ORIGINAL ARTICLE

Morphological Changes of Cortical and Hippocampal Neurons after Treatment with VEGF and Bevacizumab

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ABSTRACT

Aims: Vascular endothelial growth factor (VEGF) is a hallmark of glioblastoma multiforme (GBM) and plays an important role in brain development and function. Recently, it has been reported that treatment of GBM patients with bevacizumab, an anti-VEGF antibody, may cause a decline in neurocognitive function and compromise quality of life. Therefore, we investigated the effects of VEGF and bevacizumab on the morphology and on survival of neurons and glial cells. **Methods:** Dissociated cortical and hippocampal cell cultures of juvenile rats were treated with VEGF, bevacizumab, and VEGF + bevacizumab. Neuronal and glial cell viability was analyzed, and the morphology of neurons was objectified by morphometric analysis. **Results:** In cortical cultures, bevacizumab significantly decreased the number of neurons after 20 days and the number of glial cells subsequent 30 days. Additionally, an increase in the dendritic length of cortical neurons was obvious after 10 days of incubation with bevacizumab, but returned to control level after 30 days. In hippocampal cultures, cell viability was not affected by bevacizumab; however, dendritic length increased at day 10, but decreased after long-term treatment. **Conclusion:** Therefore, bevacizumab obviously has a cytotoxic effect in cortical cultures and decreases the dendritic length in hippocampal neurons after long-term treatment.

Introduction

GBM is a highly malignant brain tumor characterized by poorly differentiated neoplastic astrocytic tumor cells, brisk mitotic activity, extensive neo-angiogenesis, vascular thrombosis, and necrosis. GBM is often found supratentorially, but may affect all other cortical and subcortical areas such as the cerebellum, brainstem, and spinal cord [1]. Surgical resection of the tumor, if possible, is the initial treatment. However "complete resection" is not possible as tumor cells invariably infiltrate the adjacent brain parenchyma. Therefore, radiotherapy and concomitant chemotherapy with temozolomide, followed by adjuvant temozolomide for 6 cycles, are part of a multimodal standardized treatment. The prognosis is poor with an overall survival of 14.6 months and a survival rate of 10% after 5 years [2].

A hallmark of GBM is extensive neoangiogenesis to supply the highly metabolically active tumor cells with blood. One major mechanism in GBM to promote angiogenesis is the upregulation of vascular endothelial growth factor A (VEGF-A) [3].

VEGF-A, a dimeric polypeptide playing a crucial role in angiogenesis, endothelial cell proliferation, and vascular permeability, belongs to a gene family that includes placental growth factor, VEGF-B, VEGF-C, and VEGF-D [4]. VEGF-A can be induced by hypoxia-inducible factor 1 (HIF-1) [5], hormones [6,7], and

Ras- and Wnt-signaling pathway mutations [8]. It potentially binds to receptor tyrosine kinases such as VEGF receptor-1 (VEGFR-1) and VEGF receptor-2 (VEGFR-2) and in addition to coreceptors such as neuropilins 1 and 2 [4]. The main effects of VEGF are mediated by VEGFR-2. While VEGF is best known for its role in physiological angiogenesis, it is also involved in pathological conditions. An upregulation of VEGF mRNA has, for example, been shown in tumors [4] and the level of VEGF, the tumor size, as well as the density of blood vessels, correlate in GBM [9].

VEGF-A mRNA can be found in different regions of the CNS and is expressed mainly in neurons, astrocytes, and endothelial cells [10–12]. It has multiple different functions, as it promotes endothelial cell growth and survival [13], improves growth cone guidance [14], supports neurogenic protective and neurotrophic effects in cortical neurons [15], and stimulates axonal growth in the central and peripheral nervous system [14,16]. In the same way, Rosenstein et al. [17] observed an increase in neuritic growth after VEGF treatment in organotypic cortical explants. The neuroprotective effect of VEGF is also present during brain aging as it promotes larger baseline hippocampal volume, less hippocampal atrophy, and less cognitive decline over time [18]. In cultured astrocytes, VEGF increases gap junction intercellular communication, as well as migration and proliferation [19].

Targeting neo-angiogenesis by blocking VEGF pathways has become a promising oncological therapeutic principle. Inhibiting VEGF activity in human tumors may reduce tumor growth and may prolong survival in some cases [20,21]. In 2009, bevacizumab, an antibody directed to VEGF-A, obtained an accelerated conditional food and drug administration approval for treatment of recurrent GBM. The "BRAIN study" and several other phase II trials reported on tumor responses in recurrent GBM according to magnetic resonance imaging criteria and on the improvement of neurological function in responding patients [22]. Recently, the AVAglio study and the RTOG0825 study demonstrated that bevacizumab prolongs progression-free survival in newly diagnosed GBM patients, but not overall survival [23,24].

With regard to the above-mentioned physiological functions of VEGF in the CNS, findings on cognitive function and quality of life in the RTOG0825 trial and the AVAglio trial are of clinical importance. While the AVAglio study reported on an improvement in patient's quality of life, the RTOG0825 trial detected a decline in neurocognitive function affecting different cognitive domains such as oral word association, verbal learning and memory, and mental flexibility [24, JS. Wefel et al., 2013, J. Wefel, S. Pugh, T. Armstrong, M. Gilbert, M. Won, M. Wendland, D. Brachman, P. Brown, I. Crocker, H.I. Robins, R.J. Lee, M. Mehta, unpublished data]. This suggests that inhibition of VEGF may lead to a neurotoxic environment in the brain.

It is known that blocking VEGF may negatively affect neurons [25,26]. This was confirmed by Cvetanovic et al. [27], who demonstrated an increased cell death of cerebellar Purkinje cells along with a decrease in number and length of their dendrites after addition of VEGFR-2 inhibitors or VEGF antibodies in cerebellar cultures containing granule neurons and Purkinje neurons. In addition, loss-of-function of VEGF leads to neuronal cell death in the embryonic olfactory bulb, reduced spine density in newborn granule cell, and reduced dendritic length and node count of newborn periglomerular neurons in transgenic mice [28]. Finally, in transgenic mice, the repression of VEGF signaling in the hippocampus impaired memory by decreasing long-term potentiation [29].

