.4 and 3.2 IVI respectively for non-glaucomatous and
22 glaucomatous patients during the following 9 months).

23 In contrast, the RNFL thickness of the fellow control eye was stable (Fig. 4D). This
24 decrease was not associated with significant changes in intra-ocular pressure (IOP) levels
25 in either the injected or control eyes (Fig. 4C). There were no differences in RNFL thickness

1 or IOP in either eye of the control group (non-glaucomatous patients; Fig. 4E, 4C). These
2 observations suggest that anti-VEGF therapies could affect RGC survival in patients with
3 already injured RGCs.

4

1

2 **Discussion**

3 This study provides evidence that VEGF is a neuroprotective factor for RGCs through
4 an autocrine mechanism. We demonstrate *in vitro* that enriched adult rat RGCs cultured
5 under serum-deprivation – a situation that mimics ischemic conditions – can release VEGF
6 to promote their own survival. To our knowledge, such ability of RGCs to produce
7 themselves VEGF has never been described yet for these retinal neurons, except in one
8 study showing detectable VEGF amounts in supernatant harvested from RGC-5 cell line²²;
9 the nature of this cell line remaining controversial. Hence, this autocrine mechanism of
10 neuroprotection may underlie the VEGF-elicited neuroprotection of RGCs, as observed *in*
11 *vivo* following optic nerve transection¹⁵ and in glaucomatous animal models¹⁶. In retina,
12 such neuroprotective action of VEGF involving an autocrine mechanism was already
13 described for Müller cell line (MIO-M1) and retinal pigment epithelium (ARPE-19) which can
14 secrete VEGF to promote their own survival^{8,9}. An autocrine mechanism was also
15 suggested to explain the neuroprotective action of VEGF on embryonic cortical neurons²³,
16 and may be also involved in the VEGF neuroprotection of motoneurons²⁴.

17 In addition, we found that VEGF is released by microglial cells suggesting that VEGF
18 also exert neuroprotection through paracrine pathway. Other studies attributed the paracrine
19 action VEGF by a secretion from retinal pigment epithelium (RPE) or from Müller cells.
20 Indeed, VEGF is particularly expressed by RPE²⁵ and was also found in inner retina (see
21 Fig. 4S). In human retina, VEGF immunoreactivity was specifically expressed in Müller cell
22 bodies²⁶. Moreover, we demonstrate here that VEGF is among the essential factors of
23 MSC secretome conferring a direct RGC protection. A paracrine action of VEGF may also
24 explain part of the therapeutic effect provided by bone marrow-derived MSCs injected into
25 eyes following optic nerve injury or glaucoma^{27,28}.

1 Our pharmacological investigations reveal that VEGF-elicited RGC survival is
2 mediated through the VEGF-R1 stimulation. Indeed, VEGF neuroprotection was not blocked
3 by the addition of VEGF-R2 antagonist, while it was reproduced by incubation of VEGF-B
4 which only stimulates VEGF-R1 (see ⁴). VEGF-B was shown to stimulate the survival of
5 RGC-5 subjected to oxidative-stress, and it inhibits the apoptosis in the retina induced by
6 axotomy, NMDA intra-vitreous injection or ischemia ²⁹, suggesting the neuroprotective effect
7 of the VEGF-R1 signaling. On the other hand, it has been shown that VEGF can protect
8 hippocampal neurons against glutamate excitotoxicity through the activation of VEGF-R2
9 signaling ^{5,6}. Furthermore, VEGF-R2 blockade aggravates neuronal degeneration in brain
10 ^{22,30}, supporting the involvement of VEGF-R2 in VEGF neuroprotection. VEGF-R1 and
11 VEGF-R2 belong to the tyrosine kinase receptor family and both trigger the PI3K/AKT and
12 the MEK/ERK pathways involved in the VEGF neuroprotection⁵. Accordingly, VEGF-R1 and
13 VEGF-R2 can be differentially implied in the neuroprotective effect of VEGF according to
14 the cell types, but in RGCs, VEGF-R1 seems to be preferentially implied.

