VEGF$_{165}$b is an endogenous neuroprotective splice isoform of VEGF-A *in vivo* and *in vitro*.

[1]Nicholas Beazley-Long[†], [1]Jing Hua[†], [2]Thomas Jehle, [3]Richard P Hulse, [4]Rick Dersch, [2]Christina Lehrling, [1]Heather Bevan, [1]Yan Qiu, [2]Wolf A. Lagrèze, [4]David Wynick, [5]Amanda J. Churchill, [6]Patrick Kehoe, [1]Steven J. Harper, [1*]David O. Bates, [3*]Lucy F. Donaldson

[1]Microvascular Research Laboratories, Bristol Heart Institute, Department of Physiology and Pharmacology, School of Veterinary Sciences, University of Bristol, Southwell Street, Bristol BS2 8EJ, United Kingdom, [2]University Eye Hospital, Albert-Ludwigs University, Killian Strasse 5, [3]Department of Physiology and Pharmacology, School of Medical Sciences, University of Bristol, University Walk, Bristol BS8 1TD, United Kingdom, [4]79106 Freiburg, Germany, University Hospital Freiburg, Department of Neurology, Breisacher Straße 64, 79106 Freiburg [5]Bristol Eye Hospital, Lower Maudlin Street, Bristol BS1 2LX, United Kingdom. [6]Dementia Research Group, John James Laboratories, School of Clinical Sciences, University of Bristol, Frenchay Hospital, BS16 1LE

† Joint First Author

* These authors contributed equally to this paper.

Abbreviated title. VEGF$_{165}$b is an endogenous neuroprotective agent

**Correspondence to:

David Bates PhD or Lucy F Donaldson PhD,

Email: Dave.Bates@bris.ac.uk or Lucy.Donaldson@bris.ac.uk

Five figures. Abstract 229 words, Introduction, 498, Discussion 993

Acknowledgements. This work was funded by the Wellcome Trust (079736, 058083) and the Richard Bright VEGF Research Trust..

Abstract

Vascular endothelial growth factor (VEGF) is generated as two contrasting isoform families in terms of their actions on vascular permeability, angiogenesis and vasodilatation, but both families are cytoprotective for epithelial and endothelial cells. The pro-angiogenic $VEGF_{165}a$ isoform has been shown also to be neuroprotective in hippocampal, dorsal root ganglia and retinal neurones, but the effect of the contrasting $VEGF_{165}b$ isoform is unknown. We therefore tested the hypothesis that the $VEGF_{165}b$ isoform may be an endogenous neuroprotective agent for hippocampal, retinal and DRG neurons.

RESULTS. Endogenous expression of human and rat $VEGF_{165}b$ was detected in hippocampal and cortical neurons and formed a significant proportion of total VEGF in rat brain. Recombinant human (rh)$VEGF_{165}b$ exerted neuroprotective effects on several different neuronal types exposed to different insults, including glutamatergic excitotoxicity in hippocampal neurons, chemotherapy induced cytotoxicity of dorsal root ganglion cells and retinal ganglion cells in rat retinal ischemia-reperfusion injury *in vivo*. Protection against excitotoxic damage was dependent on VEGFR2 activation, and p42/p44MAPK.

CONCLUSIONS. rh$VEGF_{165}b$ is a neuroprotective agent that effectively protects both peripheral and central neurons *in vivo* and *in vitro,* through VEGFR2, MEK1/2 and inhibition of caspase-3 induction, with no requirement for neuropilin binding. $VEGF_{165}b$ may be therapeutically useful for pathologies involving neuronal damage, including hippocampal neurodegeneration, glaucoma diabetic retinopathy and peripheral neuropathy, whilst non-isoform specific inhibition of VEGF (for anti-angiogenic reasons) may be damaging to retinal and sensory neurons.

Introduction.

VEGF-A, originally described as a potent vascular permeability and growth factor for endothelial cells is upregulated in the brain during stroke and ischaemic episodes (Z. Kovacs et al., 1996) and has been linked with many neuronal diseases. The most widely studied isoform of VEGF, $VEGF_{165}a$, is upregulated in hypoxia, induces increased vascular permeability in neuronal vasculature, and can stimulate angiogenesis after ischemic episodes. The resulting oedema and hyperaemia can be damaging, but $VEGF_{165}a$ has also been shown to have direct anti-cytotoxicity effects on neurons, raising the possibility that it may act as an endogenous neuroprotective agent. VEGF also exerts neurotrophic (survival), and neurotropic (neurogenesis, axon outgrowth) actions, which, although initially thought to be a function of increased angiogenesis and perfusion after neuronal injury (M. I. Hobson et al., 2000), are now appreciated as direct effects of VEGF on neurones.

The *vegf-a* gene encodes numerous products by differential splicing, but not all isoforms exert the same effects (S. J. Harper and D. O. Bates, 2008). Alternative splicing of exon 8 leads to two functionally distinct families - the pro-angiogenic $VEGF_{xxx}a$ family and the counteracting $VEGF_{xxx}b$ family (D. O. Bates et al., 2002; J. Woolard et al., 2004). $VEGF_{165}b$ prevents the $VEGF_{165}a$ effects on increased vascular permeability, blood vessel growth and vasodilatation. However, $VEGF_{165}b$ acts in the same way as $VEGF_{165}a$ on epithelial and endothelial survival, being cytoprotective for retinal-pigmented epithelial cells, visceral glomerular epithelial cells (podocytes), and umbilical vein endothelial cells (H. S. Bevan et al., 2008; A. L. Magnussen et al., 2010).

The therapeutic potential of VEGF and anti-VEGF treatments are now widely recognized and anti-VEGF treatments are available in ophthalmology and oncology. The findings that VEGF is implicated in neuronal disorders e.g. Alzheimer's disease, Parkinson's disease, Huntington's disease, diabetic neuropathy, and ALS (see

review (E. Storkebaum et al., 2004)), provide a rationale for the use of VEGF as a therapeutic in neurodegenerative conditions. Although this is supported by preclinical evidence (M. Sondell et al., 1999) the identification of the $VEGF_{xxx}b$ family requires re-examination of VEGF isoforms in these contexts, to allow for the clear evidence that VEGF splicing variants are not functionally equivalent (S. J. Harper and D. O. Bates, 2008), and to determine whether augmentation of the pro-angiogenic isoform family ($VEGF_{xxx}a$) alone may have deleterious effects (eg in occult malignancy, carcinoma *in situ* etc).