These findings suggest that VEGF plays a crucial role in brain development and neuronal integrity. The clinical observation that GBM patients treated with bevacizumab may suffer from a decline in neurocognitive function in the RTOG0825 study prompts to further investigate the role of VEGF and its blockade in preclinical models. This study focuses on neuronal morphology as well as on cell viability to support the hypothesis of a direct neurotoxic effect of the VEGF blockade. Measurement of the dendritic length and cell counting were done after treatment with VEGF, bevacizumab and the combinations of these substances in rat cortical and hippocampal neuronal cultures after 10, 20, and 30 days of incubation.

Materials and Methods

Primary Dissociated Neuronal Culture

Primary dissociated neuronal cultures were obtained from postnatal day 1 Wistar rats as previously described with slight modifications [30,31]. Briefly, rats were anesthetized by hypothermia, and the cerebral cortices and hippocampus were prepared out of the cranium in Hanks' solution and stripped out from the meninges and blood vessels. After 5 minutes of trypsinization (0.05% trypsin Thermo Fischer Scientific, Darmstadt, Germany) with a Teflon-covered magnetic stirring bar, the clear phase containing the dissociated neurons was collected. To stop trypsin activity, cells were transferred into minimal essential medium (MEM, M2279; Sigma-Aldrich, Schnelldorf, Germany) supplemented with 10% horse serum (S9135; Biochrom, Berlin, Germany), 1% L-glutamine (G7513; Sigma-Aldrich), and 1% penicillin (Sigma-Aldrich). After addition of fresh trypsin solution, the procedure was repeated four times for a total of twenty minutes. Then, the cell suspension was centrifuged for 15 minutes at 2398 *g*, and the pellet was resuspended in neuronal medium containing MEM supplemented with 10% horse serum, 0.6% glucose, 1% chicken embryo extract, 1% L-glutamine, 2% nerve growth factor-7S (N0513; Sigma-Aldrich), and 1% penicillin. Cells were plated on coverslips (ø 32 mm, 02R321-D; Kindler, Freiburg, Germany), precoated with collagen, and incubated at 37°C in a modified atmosphere of 5% CO_2 in air (90% humidity). This study has been performed under the terms of the German animal protection law.

Drug Incubation

The effects of VEGF and bevacizumab on neurons and glial cells were investigated by addition of 0.1 *μ*g/mL VEGF-165 (SRP4365; Sigma-Aldrich), 0.25 mg/mL bevacizumab (Avastin, B7106; Roche, Grenzach-Wyhlen, Germany), or combination of VEGF with bevacizumab to the nutrient medium for periods of 10, 20, and 30 days starting at day 4 in culture. The role of VEGF receptors was experimentally examined via the use of the inhibitor axitinib (S1005; Selleckchem, Houston, TX, USA). This was used at a final concentration of 10 *μ*M. The medium was changed twice a week.

Immunocytochemistry

At selected time points, coverslips for each condition were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 30 minutes. After permeabilization with 0.05% Triton (T8532; Sigma-Aldrich) and blockade of nonspecific binding sites with 15% goat serum in PBS for 30 minutes, cultures were incubated with primary antibodies, dissolved in PBS, at 4°C overnight. Cultures were stained with polyclonal rabbit antibodies against neurofilament H (1:200, AB1987; Millipore, Darmstadt, Germany), polyclonal rabbit antibodies against VEGFR-2 (1:200, ab39256; Abcam, Cambridge, UK), monoclonal rabbit antibodies against microtubule-associated protein 2 (MAP2, 1:200, M3696; Sigma-Aldrich), and monoclonal mouse antibodies against glial fibrillary acidic protein (GFAP, 1:200, G3893; Sigma-Aldrich). After intensive washing with PBS, cultures were incubated with secondary antibodies such as anti-rabbit IgG FITC (1:1000, F6005; Sigma-Aldrich), anti-rabbit IgG TRITC (1:1000, T5268; Sigma-Aldrich), anti-mouse IgG FITC (1:1000, F0257; Sigma-Aldrich), and anti-mouse IgG TRITC (1:1000, T5393; Sigma-Aldrich) at room temperature for 2 hours. After

washing with PBS, nuclear staining was performed by incubation with bisBenzimide H 33342 trihydrochloride (DAPI, B2261; Sigma-Aldrich) for 15 minutes. Finally, samples were rinsed in PBS and cover-slipped in mounting medium (S3023, Dako; F6937, Fluoroshield; Sigma-Aldrich).

Cell Viability

To evaluate the effect of bevacizumab on cell viability, cultures labeled with MAP2 antibody, GFAP antibody, and DAPI were imaged with the aid of a confocal laser scanning microscope (LSM 510, Zeiss, Oberkochen, Germany) in combination with Zeiss 20× (Pan-Neofluar, NA 0.4) lenses. Counting the total number of cells (neurons and glial cells) was carried out with a click counter software (Click Counter by MurGee.com) and with the aid of the ImageJ software. At least eight areas of three different cultures for each condition and each time point were investigated.

Morphometric Analysis of Dendritic Length

To investigate the effects of VEGF and bevacizumab on dendritic length, cultures labeled with MAP2 antibody were imaged with the aid of a confocal laser scanning microscope (LSM 510, Zeiss) in combination with Zeiss 40× (Plan-Neofluar, NA 1.3) oil immersion lenses. Measurement of the summation of dendritic length was achieved with the aid of the Zeiss physiology kit available on the

LSM. At least 40 neurons for each condition and each time point of at least three different cultures were measured (Figure.S1).

Statistical Analyses

The experiments were repeated at least three times for each treatment and each time point, so that finally more than 120 neurons and 24 areas per condition were analyzed. The effects between groups were calculated with a Student's *t*-test (two-tailed). *P*-values <0.05 were considered to be significant, and *P*-values <0.001 were considered to be highly significant.