15 VEGF production and release by adult rat RGCs demonstrate its role as an autocrine
16 factor for these spiking retinal neurons. In vivo, VEGF is highly expressed by healthy RGCs
17 as observed here by immunolabeling, while a lower expression is found in other cell types
18 (Fig. S4). VEGF release could play a major physiological role because the extracellular
19 space is small and RGC density is very high in the RGC layer. During development, the
20 release of VEGF by RGCs may contribute to the development of the inner retinal
21 vasculature²⁰.

22 We previously showed that cultured RGCs in low nutritive conditions (without B27
23 serum) have features highly reminiscent of ischemic conditions¹⁷. Therefore, such culture
24 conditions can be considered as representative of glaucomatous ischemic conditions. As a
25 consequence, these results suggest that the VEGF synthesis is also occurring in RGCs

1 from glaucomatous patients. However, VEGF immunolabeling also demonstrated VEGF
2 synthesis in healthy RGCs^{14,15}. Although VEGF concentrations in cell cultures and vitreous
3 are difficult to compare, it is surprising to note that VEGF concentrations released by our
4 cultured RGCs were in a similar range or higher than (<600pg/ml) those measured in
5 vitreous of control patients (<150pg/ml)³¹⁻³³. It remains unclear if retinal pathologies affecting
6 RGC survival like glaucoma alter VEGF release, thus participating in the dysregulation of
7 ocular blood flow³⁴. Apart from its effect on blood flow, reduced VEGF release due to RGC
8 loss could affect the survival of the remaining RGCs in a vicious cycle. In fact, VEGF
9 contents in the vitreous of glaucomatous patients were only measured in a recent study on
10 patients affected by neovascular glaucoma, showing vitreous VEGF concentrations raising
11 to 220 pg/ml³⁵.

12 VEGF contents were higher in vitreous from patients affected by retinal
13 neovascularization, like proliferative diabetic retinopathy, going up to 500 pg/ml, and more
14 (> 1000 pg/ml) according to various studies^{31-33,36}. Interestingly, anti-VEGF treatment
15 allowed to drastically reduce the VEGF concentrations in vitreous of these patients³³.
16 Regarding the healthy subjects, the vitreous VEGF concentration does not exceed 150
17 pg/ml (from 1.7 pg/ml to 146 pg/ml). Although, these VEGF concentrations are in the same
18 range or below those measured in RGC culture media (up to 600 pg/ml), it is unlikely that
19 RGCs provide the only source of VEGF. Indeed, we showed that microglial cells can also
20 synthesize VEGF (Fig. S3) and we previously confirmed other studies showing VEGF
21 production by RPE cells³⁷. In addition, others reported that a Müller cell line can also
22 secrete VEGF amounts reaching more than 1000 pg/ml⁹. Therefore, vitreous VEGF is likely
23 resulting from the production by all these retinal cells in vivo.

24 The chronic administration of anti-VEGF therapies may enhance VEGF loss, leading
25 to deleterious consequences for vulnerable RGCs, such as in glaucoma. This safety

1 concern for anti-VEGF therapies is consistent with a previous study showing a significant
2 loss of RNFL thickness after 12 months of intravitreal anti-VEGF injections in AMD
3 patients¹³. This conclusion is further supported by a recent retrospective study showing that
4 patients with continuous unilateral anti-VEGF treatment showed a significant decrease in
5 both RNFL and RGC layer thickness during at least 24 months of follow-up³⁸. However,
6 other studies in patients with only AMD have not found any correlation between RNFL
7 changes and anti-VEGF therapy³⁹⁻⁴¹. In a recent study⁴² which looked at patients with both
8 glaucoma and AMD, the authors did not find a correlation between decrease nerve fiber
9 layer and treatment. However, the main concern with this study is that they used a macular
10 analysis of ganglion cells or RNFL, and the macular edema due to AMD may induce
11 artifacts on the measures of thickness for each macular layer (ganglion cells, RNFL) with
12 artificial increase, thus probably masking a thinning of these layers. Given these overall
13 contradictory data, future clinical trials are needed to assess further if an anti-VEGF
14 therapies sustainably affect RGC survival and thus the RNFL thickness in patients.