The neuroprotective profile of the exon 8 alternatively spliced isoforms $VEGF_{xxx}b$ remains unexplored. Interestingly, $VEGF_{xxx}b$ isoforms do not exhibit the vascular effects seen with $VEGF_{xxx}a$ isoforms, such as a sustained increase in capillary permeability or hypotension(J. Woolard et al., 2004; C. A. Glass et al., 2006) The lack of these potential adverse effects may make $VEGF_{xxx}b$ isoforms more amenable as therapeutic agents in neurodegenerative diseases.

We therefore tested the hypothesis that $VEGF_{165}b$ is neuroprotective for central and peripheral neurones. We show that $VEGF_{165}b$ is expressed in central neurones, and is neuroprotective *in vitro*, and *in vivo*. This indicates that $VEGF_{165}b$ may prove to be a suitable therapeutic agent in neurodegenerative disorders, exhibiting fewer adverse effects than $VEGF_{165}a$.

Materials and Methods

All reagents were sourced from Sigma-Aldrich (Dorset, UK) unless otherwise stated. Antibodies were sourced from R&D systems (Carlsbad, CA): αVEGF$_{165}$b, (MAB 3045); α–caspase-3; αTrkA and αNF-200. Recombinant human (rh)VEGF$_{165}$b was provided by Philogene, New York or from R&D Systems (Carlsbad). Computer-aided analysis of immunohistochemistry, retinal Fluorogold staining, and cytotoxicity was performed using Macintosh computers running public domain Image J plus Cell Counter Plugin (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

VEGF protein was localized in fixed or frozen tissue sections using standard immunohistochemical/immunofluorescent techniques (H. S. Bevan et al., 2008) or was measured in tissue extracts by commercially available ELISA, or Western blotting as previously described (J. Woolard et al., 2004). Total VEGF and VEGF$_{165}$b were detected using validated, commercially available antibodies. The VEGF$_{165}$b antibody detects the unique C-terminal of the alternatively spliced VEGF$_{xxx}$b family (J. Woolard et al., 2004; A. H. Varey et al., 2008).

Human embryonic and adult tissues were obtained under ethical approval by North Bristol NHS Trust or University of Leiden. All procedures using animals were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 and with University of Bristol Ethical Review Panel approval.

Glutamate-induced hippocampal neuronal excitotoxicity was assessed in cultures of neonatal hippocampal neurons from twelve 2-day old CD1 mouse pups as previously described (C. R. Elliott-Hunt et al., 2004). Neurons were cultured on poly-lysine coated glass cover slips in 6 well plates (37°C, ambient oxygen and 5% CO_2,

400,000 cells/ plate) in neuronal growth media plus B-27 supplement, (GIBCO-Invitrogen, Paisley, UK) with penicillin and streptomycin and 1% BSA. After 24 hours cultures were supplemented with 10μg/ml 5-fluoro-2'-deoxyuridine to inhibit growth of non-post-mitotic cells. Excitotoxicity assays started on day 10 of culture. Cultures were exposed to 3mM L-glutamic acid for 24 hours in the presence of $VEGF_{165}b$ (0.01, 0.1, 1 and 10nM) or 50nM galanin (Bachem). To determine the mechanism of $VEGF_{165}b$-mediated effects, additional cultures were incubated with $VEGF_{165}b$ in the presence of VEGF receptor, and downstream signalling molecule inhibitors PTK787 (Novartis), SU5416 (Sugen), ZM323881 (Astra Zeneca) and PD98059 (Calbiochem). Cytotoxicity was assessed by a trained observer blinded to treatment using a Live/Dead® Viability/Cytotoxicity Kit (Molecular Probes-Invitrogen, Paisley, UK). The cells were fixed and nuclei stained (Hoechst 33258; 1:2300) and mounted to a glass slide. Ten random images of each cover slip were taken.

Oxaliplatin-induced dorsal root ganglion (DRG) neuronal death was assessed in primary cultures of adult rat dorsal root ganglion neurons. DRG were dissected, enzymatically and mechanically dissociated, and cells plated onto poly-L-lysine- and laminin-coated coverslips at ~2,850 cells/cm^2. 5-Fluoro-2'-deoxyuridine (30μg/mL) was added to prevent non-post-mitotic cells from proliferating. DRG neurons were cultured for 2 days before overnight pre-treatment with test compounds (2.5nM $VEGF_{165}b$ or vehicle). Neurons were then treated with oxaliplatin for 24 hours at 0, 5, 10 or 20μg/mL with 2.5nM $VEGF_{165}b$ or vehicle. Following treatment, neurons were fixed in 4% PFA and subjected to immunofluorescence for the detection of activated caspase 3 (rab mAb, 200ng/mL, overnight, Cell Signaling). Neurons were identified by co-staining with markers NeuN, βIII-tubulin or neuron-specific enolase-1 (NSE-1). The percentage of caspase-3-positive neurons were analysed by a trained observer blinded to treatment. In separate experiments VEGFR2 expression was detected

using a rabbit VEGFR2 mAb (1:500, overnight, Cell Signaling, 50b11). The primary antibody was replaced by concentration- and species-matched IgG to control for each immunofluorescence experiment.

The effect of $VEGF_{165}b$ on neurite outgrowth in cultured rat DRG neurones was assessed as previously described (F. E. Holmes et al., 2000). Adult DRG neurones were cultured with or without 2.5nM $VEGF_{165}b$ for 24 hours, and the length of neurite outgrowth assessed.