Results

Dissociated Neuronal Cultures are Suitable to Analyze Neuronal Morphology

Primary cortical and hippocampal neurons were cultured for up to 30 days to study the effects of VEGF and bevacizumab on neuronal morphology. With the aid of a phase-contrast microscope, neurons were followed over time to record their development and morphology. After 10 days *in vitro*, details of neuronal cells were visible, like the soma, the axon and several dendrites surrounded by fibroblasts from the superficial cortical veins and the deep veins [32]. Moreover, the expression of VEGFR-2 in neurofilament H positive cortical and hippocampal neurons was confirmed after 10 days in culture (Figure S1).
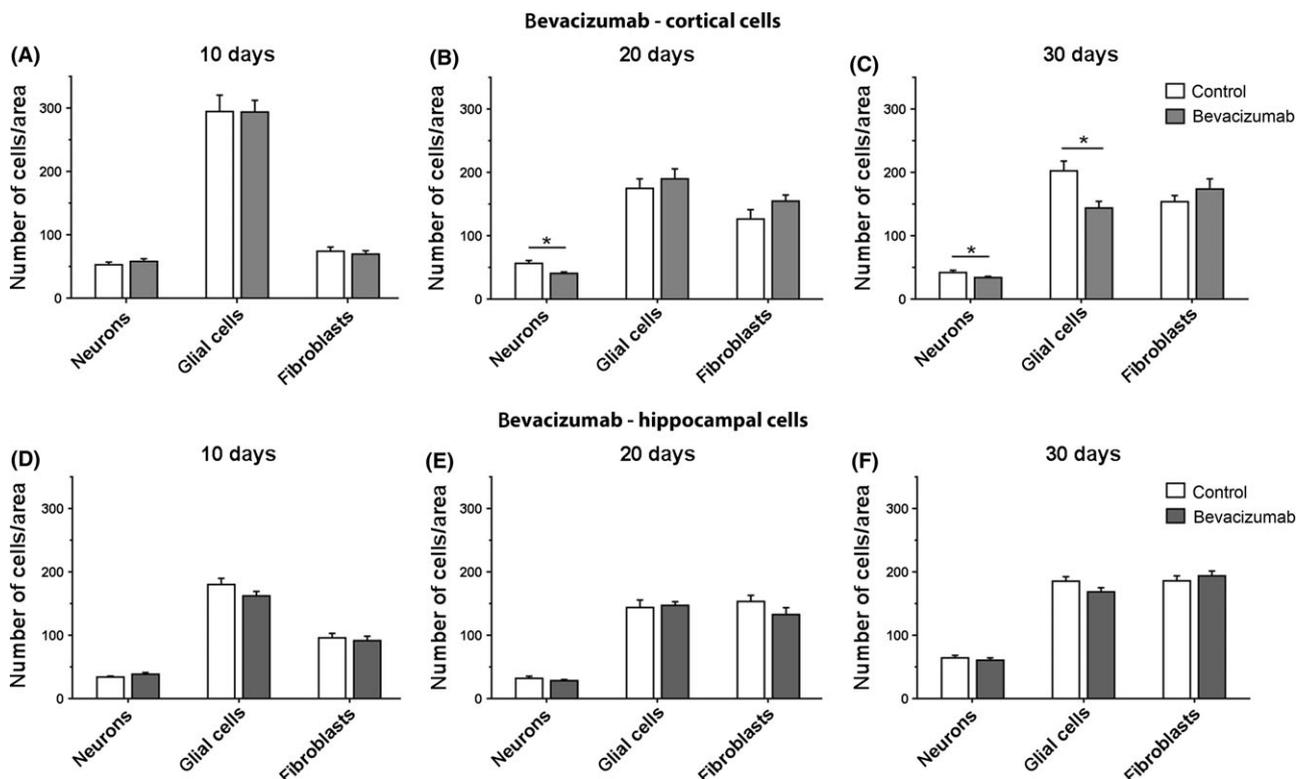

Figure 1 Cell viability in cortical and hippocampal cultures after incubation with bevacizumab. (**A–C**) Quantitative analysis of cell viability after incubation with bevacizumab in cortical cultures. (**D–F**) Quantitative analysis of cell viability after incubation with bevacizumab in hippocampal cultures. Results are the mean ± SEM, *P < 0.05 vs. control.

Effect of Bevacizumab on Neuronal Viability

In cortical cultures, bevacizumab induced changes regarding cell viability. After 10 days of incubation, no changes were observed for neurons, glial cells, and fibroblasts. After 20 days, cultures showed a decrease in the number of neurons ($P < 0.05$), and after 30 days, the number of neurons and glial cells decreased compared to control ($P < 0.05$) (Figure 1A–C).

Hippocampal cultures incubated with bevacizumab demonstrated no significant changes regarding cell viability after 10, 20, or 30 days (Figure 1D–F).

Effect of VEGF on Neuronal Morphology

After incubation of cortical neurons with VEGF for 10 days, the dendritic length significantly increased to 50.37 μm $\pm$ 1.52 ($P < 0.0001$) compared to control (43.09 μm $\pm$ 0.92) (Figure 2A,J). Similarly, after 20 days of incubation, VEGF (68.76 μm $\pm$ 1.82; $P < 0.0001$) induced an increase in dendritic length compared to control condition (49.87 μm $\pm$ 1.13) (Figure 2B,J). After 30 days of incubation, the average dendritic length was 49.64 μm $\pm$ 1.74 in control neurons. There was still an increase with subsequent VEGF treatment (56.93 μm $\pm$ 2.07; $P < 0.05$) (Figure 2C,J).

Hippocampal neurons showed an average summation of the dendritic length of 44.13 μm $\pm$ 1.06 after 10 days. Like cortical neurons, there was an increase in dendritic length in hippocampal neurons incubated with VEGF (61.79 μm $\pm$ 2.26; $P < 0.0001$) (Figure 3A,J). In the same manner, VEGF (60.10 μm $\pm$ 2.54; $P < 0.0001$) induced an increase in dendritic length in comparison with control condition (43.95 μm $\pm$ 2.13) after 20 days of incubation (Figure 3B,J). This increase in dendritic length was maintained after 30 days of incubation with VEGF (59.82 μm $\pm$ 2.32; $P < 0.0001$) (Figure 3C,J).