15 Here, we provide further clinical evidence for this deleterious effect of anti-VEGF
16 therapy, but in a glaucomatous population with already compromised RGCs. Repeated
17 injection of anti-VEGF for AMD or DME induced a significant reduction of RNFL thickness
18 over a period of 12 months in these glaucomatous patients. These results strongly suggest
19 that VEGF is a major autocrine factor for adult human RGCs. Further studies are needed to
20 demonstrate the potential therapeutic interest of VEGF receptor agonists in retinal diseases.

21

1 **Materials and methods**

2 ***Further details of methods are provided in Supplement.***

3

4 ***Primary cell cultures from adult rat retinas***

5 Cell culture experiments were performed with retina isolated from 8-week-old Long-Evans
6 rats, purchased from Elevage Janvier (France). Animals were housed in the same room of
7 animal facility under controlled conditions ($21\pm 1^{\circ}\text{C}$, 12h/12h light/dark cycles, light on at 8
8 a.m., and food and water available *ad libitum*). All experiments were in accordance with the
9 European Union Directive (2010/63/EU) and with the ARVO (Association for Research in
10 Vision and Ophthalmology) statement for the use of animals in ophthalmic and visual
11 Research. Experimental protocols were approved by the local ethics committee and the
12 Ministère de la Recherche (permission number #A-75-1739 for NF).

13 Mixed retinal cultures were maintained until confluence (10-12 DIV) and then used to
14 prepare conditioned medium from retinal mixed cultures (CM-M). Primary cultures of RGCs
15 were isolated using an immunopanning technique, according to the protocol adapted from
16 that used on adult rats^{43,44}. Purified RGCs were seeded at various densities and cultured in
17 Neurobasal-A medium containing glutamine and supplemented with B27.

18

19 ***Mesenchymal stem cell (MSC) collection***

20 Bone marrow was obtained from the femur cavities of rats after flushing with minimal
21 essential medium (α -MEM) containing 10% fetal calf serum. MSCs were seeded at 200,000
22 cells/cm² and cultured until confluence.

23

24 ***Cell treatments***

1 Enriched RGC were subjected to different treatment during the whole period of the RGC
2 cultures (6 DIV). Conditioned media were applied at the dilution of 1:2 directly into RGC
3 culture medium. The monoclonal mouse anti-VEGF-A (anti-pan against all VEGF-A
4 isoforms, Santa-Cruz Biotechnology, Santa-Cruz, CA, USA), the polyclonal rabbit anti-
5 murine VEGF-A₁₆₄ (Abcam), ranibizumab (Lucentis®, Novartis; gift from Pr Matonti), a Fab
6 fragment of the humanized monoclonal VEGF antibody (neutralizes all VEGF isoforms) and
7 a polyclonal Ig-G (rabbit polyclonal anti-NF200; Sigma-Aldrich) were incubated directly into
8 the culture medium of RGC cultures. Recombinant rat VEGF-A₁₆₄ protein (R&D system,
9 Minneapolis, MI, USA), recombinant mouse VEGF-B₁₆₇ protein (R&D system) and
10 recombinant rat VEGF-D₁₆₄ protein (R&D system) were also directly added into the medium.
11 The antagonist at VEGF-R2 receptor, ZM 323881 (Tocris Biosciences, Ellisville, MO, USA)
12 was co-incubated with VEGF-A₁₆₄. ZM 323881 (5-[[7-(benzyloxy) quinazolin-4-yl]amino]-4-
13 fluoro-2-methylphenol) is a potent and selective inhibitor of VEGF-R2 tyrosine kinase in vitro
14 ($IC_{50} < 2$ nM), compared with other receptor tyrosine kinases, including VEGF-R1, a range
15 of other receptor tyrosine kinases such as PDGFR β , FGFR1, EGFR and erbB2 ($IC_{50} > 50$
16 μ M)⁴⁵.