A total of 27 male Wister rats (250-350g, Charles River, Germany) were used to assess the effect of $VEGF_{165}b$ on retinal ganglion cell (RGC) death following ischemia. One week before ischaemia/reperfusion injury, retinal ganglion cells were retrogradely labelled with fluorogold using a previously described method (I. Selles-Navarro et al., 1996; T. Jehle et al., 2008). Briefly, Fluorogold (4% in PBS, Fluorochrome Inc., Denver, CO) was injected into bilateral superior colliculi (0.6μl at 4.2mm and 0.7μl at 4.7mm depth) under isoflurane inhalation anaesthesia (one minute induction at 5% in O_2, reduced to 3% for maintenance) and sterotaxic guidance. Fluorogold is retrogradely transported in RGCs and somatic labelling is maintained for at least three weeks (A. K. Ahmed et al., 1995). Animals were randomly assigned to one of three groups: 12 animals received an intravitreal injection of 10ng $VEGF_{165}b$ in 5μl HBSS, 7 received the same volume of HBSS and 8 were untreated. 24 hours after intraocular injection animals were subject to retinal ischemia as previously described (I. Selles-Navarro et al., 1996; T. Jehle et al., 2008). Under Isoflurane anaesthesia and stereotaxic guidance, the pupil was dilated (1% tropicamide and 2.5% phenylepherine eye drops), and anterior chamber pressure was maintained for 60 minutes with 0.9% NaCl infusion that elevated the intraocular pressure to 120 mm Hg. The cessation of the retinal blood flow was

observed using direct fundoscopy during the procedure. Reperfusion of retinal vessels was observed as intraocular pressure reduced after cessation of perfusion. Twenty-four hours after ischemia, retinas from three animals from each group were stained for activated caspase-3 immunofluorescence to assess the extent of ischemic damage (T. T. Lam et al., 1999). Caspase-3 positive cells were counted independently in the retinal ganglion and inner nuclear layers. The remaining animals were killed with isoflurane overdose ten days after ischemia and the numbers of surviving Fluorogold labelled neurones counted. Cell counts were performed by an operator blinded to treatment.

Partial saphenous nerve injury (PSNI) on C57/Bl6 mice was carried out as previously described(J. S. Walczak et al., 2005) under isofluorane anesthesia (2-3% in O_2). A ~5mm incision was made in the inguinal fossa region of the right hind leg. 50% of the saphenous nerve was tightly ligated using a size 6.0 sterile silk suture and the wound was closed with size 4.0 sterile silk suture. Sham-operated animals (n=5) underwent anesthesia and surgery involving solely an incision in the inguinal fossa region of the right hind limb. Each PSNI experimental group received biweekly $VEGF_{165}b$ (n=6, 20ng/g) or PBS vehicle (n=16, 200µl). Sham surgery involved the exact same procedure, except without the tight ligation of the saphenous nerve. Sham operated controls (n=5) received i.p. PBS at the same times. Injections were given immediately after surgery, and animals were killed after 10 days by overdose of anaesthetic and perfuse fixation with 4% paraformaldehyde. DRG were dissected out, frozen, sectioned at 10µm thickness and stained for ATF3 (cat no sc188, Santa Cruz).

Statistical analysis

Numbers of repetitions/animals are given in the text and/or Figure legends. All data were analysed using or t-tests (2 groups), 1 way ANOVA and appropriate post-

hoc tests (3 or more groups), unless data were ordinal or obviously non-Gaussian, in which case non-parametric equivalents were used.

Results

$VEGF_{xxx}$b: Endogenous neuronal expression.

Immunohistochemistry of human brain samples demonstrated that $VEGF_{165}$b was strongly expressed in the human hippocampal region, throughout all three pyramidal regions (CA1, CA2 and CA3), and the dentate gyrus, in neurons as well as being expressed in occasional neurons scattered through the cortex (Figure 1A). To quantify expression, protein was extracted from rat brains, and $VEGF_{165}$b and total VEGF levels measured by ELISA. $VEGF_{165}$b levels averaged 45% and 41% of total VEGF in the cortex and hippocampus respectively (Figure 1Bi-ii).

As $VEGF_{xxx}$a splice variants are known to be neuroprotective, we assessed the effectiveness of $VEGF_{xxx}$b splice variants as neuroprotective agents in hippocampal neurons, CNS neurons that are particularly vulnerable to damage.

Recombinant human $VEGF_{165}$b reversed glutamate-induced hippocampal neuronal death in a concentration-dependent manner (Figure 2A, B), and was more potent than galanin, which has previously been described as a potent hippocampal neuroprotective agent (C. R. Elliott-Hunt et al., 2004) (Figure 2B). At 10nM, the maximum $VEGF_{165}$b concentration used, cell death was the same as untreated cells, showing complete inhibition of excitotoxicity. $VEGF_{165}$b is a weak partial agonist at VEGF receptor 2 (VEGFR2, a.k.a. Flk1) *in vitro* (H. Kawamura et al., 2008a). In hippocampal neurones, the neuroprotective action of $VEGF_{165}$b was dependent on VEGFR2 activation (Figure 2C), as has been described for $VEGF_{165}$a (H. Matsuzaki et al., 2001). The partial reversal of excitotoxic cell death seen in the presence of $VEGF_{165}$b was blocked by the VEGFR blockers PTK787 (100nM, blocks both VEGFR1 and VEGFR2, (J. M. Wood et al., 2000)) and ZM323881 (10nM, blocks VEGFR2, (C. E. Whittles et al., 2002)) but was unaffected by SU5146 (at 100nM has specificity for VEGFR1, (C. A. Glass et al., 2006)). Hippocampal neurons in culture expressed VEGFR2 (Figure 2D). Hippocampal neuroprotection elicited by $VEGF_{165}$b

was dependent on downstream signalling of the VEGFR through the p42/p44MAPK pathway (Figure 3A), as it was also blocked by the MEK2 inhibitor, PD98059 (10μM), as is also the case for $VEGF_{165}$a. Neuroprotection was not affected by blockade of either p38 MAP kinase by SB203580, or PI3 kinase (PI3K) with LY294002 (Figure 3A, B).