Effect of Bevacizumab on Neuronal Morphology

In cortical neurons, the average summation of the dendritic length in control neurons was 43.09 μm $\pm$ 0.92 after 10 days in culture. Incubation with bevacizumab and VEGF + bevacizumab induced a significant increase in the dendritic length (62.35 μm $\pm$ 2.06 and 74.02 μm $\pm$ 2.31, respectively; $P < 0.0001$) (Figure 2A,D,G, J). After 20 days of incubation, there was still a significant increase in the dendritic length in cultures treated with bevacizumab (68.60 μm $\pm$ 2.43; $P < 0.0001$) and with VEGF + bevacizumab (70.29 μm $\pm$ 3.07; $P < 0.0001$) (Figure 2B,E,H,J). This increase in dendritic length also persisted in long-term cell cultures (30 days) incubated with VEGF + bevacizumab (57.85 μm $\pm$ 2.62; $P < 0.05$), whereas this effect was not significant after incubation with bevacizumab alone (51.96 μm $\pm$ 2.07) (Figure 2C,F,I,J).

Hippocampal cultures showed similar results to cortical neurons after 10 days in culture. Indeed, hippocampal neurons treated with bevacizumab (64.57 μm $\pm$ 2.48; $P < 0.0001$) and VEGF + bevacizumab (68.62 μm $\pm$ 3.16; $P < 0.0001$) showed a significant increase in dendritic length compared to control cultures (44.13 μm $\pm$ 1.06) (Figure 3A,D,G,J). However, after 20 days of incubation, the average summation of dendritic length

in neurons treated with VEGF + bevacizumab (45.45 μm $\pm$ 2.51) reached the level of control cultures (43.95 μm $\pm$ 2.13) and decreased significantly with bevacizumab alone (34.79 μm $\pm$ 1.31; $P < 0.001$) (Figure 3B,E,H,J). After 30 days of incubation, the decrease in dendritic length persisted with bevacizumab (33.55 μm $\pm$ 1.35; $P < 0.001$), whereas neurons incubated with VEGF + bevacizumab (40.64 μm $\pm$ 1.84) showed no significant changes compared to control neurons (44.27 μm $\pm$ 1.67) (Figure 3C,F,I,J).

Effect of Axitinib on Cell Viability and Neuronal Morphology

After 10, 20, and 30 days of axitinib incubation in cortical and hippocampal cultures, a decreased number of neurons, glial cells, and fibroblasts compared to control cultures were observed ($P < 0.0001$) (Fig. 4A–F). Besides this, the dendritic length of cortical neurons significantly increased during axitinib exposure (106.26 μm $\pm$ 4.62 after 10 days; 82.29 μm $\pm$ 3.13 after 20 days; 58.44 μm $\pm$ 3.13 after 30 days; $P < 0.0001$) and VEGF + axitinib (97.64 μm $\pm$ 7.12 after 10 days; 122.78 μm $\pm$ 4.5 after 20 days; 67.09 μm $\pm$ 3.37 after 30 days; $P < 0.0001$) compared to control (43.09 μm $\pm$ 0.92 after 10 days; 49.87 μm $\pm$ 1.13 after 20 days; 49.64 μm $\pm$ 1.74 after 30 days) (Fig. 4G). Similarly, hippocampal neurons incubated with axitinib (114.09 μm $\pm$ 5.37 after 10 days; 102.71 μm $\pm$ 4.93 after 20 days; 71.10 μm $\pm$ 4.33 after 30 days; $P < 0.0001$) and VEGF + axitinib (173.53 μm $\pm$ 8.52 after 10 days; 111.33 μm $\pm$ 4.67 after 20 days; 94.34 μm $\pm$ 5.21 after 30 days; $P < 0.0001$) for 10, 20, and 30 days showed an increase in dendritic length in comparison with control neurons (44.13 μm $\pm$ 1.06 after 10 days; 43.95 μm $\pm$ 2.13 after 20 days; 44.27 μm $\pm$ 1.67 after 30 days) (Fig. 4H).

Conclusion

In this study, we investigated the effect of VEGF and bevacizumab on neuronal morphology and viability. Therefore, we morphometrically analyzed the length of neuronal dendrites in dissociated rat cortical and hippocampal cell cultures after 10, 20, and 30 days of incubation. In addition, cell survival was evaluated by counting the number of neurons, glial cells, and fibroblasts after 10, 20, and 30 days of incubation.

As we visualized single rat neurons to record their overall morphology, cortical and hippocampal primary cell cultures were particularly suitable for a detailed morphological analysis. In the cortex, pyramidal neurons of different size, stellate cells, Cajal–Retzius cells, interneurons, and subplate cells were found and first described at the end of the 19th century. In the hippocampus, pyramidal and granular cells were mostly present. As all kinds of different neurons are responsible for brain function, we measured the dendritic length in all types of neurons.