17

18 ***Automated counting of viable RGCs***

19 RGC counting was realized after 6 DIV on alive cells labeled with calcein-AM (producing a
20 green fluorescence; Invitrogen). Briefly, RGGs cultured in 96-well plate, were incubated in a
21 calcein-AM solution (1.3 μ g/ml, diluted in Neurobasal-A medium) for 1 hour into the
22 incubator (humidified chamber, 37°C, 5%CO₂). Calcein-positive RCGs were then
23 automatically counted after a frame per frame scanning of the full well surface (objective
24 X10), by using an inverted epifluorescence microscope (TiE, Nikon, Champigny-sur-Marne,

1 France) equipped with a motorized stage and a CCD camera (Roper Scientific, Evry,
2 France).

3

4 ***Measurement of growth factors***

5 A microparticle bead-based multiplex assay was used to measure the growth factors VEGF,
6 BDNF, G-CSF, and GM-CSF in supernatants from mixed retinal cells, using the Luminex
7 200 technology. VEGF-A₁₆₄ was specifically measured in supernatants from cell cultures
8 using ELISA technique. Supernatants were harvested from mixed, glial or microglial or RGC
9 cultures and frozen at -80°C until use. VEGF-A164 was then measured in supernatants
10 using the “Rat VEGF Quantikine ELISA Kit” (R&D system) according the instructions of the
11 kit.

12

13 ***Patients and clinical examinations***

14 We conducted a prospective single-center real-life study on 20 patients (10 non-
15 glaucomatous and 10 stabilized glaucomatous patients) treated by intravitreal injections
16 (IVI) of VEGF traps (ranibizumab or aflibercept; at least six intravitreal injections - 6.4 vs 6.2
17 - during 12 months). Patient characteristics are summarized in table 2. The eyes receiving
18 IVI were included in the "injected" group, whereas the follow eyes (without IVI) were
19 included in the "non-injected" group. Informed consent was obtained from all patients in
20 agreement with the Declaration of Helsinki for research involving human subjects.

21 This study did not include a placebo group because it consists in a real-life study, and not a
22 randomized, placebo-controlled and double-blind trial.

23 The study was approved by the Ethics Committee of the French Society of Ophthalmology
24 (IRB 00008855 Société Française d’Ophtalmologie IRB#1).

25

1 **Statistics**

2 All data are expressed as means \pm SEM. In ELISA experiments for detection of VEGF in
3 culture medium, undetectable values (below the detection threshold) were defined as zero
4 and included in the mean calculations.

5 The Mann Whitney test was used to compare means of 2 groups. When more than 2 groups
6 were compared, a non-parametric Kruskal-Wallis analysis of variance was performed and
7 followed, in case of significance by a Dunns post-hoc test to compared means between
8 each group. For clinical observations, the Friedman test was used for analysis of variance
9 for repeated measures and followed by a Dunns *post-hoc* test to compared means between
10 each group. For all statistical analysis, differences were considered significant at * $p \leq 0.05$,
11 ** $p \leq 0.01$ and *** $p \leq 0.001$.

12

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11

12 **Competing interest statement**

13 Christophe Baudouin has received consultancies from Allergan Alcon, Dompe, Horus
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21 Other authors declare that no conflict of interest exists.

22

23 **Author contributions**

24 NF, FM, CR, VF, CB, JAS and SP designed research.

25 NF, FM, CR, VF, II and NB performed experiments

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1 NF, VF, II and NB contributed new reagents or analytic tools

2 NF, FM, CR, VF, and SP analyzed data.

3 NF, FM, CR, VF, CB and SP wrote the manuscript

4

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- 32

1 **Figure legends**

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Figure 1: VEGF-elicited survival of retinal ganglion cells (RGCs).