$VEGF_{165}$b also exerted a neuroprotective action on retinal neurones *in vivo* (Figure 4). In retinal ischaemia a reduction in retrograde transport of fluorescent tracer was seen (Figure 4Aii). This ischaemic neuronal loss was reversed by prior intraocular $VEGF_{165}$b treatment (Figure 4Aiii). To determine whether this was due to apoptosis, retinae were stained for active caspase-3 (Figure 4B). Un-injected and HBSS-injected ischaemic eyes showed significant retinal neuronal loss through apoptosis, as assessed by fluorogold labelling of live neurones (Figure 4C-D), and caspase-3 staining respectively (Figure 4E). The reduction in neuronal retrograde transport was significantly reversed by $VEGF_{165}$b (Figure 4A,C-D), when compared to the un-injected contralateral eye, and reduced apoptosis in both retinal ganglion cells and inner nuclear layer cells (Figure 4B, D).

To determine whether neuroprotection was confined to central neurons or also affected peripheral neurons, cultured primary DRG neurones were investigated. The addition of 1nM $VEGF_{165}$b significantly increased the length of neurite outgrowth over 24 hours from 41.7±2.2μm to 73.8±20μm ($p<0.05$, Mann Whitney U test) but did not affect the number of DRG neurones in treated and control cultures. Staining of dispersed DRG neurons with VEGFR2 showed strong expression on the cell membrane (Figure 5A). $VEGF_{165}$a has been shown to be cytoprotective against injury induced by a variety of cellular insults to neurones (D. Lambrechts et al., 2003). $VEGF_{165}$b (2.5nM) significantly prevented an increase in activated caspase-3 in primary DRG neurons induced by 24 hours treatment with the chemotherapeutic oxaliplatin. Treatment with oxaliplatin alone (5, 10 or 20μg/mL) significantly increased

the percentage of caspase-3-positive DRG neurons compared to untreated neurons, and $VEGF_{165}b$ prevented this (Figure 5C).

To investigate the receptor mediating the neuroprotective response to $VEGF_{165}b$ DRG cultures were treated with the specific VEGFR2 inhibitor ZM323881 (10nM) or vehicle during oxaliplatin treatment and stained for NeuN and activated caspase-3 (Figure 5D). Treatment with 20μg/mL oxaliplatin, either alone or with ZM323881, induced a significant increase in the percentage of activated caspase-3-positive, NeuN-positive cells compared to media (51.4±3.0% and 55.7±1.9% vs. 37.8±1.3% and 42.5±2.6% respectively) (Figure 5D). Concurrent treatment with recombinant human $VEGF_{165}b$ (2.5nM) without ZM323881 significantly reduced the percentage of activated caspase-3-positive, NeuN-positive cells compared to vehicle (35.4±3.1%) (Figure 5D) and this effect was blocked by treatment with the VEGFR2 inhibitor ZM323881 (54.3±3.3%).

To determine whether systemic administration of $VEGF_{165}b$ could be neuroprotective we used a mouse model of peripheral traumatic nerve injury that results in activation of injury-response genes in DRG neurons, such as galanin(R. Hulse et al., 2008), and ATF3 (Figure 6A). Treatment with $rhVEGF_{165}b$ biweekly reduced the intensity of ATF3 expression in L3/4 DRG compared to sham surgery controls after 10 days (Figure 6A & B).

Discussion

We show here that, like $VEGF_{165}a$, the splice isoform $VEGF_{165}b$ is neuroprotective in central and peripheral neurones, *in vitro* and *in vivo*, and mediates this neuroprotective effect through VEGFR2 and MEK1/2 activation. In addition to its neuroprotective effects, $VEGF_{165}b$ also has neurotropic actions on neurones.

$VEGF_{165}a$ has been shown to play a key role in neuronal protection, in addition to its actions on the vasculature, directly protecting motoneurons under conditions of hypoxia, oxidative stress, and serum deprivation (B. Oosthuyse et al., 2001). Disturbance of VEGF expression contributes to the development of amyotrophic lateral sclerosis in man (D. Lambrechts et al., 2003). VEGF and its receptors are also expressed in central and peripheral nervous system support cells such as astrocytes and Schwann cells, thereby also contributing to neuronal survival and growth (for reviews see (E. Storkebaum et al., 2004; I. Zachary, 2005)).

In the CNS, $VEGF_{165}a$ protects hippocampal, cortical, and cerebellar granule neurones against numerous insults (K. L. Jin et al., 2000b, a; J. M. Rosenstein et al., 2003; J. N. Nicoletti et al., 2008), through VEGFR2, signalling through activation of multiple intracellular pathways including PLC, PI3K and MEK1/2 (I. Zachary, 2005), whereas effects on supporting cells, such as Schwann cells, and astrocytes, are generally mediated through VEGFR1 (E. Storkebaum et al., 2004). In contrast, our data show that neuroprotection by $VEGF_{165}b$ in hippocampal neurons, although also mediated through VEGFR2, does not involve either PI3K or p38 MAPK. The $VEGF_{xxx}b$ isoforms compete for and inhibit $VEGF_{xxx}$ binding at VEGFR2 (J. Woolard et al., 2004; S. Cebe Suarez et al., 2006; H. Kawamura et al., 2008b), but the $VEGF_{xxx}b$ isoforms are not simply competitive inhibitors of the VEGFR2 as binding of $VEGF_{165}b$ results in differential tyrosine residue phosphorylation of VEGFR2 (H. Kawamura et al., 2008a). Neuroprotective and neurotrophic actions by $VEGF_{165}a$ may also involve the VEGF-co-receptor, neuropilin-1 (NP-1) (I. Zachary, 2005);

$VEGF_{165}b$ binds weakly to NP-1 , and does not require NP-1 binding to phosphorylate and activate VEGFR2 (H. Kawamura et al., 2008a). Our data support the conclusion that the differential VEGFR2 phosphorylation and MEK1/2 activation exerted by $VEGF_{165}b$ is sufficient to protect central and peripheral neurons, without NP-1 binding. Our data on both CNS and peripheral sensory neurons show that the neuroprotective mechanism of $VEGF_{165}b$, is like $VEGF_{165}a$, in that neuroprotection occurs through prevention of caspase-3 induction (I. Zachary, 2005).