In summary, this study shows that direct inhibition of VEGF by bevacizumab decreased neuronal and glial cell numbers in cortical cultures after both 20 and 30 days. In addition, bevacizumab increased the cortical dendritic length for up to 20 days, as well as the hippocampal dendritic length for as long as 10 days. After a prolonged time of incubation, bevacizumab decreased the den-
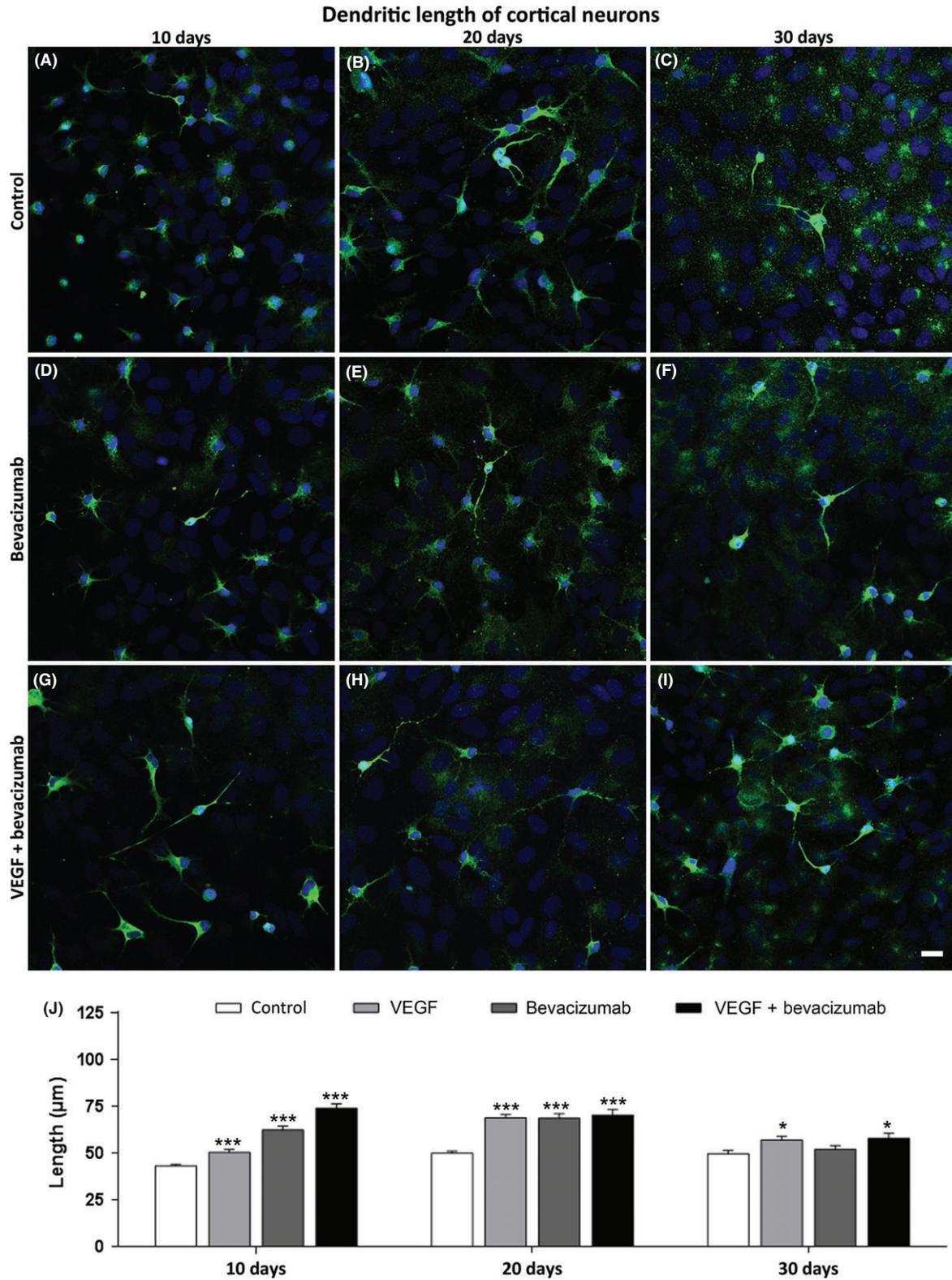
Dendritic length of cortical neurons