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A-C: Calcein-positive RGCs cultured for 6 DIV in control medium (A), conditioned medium from mesenchymal stem cells (CM-MS) (B), or CM-MS containing a rabbit polyclonal anti-VEGF-A₁₆₄ antibody (C). D: RGC survival in CM-MS, with or without the rabbit polyclonal anti-VEGF-A₁₆₄ antibody. E: Linear correlation between VEGF-A₁₆₄ concentration in CM-MS and RGC survival. Each point corresponds to an independent experiment. F: RGC survival in the control medium, conditioned medium from mixed retinal cells (CM-M), CM-M plus the anti-VEGF antibody, or the anti-VEGF antibody alone (in control medium). G: Survival of RGC cultured in control medium, CM-M, CM-M plus an anti-NF200 antibody, or control medium plus the anti-NF200 antibody. Data (means ± SEM) are normalized to the control condition in independent cultures (n=4 in D, E; n=11 in F; n=6 in G). RGCs were seeded at initial density of 8,000 cells/well. ***p<0.001, **p<0.01, and *p<0.05 as compared to the control group, and ###p<0.01 compared between indicated groups (Kruskal-Wallis ANOVA followed by a Dunns *post-hoc* test).

1

2 **Figure 2: Autocrine VEGF release by retinal ganglion cells (RGCs)**

3 A-B: Representative confocal images showing live calcein-positive RGCs (6 DIV) with an initial low seeding
4 density (8,000 cells/well; A) vs. high density (30,000 cells/well; B). Scale bar represents 50 μ m. C: VEGF-A₁₆₄
5 concentrations in supernatants from cultured RGCs after 6 DIV, depending on the seeding density. Data are
6 expressed as pg/ml and the means \pm SEM from independent cultures with n/N referring to the number of
7 experiments with detectable VEGF A₁₆₄ (n) over the total number of experiments (N). *p<0.05, **p<0.01 and
8 ***p<0.001 as compared to the seeding density of 8×10^3 . #p<0.05 and ##p<0.01 between indicated groups. D:
9 Correlation between the measured VEGF-A₁₆₄ concentration and the actual number of surviving RGCs. The
10 initial seeding densities were 8,000 cells/well (green, n=22); 18,000 cells/well (blue, n=15); 30,000 cells/well
11 (red, n=15), or 50,000 cells/well (purple, n=5). The non-linear regression curve is fitted with an exponential
12 growth equation ($r^2=0.65$). p<0.001, Spearman test. E: Suppression of RGC neuroprotection by VEGF-A traps
13 added into the control culture medium: non-selective anti VEGF-A (pan antibody for VEGF isoforms), selective
14 anti-VEGF-A₁₆₄ antibody, and the anti-VEGF Fab fragment ranibizumab. N refers to the total number of
15 experiments in each group. F: VEGF-A₁₆₄ concentrations (in pg/ml) in supernatants from RGC cultures (6 DIV)
16 in the presence of various concentrations of the 3 VEGF traps: anti-pan VEGF-A, anti-VEGF-A₁₆₄, or the
17 VEGF trap ranibizumab. n/N refers to the number of experiments with detectable VEGF A164 (n) over the total
18 number of experiments (N). *p<0.05 as compared to the control group (C) (Kruskal-Wallis ANOVA followed by
19 the Dunn's *post-hoc* test).
20 RGCs were seeded at initial density of 30,000 cells/ well in E & F.
21

1 **Figure 3: Recombinant VEGF-A₁₆₄ directly stimulates *in vitro* the RGC survival**
2 **through VEGF-R1 activation.**

3 A: Quantification, by automatic counting, of densities of calcein-positive RGCs cultured for 6 DIV either in the
4 low-nutritive condition (Control; white bar), or with application of two concentration of VEGF-A (1 ng/ml; grey
5 bar and 10 ng/ml, black bar), or with application of the B27 supplement (2%, hatched bar), taken as positive
6 control.