In the eye, endogenous VEGF is a survival factor for retinal ganglion cells, protecting against ischaemia-reperfusion injury (K. Nishijima et al., 2007), and preventing neuronal apoptosis without the necessity for NP-1 binding, as $VEGF_{120}$ (which lacks the NP-1 binding domain (H. Kawamura et al., 2008c)) also exerted neuroprotective effects. Our findings show that $VEGF_{165}b$ can reduce both retinal ganglion cell and inner nuclear cell loss, through VEGFR2 activation. The loss of neuronal retrograde transport is seen clinically in diabetic retinopathy as cotton wool spots under fundus examination, and the loss of ganglion cells contributes to vision loss in glaucoma(M. Almasieh et al.). We have previously shown that $VEGF_{165}b$ is cytoprotective for endothelial cells (A. L. Magnussen et al., 2010), while being anti-angiogenic in the eye (J. Hua et al., 2010). Prevention of the ischemia-induced damage to neurones in the retina by $VEGF_{165}b$ would therefore also be a substantial advantage in therapeutic approaches for diabetic retinopathy, and glaucoma.

In hippocampal and peripheral sensory cultured neurons, $VEGF_{165}b$ also enhances neurite outgrowth, demonstrating that $VEGF_{165}b$ exerts neurotrophic effects, also through VEGFR2. In CNS and PNS peripheral sensory and autonomic neurons, $VEGF_{165}a$ enhances neuronal neurite outgrowth in culture (M. Sondell et al., 1999; I. Zachary, 2005; G. Lin et al., 2010). VEGFR2 is expressed in both the central and peripheral nervous systems, but often in different neuronal populations from VEGF-A proteins (M. Sondell and M. Kanje, 2001; T. Licht et al., 2011). These

different distributions suggest that VEGF isoforms exert paracrine actions on neurons, through which neuroprotective and neurotrophic effects may be mediated. We demonstrate that $VEGF_{165}$b is a major VEGF-A splice variant commonly found in central and peripheral neurones, where it is well placed to exert such paracrine effects. $VEGF_{165}$b is also expressed in human skin, prostate, and kidney, amongst other tissues (J. Woolard et al., 2004; R. O. Pritchard-Jones et al., 2007), where it could also exert paracrine effects on peripheral sensory neurons.

$VEGF_{165}$b has clear inhibitory effects on tumour growth (J. Woolard et al., 2004; E. Rennel et al., 2008; A. H. Varey et al., 2008) but does not result in hypertension, angiogenesis and proteinuria (J. Woolard et al., 2004; E. S. Rennel et al., 2008; Y. Qiu et al., 2010). Our data show that $VEGF_{165}$b has neuroprotective effects similar to those of $VEGF_{165}$a, on central and peripheral neurones. VEGF-dependent neovascularisation is key in the pathophysiology of many conditions, and anti-VEGF therapies have entered clinical practice in oncology (H. Hurwitz et al., 2004), and ophthalmology (P. J. Rosenfeld et al., 2006). Following successful pre-clinical studies of VEGF administration in neurodegenerative disease (M. Sondell et al., 1999; P. Schratzberger et al., 2000; P. Schratzberger et al., 2001; D. Lambrechts et al., 2003; T. Yasuhara et al., 2005), VEGF supplementation is now under trial for treatment of neuronal degenerative diseases, for instance in diabetic neuropathy (A. H. Ropper et al., 2009). Although effective in some patients, there has to be some concern about the safety profile of these strategies in relation to the potential compromise of non-endothelial tissue/cell function in which VEGF has been shown to have cytoprotective properties, particularly neurons (E. Storkebaum et al., 2004) and podocytes (R. R. Foster et al., 2003). We show that $VEGF_{xxx}$b can exert similar neuroprotective and neurotropic effects with $VEGF_{xxx}$a both centrally (e.g. hippocampal neurons in culture, and retinal neurons *in vivo*) and peripherally.

With the requirement that therapy for pathological conditions in which VEGF has been implicated should not adversely affect the function of the normal vasculature, or other cell types, we suggest that $VEGF_{165}b$ may be an alternative therapeutic agent in neurodegenerative conditions with fewer adverse vascular side effects.

Figure legends

Figure 1. $VEGF_{165}b$ is expressed in human and rat hippocampus.

Human cortical sections from the Human Tissue Authority licensed South West Dementia Brain Bank were stained with an anti-$VEGF_{165}b$ antibody. A. Hippocampal staining in CA1, CA2, CA3 and dentate gyrus (DG). B. Protein was extracted from cortex and hippocampus dissected from rat brains (n=3) and subjected to ELISA for VEGF and $VEGF_{xxx}b$. (i) $VEGF_{xxx}$ levels were estimated from the difference between total VEGF and $VEGF_{xxx}b$. (ii) percent of total VEGF that is $VEGF_{xxx}b$.

Figure 2 $VEGF_{165}b$ is protective against glutamate-induced hippocampal neuronal excitotoxicity.

A. i. Pseudocoloured image of cultured hippocampal neurons exposed to glutamic acid, with and without $VEGF_{165}b$ or galanin. Cells co-stained with Hoechst 33258 nuclear stain (blue) and Dead stain (red) show as purple, and represent ~75% of glutamic acid treated neurones under control conditions. ii. Effect of treatment with 10nM $VEGF_{165}b$ on excitotoxicity in neurons co-incubated with 3nM glutamate. B. $VEGF_{165}b$ has a concentration-dependent inhibitory effect on glutamatergic excitotoxicity in hippocampal neurons. $VEGF_{165}b$ was a more potent neuroprotective than galanin ($p<0.0001$, n=4, ANOVA + Newman-Keuls post-hoc test *$p<0.01$, **$p<0.001$ ns=non-significant). C. Effect of VEGF receptor inhibitors on the hippocampal neuroprotective effect of $VEGF_{165}b$. (100nM PTK787 (nonspecific VEGFR2 antagonist), 10nM ZM323881 (VEGFR2 specific antagonist), 100nM SU5416 (VEGFR1 specific antagonist) **=$p<0.01$, *=$p<0.05$ compared with control, n=4/group). D. VEGFR2 (red) expression in β3 tubulin positive (green) neurons.

Nuclei are stained blue. Arrows show membrane staining shown in higher power in lower panel.

Figure 3. The effect of intracellular kinase-pharmacological inhibition on VEGF$_{165}$b hippocampal neuroprotection against L-glutamic acid-induced excitotoxicity.