Figure 2 Morphological alterations of cortical cultures after incubation with VEGF and bevacizumab. (**A–I**) Representative images of cortical neurons immunostained for MAP2 (green) and Hoechst (blue) after 10, 20, and 30 days of incubation with nutrient medium alone or supplemented with bevacizumab or VEGF + bevacizumab. (**J**) Quantitative analysis of cortical dendritic length. Results are the mean $\pm$ SEM, *$P < 0.05$, ***$P < 0.0001$, vs. control. Scale bar = 20 μm.
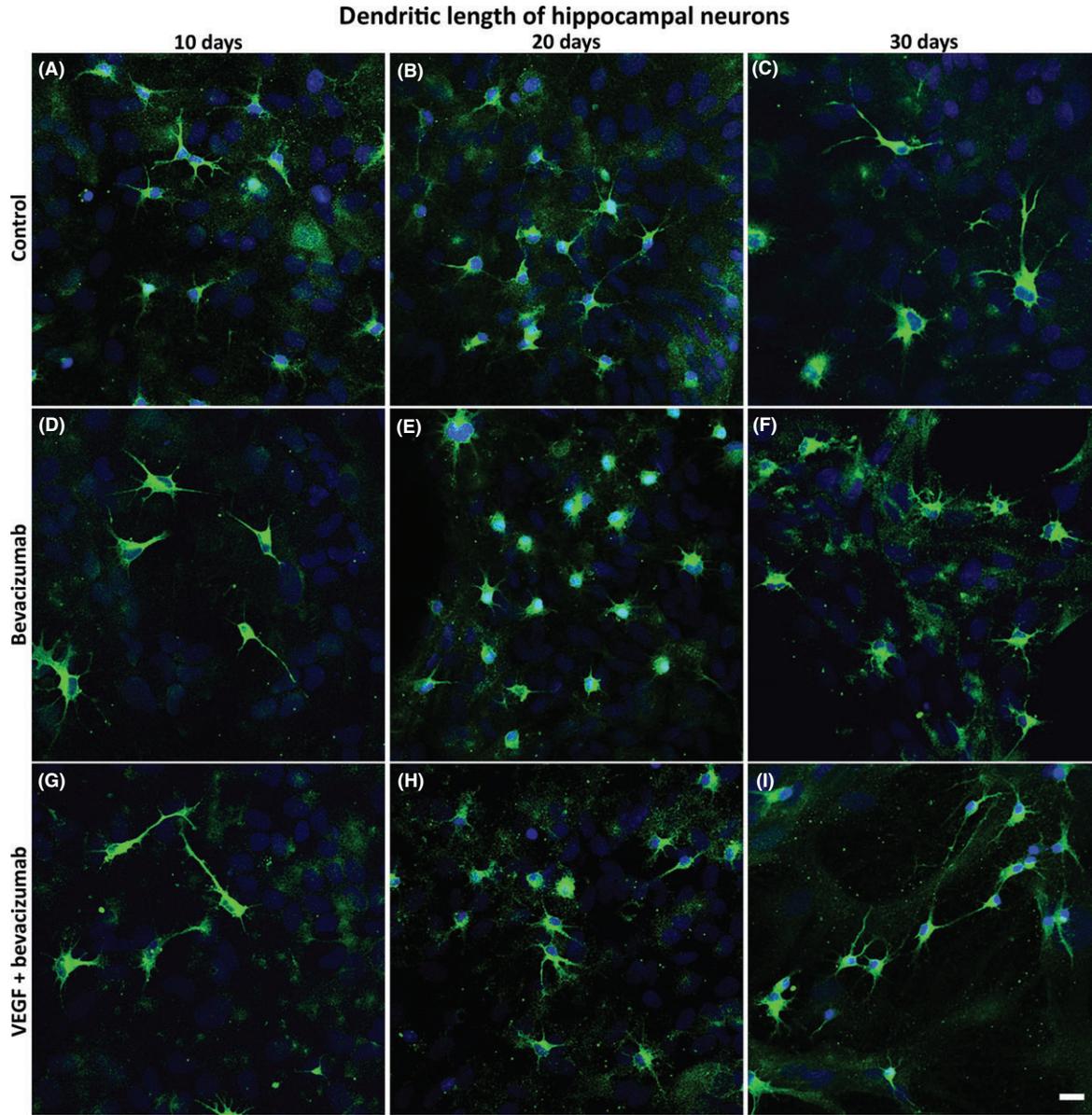
Figure 3 Morphological alterations of hippocampal cultures after incubation with VEGF and bevacizumab. (**A–I**) Representative images of hippocampal neurons immunostained for MAP2 (green) and Hoechst (blue) after 10, 20, and 30 days of incubation with nutrient medium alone or supplemented with bevacizumab or VEGF + bevacizumab. (**J**) Quantitative analysis of hippocampal dendritic length. Results are the mean ± SEM, **$P < 0.001$, ***$P < 0.0001$, vs. control. Scale bar = 20 μm.
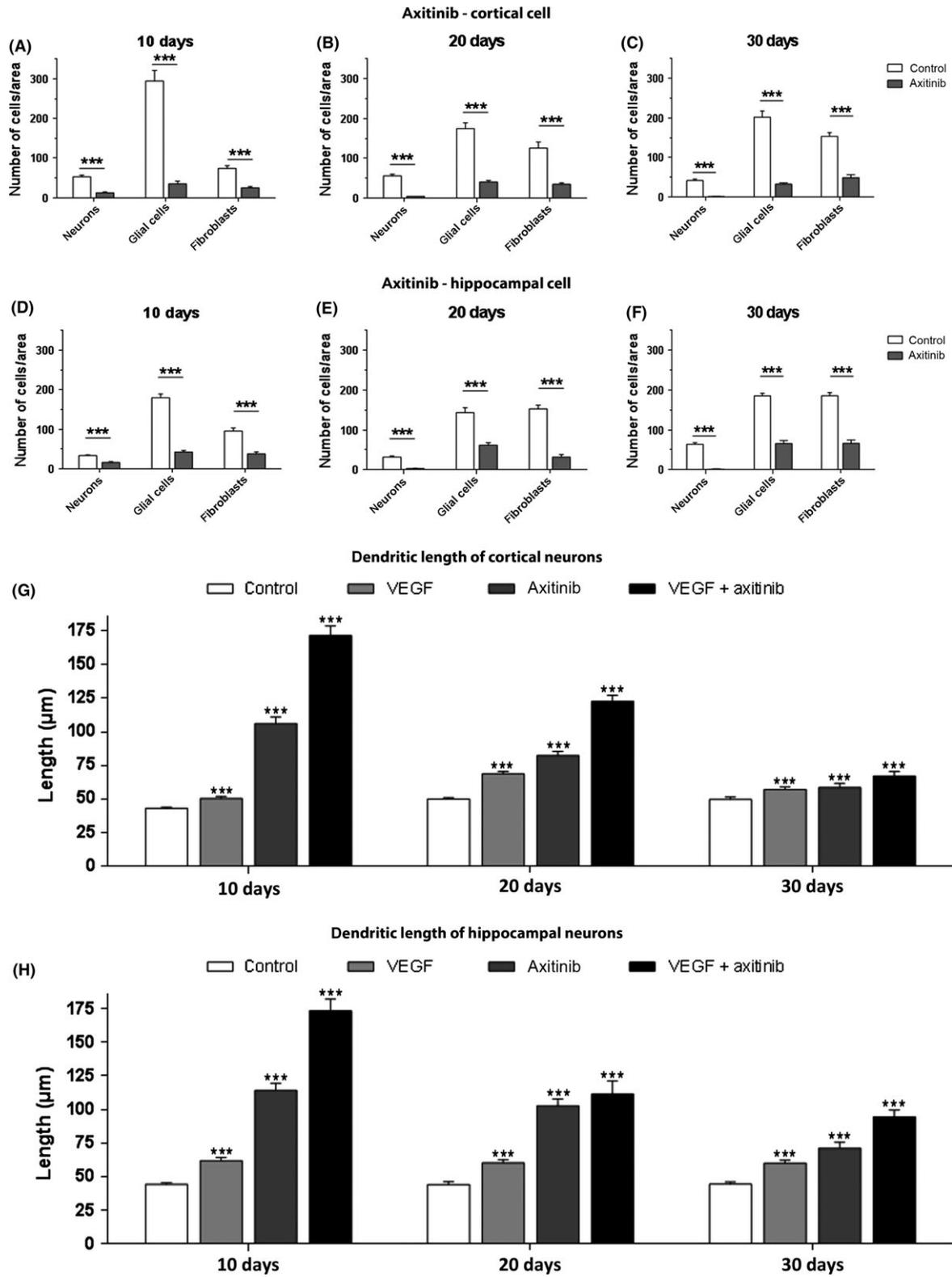
Figure 4 Cell viability and morphological alterations in cortical and hippocampal cultures after incubation with axitinib. (**A–C**) Quantitative analysis of cell viability after incubation with axitinib in cortical cultures. (**D–F**) Quantitative analysis of cell viability after incubation with axitinib in hippocampal cultures. (**G**) Quantitative analysis of cortical dendritic length. (**H**) Quantitative analysis of hippocampal dendritic length. Results are the mean ± SEM, ***$P < 0.0001$ vs. control.