7 B: Quantification of densities of calcein-positive RGCs cultured for 6 DIV either in the low-nutritive condition
8 (Control; white bar), or with application of 10 ng/ml VEGF (black bar), or with 10 ng/ml VEGF plus 5 nM ZM
9 323881, a selective VEGF-R2 antagonist (ZM, grey bar) or with 5 nM of ZM 323881 alone (oblique hatched
10 bar).

11 C: Graph representing the densities of alive calcein-positive RGC cultured for 6 DIV either in the low-nutritive
12 control condition (Control; white bar), or with application of 10 ng/ml VEGF-B (black bar) or 0.5 ng/ml VEGF-D
13 (grey bar).

14 D-E) Gene amplification (PCR) of VEGF-Receptor1 (VEGF-R1, C) and VEGF-Receptor2 (VEGF-R2; D)
15 performed on total cDNAs, previously obtained through a reverse transcription (RT) of total RNA extracted
16 from freshly purified rat retinal ganglion cells (RGC) and rat full retina (Ret), whereas RNA from rat brain tissue
17 (Br), was taken as positive control. These data revealed a high expression of these two-receptor transcripts in
18 enriched RGCs.

19 For each experiment, RGCs were seeded at initial density of 8,000 cells/well. The respective RGC densities at
20 6 DIV were expressed as a percentage of the control. Data are means±SEM from independent cultures (n=20
21 in A, n=18 in B, n=11 in C). ***p<0.001, **p<0.01 and *p<0.05 as compared to control (Kruskal-Wallis ANOVA,
22 followed by a Dunns *post-hoc* test).

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1 **Figure 4: Treatment with VEGF trap reduces RNFL thickness in glaucomatous**
2 **patients**

3 A: Circular peripapillary OCT scan analysis: An abnormal left eye with its corresponding fundus image (upper
4 left). Red lines in the OCT B-Scan (upper right) indicate the inner and outer borders of the RNFL found by the
5 algorithm. RNFL thicknesses (lower right) plotted over the thickness values measured in healthy subjects of
6 the same age. Mean thickness of the RNFL in the six sectors (lower left). This scan shows a significant
7 decrease (red segment) in the inferior temporal quadrant. B-C: IOP levels in eyes injected with VEGF traps
8 (blue curves, n=10) or non-injected control eyes (red curves, n=10) from glaucomatous (B) or non-
9 glaucomatous patients (C). D-E: RNFL thickness of eyes injected with VEGF trap (blue curves, n=10) or non-
10 injected control eyes (red curves, n=10) from glaucomatous (D) or non-glaucomatous patients (E). **p<0.01
11 compared to the baseline value in the treated group (Friedman test followed by a Dunn's *post hoc* test).
12

1 **Table 1: Growth factors in conditioned medium harvested from mixed retinal cultures**

	Growth factors (pg/ml)			
	VEGF	BDNF	G-CSF	GM-CSF
Control medium	ND	ND	ND	ND
CM-M	86.8 ± 13.2 (4/4)	ND	ND	ND

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Growth factors were measured in control and conditioned medium harvested from mixed retinal cultures (CM-M). Concentrations are expressed as pg per ml and the means ± SEM from independent preparations (n = 4), all showing detectable VEGF levels. ND: no-detectable.

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2 **Table 2: Patient characteristics**

	Age (mean±S.D.)	Gender (% female)	Glaucoma treatments* (numbers of drugs; mean±S.D)
Glaucoma group (n=10)	73.8 ± 7.9	60	1.8 ± 0.6
Non glaucoma group (n=10)	73.0 ± 9.0	70	-

3 *Glaucomatous patients received anti-glaucomatous treatment through eye-drop consisting in either
4 monotherapy, bi-therapy, tri-therapy, or quadri-therapy with drugs from the 4 distinct classes (Beta blockers,
5 Alpha-adrenergic agonists, Prostaglandins or Carbonic anhydrase inhibitors).
6 Non glaucomatous patients did not receive any eye treatment.