A. Panels display representative images of hippocampal neurons subjected to the Live/Dead cell viability stain after 24 hours treatment with 3mM L-glutamic acid in the presence of test compounds or respective vehicles. Neurons treated in culture media alone (neurobasal media) or L-glutamic acid + 2.5nM VEGF$_{165}$b with or without 10μM SB203580 (p38 MAPK inhibitor) or 15μM LY294002 (PI3K inhibitor) maintained neurite projections (arrows). In the presence of L-glutamic acid + VEGF$_{165}$b + 10μM PD098059 (MEK1/2 inhibitor) neurons retracted neurites (arrowheads). B. The percentage of red-stained (dead) nuclei per total nuclei stained was calculated. More neurons died when treated with the MEK1/2 inhibitor + VEGF$_{165}$b (Figure 3B, PD), than when treated with VEGF$_{165}$b and vehicle (Figure 3B, Control). Neither p38 MAPK (SB) nor PI3 kinase (LY) inhibition had any effect on the neuroprotection exerted by VEGF$_{165}$b. (Data shown are mean±SEM, n=3/4 1-way ANOVA + Bonferroni post hoc comparison); glutamic acid + inhibitor/vehicle vs. media + inhibitor/vehicle, *$p<0.05$, **$p<0.01$, ***$p<0.001$; glutamic acid + VEGF$_{165}$b + inhibitor/vehicle vs. glutamic acid + inhibitor/vehicle, #$p<0.05$, ##$p<0.01$, ###$p<0.001$; glutamic acid + VEGF$_{165}$b + inhibitor vs. glutamic acid + VEGF$_{165}$b + vehicle, ^{+}p0.05.)

Figure 4. VEGF$_{165}$b protects retinal neurons from ischemia induced cell death *in vivo*.

A. Pseudo-coloured fluorescent images of retinal cells showing (i) the contralateral non-ischemic retina, (ii) ischaemic eye injected with HBSS, or (iii) ischaemic eye

injected with $VEGF_{165}b$. B. Staining of retinae of HBSS or $VEGF_{165}b$ injected rats for activated caspase 3 (red) and nuclei (blue). C. Live retinal ganglion cell counts were significantly lower in ischemic eyes compared to non-ischemic eyes. $VEGF_{165}b$ treatment resulted in more viable retinal ganglion cells (n=8 HBSS, n=12 $VEGF_{165}b$, $p<0.001$, ANOVA + Bonferroni post-hoc test). $VEGF_{165}b$ treatment increased the numbers of live fluorogold labelled retinal cells, compared to the HBSS and control untreated eyes, which can be clearly seen when D. the ratio of retinal ganglion cells per field in the ischemic/non-ischemic eyes were compared. E. Neuroprotection by $VEGF_{165}b$ was mediated through an inhibition of apoptosis, as indicated by a reduction in active caspase-3 staining, in both the retinal ganglion cells (RGC) and inner nuclear layer (INL). Data are means±SEM. *=p<0.05 **=p<0.01, ***=p<0.001 compared with contralateral and ++=p<0.01 compared with HBSS. NS=not different from contralateral.

Figure 5. $VEGF_{165}b$ is cytoprotective for primary sensory neurons

A. Primary cultured DRG were stained for βIII tubulin (green), VEGFR2 (red) and Hoechst (blue). βIII-tubulin-positive cells were also positive for VEGFR2, with the receptor detected in the soma (white arrow) and along cellular projections (arrowhead). Some VEGFR2 detection did not colocalize to βIII-tubulin (cyan arrow). The matched-species IgG negative control confirms the detection of VEGFR2 expression. B. Effect of $VEGF_{165}b$ in a model of chemotherapeutic-induced neurotoxicity. Primary adult rat DRG cultures were treated with increasing concentrations (0, 5, 10, 20 μg/mL) of the chemotherapeutic oxaliplatin for 24 hours with or without 2.5nM $VEGF_{165}b$ (following 16h pre-treatment). The percentage of activated caspase-3-positive NeuN-positive cells was determined after immunofluorescence analysis. Panels display representative images of NeuN-positive cells after treatment. NeuN negative cells (only blue or blue and red only)

were not counted. Arrows signify activated caspase-3 detected both around and in neuronal nuclei. C. The percentage of neurons positive for activated caspase-3 was determined after treatment with 20μg/mL oxaliplatin for 24 hours with or without 2.5nM $VEGF_{165}b$. $VEGF_{165}b$ treatment inhibited oxaliplatin-induced caspase-3 expression. Data shown are mean±SEM, n=3. D. Treatment of DRG cultures with oxaliplatin + $VEGF_{165}b$ and the VEGFR2 inhibitor ZM323881 (10nM) blocked the neuroprotection exerted by $VEGF_{165}b$ alone, indicating this action is mediated through VEGFR2. ZM323881 did not affect the oxaliplatin-induced caspase-3 induction. Data shown are mean±SEM, n=3, 1-way ANOVA + Bonferroni post hoc comparison; * = compared with no oxaliplatin; *=p<0.05, **=p<0.01., ***=*p*<0.001.. † = compared with concentration matched vehicle., +=p<0.05. Scale bars = 50µm for B

Figure 6. $VEGF_{165}b$ is neuroprotective for DRG neurons *in vivo*.

Partial saphenous nerve injury (PSNI), was performed on lightly anaesthetized C57BL/6 mice. Test compounds were administered biweekly by i.p. injection following surgery (20ng $rhVEGF_{165}b$ per g body weight or a matched volume of PBS) and L4 DRG were harvested 14 days later. DRG were cut into 10μm sections and for each DRG a complete cross-section profile was analyzed for ATF3 immunofluorescence intensity. A. Panels display representative images of ATF3 immunofluorescence intensities from ipsilateral L4 DRG and a species- and concentration- matched IgG negative control. B. Using Image J software the average pixel intensity was calculated for each DRG profile and expressed relative to the mean pixel intensity for sham operated L4 DRG. Results are mean±SEM, n=3 per group, statistical analysis: Kruskal-Wallis + Dunn's multiple comparison test: test group ipsilateral DRG intensity vs. sham-operated ipsilateral DRG, *p<0.05. Scale bar=500µm.