Table 1 Effects of bevacizumab on cortical and hippocampal cultures

Time of incubation	Measurement	Bevacizumab cortex	Bevacizumab Hippocampus
10 days	Number of		
	neurons	No changes	No changes
	glial cells	No changes	No changes
	fibroblasts	No changes	No changes
	neuronal dendritic length	↑↑↑	↑↑↑
20 days	Number of		
	neurons	↓	No changes
	glial cells	No changes	No changes
	fibroblasts	No changes	No changes
	neuronal dendritic length	↑↑↑	↓↓
30 days	Number of		
	neurons	↓	No changes
	glial cells	↓	No changes
	fibroblasts	↓	No changes
	neuronal dendritic length	No changes	↓↓↓

↑ significant increase compared to control. ↓ significant decrease compared to control.

dritic length of hippocampal neurons suggesting a chronic effect of this medication (Table 1).

Morphological Effect of VEGF and Bevacizumab

VEGF Increases the Dendritic Length

VEGF-incubated neurons showed an increase in dendritic length after 10, 20, and 30 days of incubation in cortical and hippocampal cultures. Similar results were demonstrated by Cvetanovic et al. (2011) in Purkinje cells and by Licht et al. (2010) in interneurons from the olfactory bulb, where VEGF showed a positive effect on dendritogenesis [27,28]. Moreover, Rosenstein et al. [17] observed an increase in neuritic growth following VEGF treatment in organotypic cortical explants. Together, these results confirm the neurotrophic role of VEGF in the brain.

Bevacizumab

No changes regarding cell viability have been denoted subsequent to bevacizumab treatment after 10 days in cortical and after 10, 20, and 30 days in hippocampal cultures. This is in accordance with another report on the effect of bevacizumab on retinal ganglion cells in rats showing no significant differences in cell number between control and bevacizumab treatment *in vivo* as well as *in vitro* [33]. Similar results were obtained after incubation of the human trabecular meshwork cells with bevacizumab [34]. On the contrary, death of retinal ganglion cells after intravitreal injection of bevacizumab was found based on apoptotic markers as well as a decrease in Purkinje cells in primary cultures of cerebellar neurons following anti-VEGF treatment [27,35]. This is in accordance with our results following 20 and 30 days of incubation with bevacizumab, where a decrease in neuronal cell number could be observed in cortical cultures.

Cortical neurons treated with bevacizumab alone or in combination with VEGF showed a strong increase in dendritic length after 10 and 20 days of incubation, and no significant increase after 30 days, except for the combination of bevacizumab with VEGF. This may be due to the fact that bevacizumab does not completely block endogenous and exogenous VEGF binding at each time point and allows neurite extension for up to 20 days. On the contrary, after an increase in dendritic length following 10 days of treatment, hippocampal neurons exposed to bevacizumab showed a significant decrease after 20 and 30 days of incubation, which is rescued by the addition of VEGF. This indicates that bevacizumab is less efficient at blocking exogenous VEGF after 20 and 30 days of treatment, but still inhibits neurite extension in hippocampal cultures.

The decrease in dendritic length in hippocampal neurons is in agreement with the results obtained by Cvetanovic et al. (2011) [27], who demonstrated that in short-term experiments, Purkinje cells exhibited short neurites after 3 days of treatment with VEGF antibodies. By secreting a soluble receptor that binds and inhibits VEGF, transgenic mice showed a decrease in dendritic length of newborn periglomerular cells after 45 days of inhibition [28]. However, 24 hours of treatment with bevacizumab induced no changes in neurons from dorsal root ganglia [36], which is comparable to our results in cortical neurons after 30 days of treatment.

Signaling Pathways

VEGF can bind to tyrosine kinase receptors VEGFR-1 and VEGFR-2 and to coreceptors, neuropilins 1 and 2. The function of VEGFR-1 is still under debate, but it seems to promote a decoy effect that prevents binding to VEGFR-2 [37]. Neuropilins 1 and 2 seem to present VEGF-165 to VEGFR-2 and enhance its binding [38]. Therefore, the main effects of VEGF are mediated by VEGFR-2, which undergoes dimerization and ligand-dependent tyrosine phosphorylation to mediate mitogenic, chemotactic, and pro-survival signals [4]. It has also been reported that VEGFR-2 mediates actin polymerization via the Rho/ROK pathway [39] and also forced cell migration via SAPK2/p38 (mitogen-activated protein

kinase MAPK), angiogenesis, and cell proliferation via Raf-Mek-Erk1-2 pathways [40].

In the nervous system, effects on morphology, particularly on the cytoskeletal reorganization, are conveyed by VEGFR-2 [14]. The important role of VEGFR-2 was also investigated through its inhibition. It was demonstrated with antisense oligodeoxynucleotides that VEGFR-2 is responsible for the neuroprotective effect of VEGF against glutamate excitotoxicity in hippocampal neurons [41]. This has been confirmed using the VEGF receptor blocker PTK787 and even more specifically using the VEGFR-2 blocker ZM323881 [42]. In cortical neurons, inhibition of VEGFR-2 by SU1498 blocked the ability of VEGF to induce neurogenesis [15]. This inhibitor also prevents the VEGF-induced calcium influx and reduced the VEGF-induced synaptic enhancement in hippocampal neurons [43]. Besides this, SU5416, a selective Flk-1 inhibitor, also blocked VEGF-induced cell proliferation in cultured hippocampal stem/progenitor cells [44]. These studies confirm the primordial role of VEGFR-2 to convey the effect of VEGF in the nervous system.

In the present study, VEGFR-2 is expressed in dissociated cortical and hippocampal neurons, which is in line with previous studies [15,32,41,42,44]. Therefore, it is very likely that the observed increase in dendritic length after VEGF incubation is mediated by VEGFR-2.

However, blocking VEGFR-1 and VEGFR-2 by the use of axitinib increased the dendritic length of cortical and hippocampal neurons but significantly reduced the number of neurons, glial cells, and fibroblasts. In comparison with our results in cortical and hippocampal cultures, a reduction in Tuj1-positive neurites has been reported by incubation with antisense oligonucleotides to VEGFR-2 mRNA in cortical explants and cortical neuronal cultures [17]. In line with this, in cortical neurons treated with SU1498, a selective Flk-1 inhibitor, the VEGF-induced neurite outgrowth was abolished [39]. However, in these studies, qualitative analysis of Tuj1-positive neurites was carried out after 3 days of culture and morphometric analysis of neurite outgrowths after 4 days *in vitro* by measuring the absorption of cresyl violet by the neurons. This is not comparable to our study, as we used a specific marker for dendrites and much longer incubation periods.