References

Ahmed AK, Sugioka K, Dong K, Yamadori T (1995) A study of double-labeled retinal ganglion cells from the superior colliculus in the developing albino rat. Brain research 85:71-79.

Almasieh M, Wilson AM, Morquette B, Cueva Vargas JL, Di Polo A The molecular basis of retinal ganglion cell death in glaucoma. Prog Retin Eye Res 31:152-181.

Bates DO, Cui TG, Doughty JM, Winkler M, Sugiono M, Shields JD, Peat D, Gillatt D, Harper SJ (2002) VEGF165b, an inhibitory splice variant of vascular endothelial growth factor, is down-regulated in renal cell carcinoma. Cancer research 62:4123-4131.

Bevan HS, van den Akker NM, Qiu Y, Polman JA, Foster RR, Yem J, Nishikawa A, Satchell SC, Harper SJ, Gittenberger-de Groot AC, Bates DO (2008) The alternatively spliced anti-angiogenic family of VEGF isoforms VEGFxxxb in human kidney development. Nephron Physiol 110:p57-67.

Cebe Suarez S, Pieren M, Cariolato L, Arn S, Hoffmann U, Bogucki A, Manlius C, Wood J, Ballmer-Hofer K (2006) A VEGF-A splice variant defective for heparan sulfate and neuropilin-1 binding shows attenuated signaling through VEGFR-2. Cell Mol Life Sci 63:2067-2077.

Elliott-Hunt CR, Marsh B, Bacon A, Pope R, Vanderplank P, Wynick D (2004) Galanin acts as a neuroprotective factor to the hippocampus. Proc Natl Acad Sci U S A 101:5105-5110.

Foster RR, Hole R, Anderson K, Satchell SC, Coward RJ, Mathieson PW, Gillatt DA, Saleem MA, Bates DO, Harper SJ (2003) Functional evidence that vascular endothelial growth factor may act as an autocrine factor on human podocytes. Am J Physiol Renal Physiol 284:F1263-1273.

Glass CA, Harper SJ, Bates DO (2006) The anti-angiogenic VEGF isoform VEGF165b transiently increases hydraulic conductivity, probably through VEGF receptor 1 in vivo. J Physiol (Lond) 572:243-257.

Harper SJ, Bates DO (2008) VEGF-A splicing: the key to anti-angiogenic therapeutics? Nature reviews 8:880-887.

Hobson MI, Green CJ, Terenghi G (2000) VEGF enhances intraneural angiogenesis and improves nerve regeneration after axotomy. J Anat 197 Pt 4:591-605.

Holmes FE, Mahoney S, King VR, Bacon A, Kerr NC, Pachnis V, Curtis R, Priestley JV, Wynick D (2000) Targeted disruption of the galanin gene reduces the number of sensory neurons and their regenerative capacity. Proc Natl Acad Sci U S A 97:11563-11568.

Hua J, Spee C, Kase S, Rennel ES, Magnussen AL, Qiu Y, Varey A, Dhayade S, Churchill AJ, Harper SJ, Bates DO, Hinton DR (2010) Recombinant human VEGF165b inhibits experimental choroidal neovascularization. Invest Ophthalmol Vis Sci 51:4282-4288.

Hulse R, Wynick D, Donaldson LF (2008) Characterization of a novel neuropathic pain model in mice. Neuroreport 19:825-829.

Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, Berlin J, Baron A, Griffing S, Holmgren E, Ferrara N, Fyfe G, Rogers B, Ross R, Kabbinavar F (2004) Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. The New England journal of medicine 350:2335-2342.

Jehle T, Wingert K, Dimitriu C, Meschede W, Lasseck J, Bach M, Lagreze WA (2008) Quantification of ischemic damage in the rat retina: a comparative study using evoked potentials, electroretinography, and histology. Invest Ophthalmol Vis Sci 49:1056-1064.

Jin KL, Mao XO, Greenberg DA (2000a) Vascular endothelial growth factor: direct neuroprotective effect in in vitro ischemia. Proc Natl Acad Sci U S A 97:10242-10247.

Jin KL, Mao XO, Greenberg DA (2000b) Vascular endothelial growth factor rescues HN33 neural cells from death induced by serum withdrawal. J Mol Neurosci 14:197-203.

Kawamura H, Li X, Harper SJ, Bates DO, Claesson-Welsh L (2008a) Vascular endothelial growth factor (VEGF)-A165b is a weak in vitro agonist for VEGF receptor-2 due to lack of coreceptor binding and deficient regulation of kinase activity. Cancer research 68:4683-4692.

Kawamura H, Li X, Goishi K, van Meeteren LA, Jakobsson L, Cebe-Suarez S, Shimizu A, Edholm D, Ballmer-Hofer K, Kjellen L, Klagsbrun M, Claesson-Welsh L (2008b) Neuropilin-1 in regulation of VEGF-induced activation of p38MAPK and endothelial cell organization. Blood.

Kawamura H, Li X, Goishi K, van Meeteren LA, Jakobsson L, Cebe-Suarez S, Shimizu A, Edholm D, Ballmer-Hofer K, Kjellen L, Klagsbrun M, Claesson-Welsh L (2008c) Neuropilin-1 in regulation of VEGF-induced activation of p38MAPK and endothelial cell organization. Blood 112:3638-3649.

Kovacs Z, Ikezaki K, Samoto K, Inamura T, Fukui M (1996) VEGF and flt. Expression time kinetics in rat brain infarct. Stroke 27:1865-1872; discussion 1872-1863.

Lam TT, Abler AS, Tso MO (1999) Apoptosis and caspases after ischemia-reperfusion injury in rat retina. Invest Ophthalmol Vis Sci 40:967-975.

Lambrechts D et al. (2003) VEGF is a modifier of amyotrophic lateral sclerosis in mice and humans and protects motoneurons against ischemic death. Nat Genet 34:383-394.

Licht T, Goshen I, Avital A, Kreisel T, Zubedat S, Eavri R, Segal M, Yirmiya R, Keshet E (2011) Reversible modulations of neuronal plasticity by VEGF. Proc Natl Acad Sci U S A 108:5081-5086.