Besides this, the reduction in cell viability observed in our study is in accordance with an increase of apoptosis observed after inhibition of VEGF receptor tyrosine kinase activity using SU1498 in cortical neurons [45]. Other than that, a decrease in cell viability in hippocampal neurons following a similar treatment, along with oxidative stress and a collapse in the mitochondrial membrane potential, was observed [26].

Therefore, we suggest that due to the decreased number of cells within the cell culture treated with axitinib, for surviving neurons, there is an enlarged space to grow and to spread their dendrites into. Phase-contrast microscopy confirmed these results, as we could clearly see the cellular debris of dead cells during prolonged axitinib exposure times, and even larger neurons in the axitinib incubated cultures in comparison with controls. It is also conceivable that the increase in dendrite length results from an enhanced NGF activity by the loss of competitive interaction with VEGF pathways. The culture medium includes NGF, which is known to activate Cdc42 by binding to its Trk A receptor [46,47]. Cdc42 is also part of the VEGF pathways, where its activation leads to cytoskeletal rearrangements similar to the Trk A pathway [48]. While VEGF pathways are specifically blocked by axitinib treatment, the NGF pathway is still able to induce an increase in dendritic length. It is also conceivable that VEGF binds to neuropilin receptors to mediate its effect as demonstrated by Hao and co-workers (2013) [26]. In fact, they denoted a loss in cell viability using SU1498, which was rescued by addition of VEGF even though VEGFR-2 was still inhibited. This indicates that VEGF can also act through activation of alternative receptors. The role of neuropilin receptors in neurons has also been investigated in regard to gonadotropin-releasing hormone (GnRH). Mice lacking the neuropilin 1 receptor showed a decrease in GnRH-positive neurons in the head, and an inhibition of NRP1 in immortalized GnRH-positive neurons abrogated the pro-survival role of VEGF independently of VEGFR-2 [49]. Furthermore, neuropilin receptors 1 and 2 are involved in the migration of GnRH-positive neurons via VEGF and semaphorin signaling [50]. In primary cultures of trigeminal ganglion neurons, Pan et al. [51] showed that inhibition of VEGFR-1, VEGFR-2, or neuropilin receptor-1 decreased neurite elongation, suggesting that multiple VEGF receptors mediate neuronal growth.

Effects on Different Cell Types

This study shows that bevacizumab leads to a decrease in the number of neuronal and glial cells. Beside this, fibroblasts seemed to be unaffected by bevacizumab in cortical and hippocampal cultures. This confirms that the effects seen in our cultures systems are cell type specific and not the consequence of a global unspecific cell toxic effect.

In our study, we investigated the effect of VEGF and bevacizumab in cortical and hippocampal neurons, both regions being implicated in cognitive functions. A difference between these regions has been detected regarding bevacizumab treatment, as an increase in the dendritic length during 20 days of incubation in cortical neurons was seen, but a decrease after 20 days in hippocampal cultures has been observed. We also demonstrated that in cortical cultures, VEGF induced an increase in dendritic length at each time points. In hippocampal cultures, VEGF-induced increases in dendritic length are more pronounced after 10 and 30 days of incubation compared to cortical cultures. This implies that VEGF and bevacizumab exert a selective biological activity depending on the neuronal cells and very likely on the developmental stage of these cells.

As our dissociated cell cultures contain neurons, glial cells as well as fibroblasts, the effects of VEGF and its related inhibitor can probably be induced by targeting neurons directly or through glial cells as mediators. Indeed, it is known that VEGF's immunoreactivity was demonstrated to be primarily astrocytic [12]. There is also experimental evidence that VEGF has a mitogenic effect on GFAP positive cells in mesencephalic explant cultures [52], as well as in cultured astrocytes where VEGF is also able to increase cell communication and cell migration through the VEGFR-2 [18]. This indicates that glial cells are sensitive to VEGF, which indirectly have the ability to influence the neuronal morphology.

As we observed a decrease in neuronal and glial cell number after bevacizumab treatment in cortical cultures, as well as a decrease in hippocampal dendritic length, we concluded that neu-

rons as well as glial cells are sensitive to bevacizumab. Neuronal sensibility to VEGF antibody was also observed by an increase in dead Purkinje cells [27]. In a similar way, glial cells are sensitive to bevacizumab as shown by Fusco et al. [53], who denoted an increase in gliosis in the presence of bevacizumab in juvenile rabbits.

This indicates that glial cells are sensitive to VEGF and bevacizumab, which can influence neuronal dendritic length through signaling mechanisms that are unknown until now. Therefore, we assume that VEGF has direct effects on the neurons, mediated at least through the VEGFR-2, but besides this it is likely that other cell type such as neighboring astrocytes may also transmit VEGF effects to the neurons.

This is the first study that showed the morphological effects of bevacizumab in cortical and hippocampal cultures. As we investigated cell viability and the dendritic length, we cannot directly conclude on the impact on neuronal function. Nevertheless, recent studies demonstrated a correlation between dendritic length and cognition. In fact, the relation between the size of the dendritic tree, the number of synapses it can receive as well as the number of presynaptic cells and synapses it can sample has been reviewed by Lefebvre et al. [54]. These authors specified that modification of dendritic size influences the function of the neuronal circuits. Besides this, Xu et al. [55] observed a decrease in cognition in stressed mice accompanied by a deterioration of dendritic morphologies in the hippocampus and cortex. Although bevacizumab showed several beneficial effects in the clinical studies of GBM treatment [22,23], the adverse effects of bevacizumab on hippocampal morphology as well as on cortical cell viability have to be considered.

Acknowledgment

Conflict of Interests

The authors declare no conflict of interest.

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Supporting Information

The following supplementary material is available for this article:

Figure S1. Dissociated neuronal cultures to analyze neuronal morphology.