Lin G, Shindel AW, Fandel TM, Bella AJ, Lin CS, Lue TF (2010) Neurotrophic effects of brain-derived neurotrophic factor and vascular endothelial growth factor in major pelvic ganglia of young and aged rats. BJU Int 105:114-120.

Magnussen AL, Rennel ES, Hua J, Bevan HS, Beazley Long N, Lehrling C, Gammons M, Floege J, Harper SJ, Agostini HT, Bates DO, Churchill AJ (2010) VEGF-A165b is cytoprotective and antiangiogenic in the retina. Invest Ophthalmol Vis Sci 51:4273-4281.

Matsuzaki H, Tamatani M, Yamaguchi A, Namikawa K, Kiyama H, Vitek MP, Mitsuda N, Tohyama M (2001) Vascular endothelial growth factor rescues hippocampal neurons from glutamate-induced toxicity: signal transduction cascades. Faseb J 15:1218-1220.

Nicoletti JN, Shah SK, McCloskey DP, Goodman JH, Elkady A, Atassi H, Hylton D, Rudge JS, Scharfman HE, Croll SD (2008) Vascular endothelial growth factor is up-regulated after status epilepticus and protects against seizure-induced neuronal loss in hippocampus. Neuroscience 151:232-241.

Nishijima K, Ng YS, Zhong L, Bradley J, Schubert W, Jo N, Akita J, Samuelsson SJ, Robinson GS, Adamis AP, Shima DT (2007) Vascular endothelial growth factor-A is a survival factor for retinal neurons and a critical neuroprotectant during the adaptive response to ischemic injury. The American journal of pathology 171:53-67.

Oosthuyse B et al. (2001) Deletion of the hypoxia-response element in the vascular endothelial growth factor promoter causes motor neuron degeneration. Nat Genet 28:131-138.

Pritchard-Jones RO, Dunn DB, Qiu Y, Varey AH, Orlando A, Rigby H, Harper SJ, Bates DO (2007) Expression of VEGF(xxx)b, the inhibitory isoforms of VEGF, in malignant melanoma. British journal of cancer 97:223-230.

Qiu Y, Ferguson J, Oltean S, Neal CR, Kaura A, Bevan H, Wood E, Sage LM, Lanati S, Nowak DG, Salmon AH, Bates D, Harper SJ (2010) Overexpression of VEGF165b in podocytes reduces glomerular permeability. J Am Soc Nephrol 21:1498-1509.

Rennel E, Waine E, Guan H, Schuler Y, Leenders W, Woolard J, Sugiono M, Gillatt D, Kleinerman E, Bates D, Harper S (2008) The endogenous anti-angiogenic VEGF isoform, VEGF165b inhibits human tumour growth in mice. British journal of cancer 98:1250-1257.

Rennel ES, Hamdollah-Zadeh MA, Wheatley ER, Magnussen A, Schuler Y, Kelly SP, Finucane C, Ellison D, Cebe-Suarez S, Ballmer-Hofer K, Mather S, Stewart L, Bates DO, Harper SJ (2008) Recombinant human VEGF165b protein is an effective anti-cancer agent in mice. Eur J Cancer 44:1883-1894.

Ropper AH, Gorson KC, Gooch CL, Weinberg DH, Pieczek A, Ware JH, Kershen J, Rogers A, Simovic D, Schratzberger P, Kirchmair R, Losordo D (2009) Vascular endothelial growth factor gene transfer for diabetic polyneuropathy: a randomized, double-blinded trial. Ann Neurol 65:386-393.

Rosenfeld PJ, Brown DM, Heier JS, Boyer DS, Kaiser PK, Chung CY, Kim RY (2006) Ranibizumab for neovascular age-related macular degeneration. The New England journal of medicine 355:1419-1431.

Rosenstein JM, Mani N, Khaibullina A, Krum JM (2003) Neurotrophic effects of vascular endothelial growth factor on organotypic cortical explants and primary cortical neurons. J Neurosci 23:11036-11044.

Schratzberger P, Schratzberger G, Silver M, Curry C, Kearney M, Magner M, Alroy J, Adelman LS, Weinberg DH, Ropper AH, Isner JM (2000) Favorable effect of VEGF gene transfer on ischemic peripheral neuropathy. Nature medicine 6:405-413.

Schratzberger P, Walter DH, Rittig K, Bahlmann FH, Pola R, Curry C, Silver M, Krainin JG, Weinberg DH, Ropper AH, Isner JM (2001) Reversal of experimental diabetic neuropathy by VEGF gene transfer. The Journal of clinical investigation 107:1083-1092.

Selles-Navarro I, Villegas-Perez MP, Salvador-Silva M, Ruiz-Gomez JM, Vidal-Sanz M (1996) Retinal ganglion cell death after different transient periods of pressure-induced ischemia and survival intervals. A quantitative in vivo study. Invest Ophthalmol Vis Sci 37:2002-2014.

Sondell M, Kanje M (2001) Postnatal expression of VEGF and its receptor flk-1 in peripheral ganglia. Neuroreport 12:105-108.

Sondell M, Lundborg G, Kanje M (1999) Vascular endothelial growth factor has neurotrophic activity and stimulates axonal outgrowth, enhancing cell survival and Schwann cell proliferation in the peripheral nervous system. J Neurosci 19:5731-5740.

Storkebaum E, Lambrechts D, Carmeliet P (2004) VEGF: once regarded as a specific angiogenic factor, now implicated in neuroprotection. Bioessays 26:943-954.

Varey AH, Rennel ES, Qiu Y, Bevan HS, Perrin RM, Raffy S, Dixon AR, Paraskeva C, Zaccheo O, Hassan AB, Harper SJ, Bates DO (2008) VEGF 165 b, an antiangiogenic VEGF-A isoform, binds and inhibits bevacizumab treatment in experimental colorectal carcinoma: balance of pro- and antiangiogenic VEGF-A isoforms has implications for therapy. British journal of cancer 98:1366-1379.

Walczak JS, Pichette V, Leblond F, Desbiens K, Beaulieu P (2005) Behavioral, pharmacological and molecular characterization of the saphenous nerve partial ligation: a new model of neuropathic pain. Neuroscience 132:1093-1102.

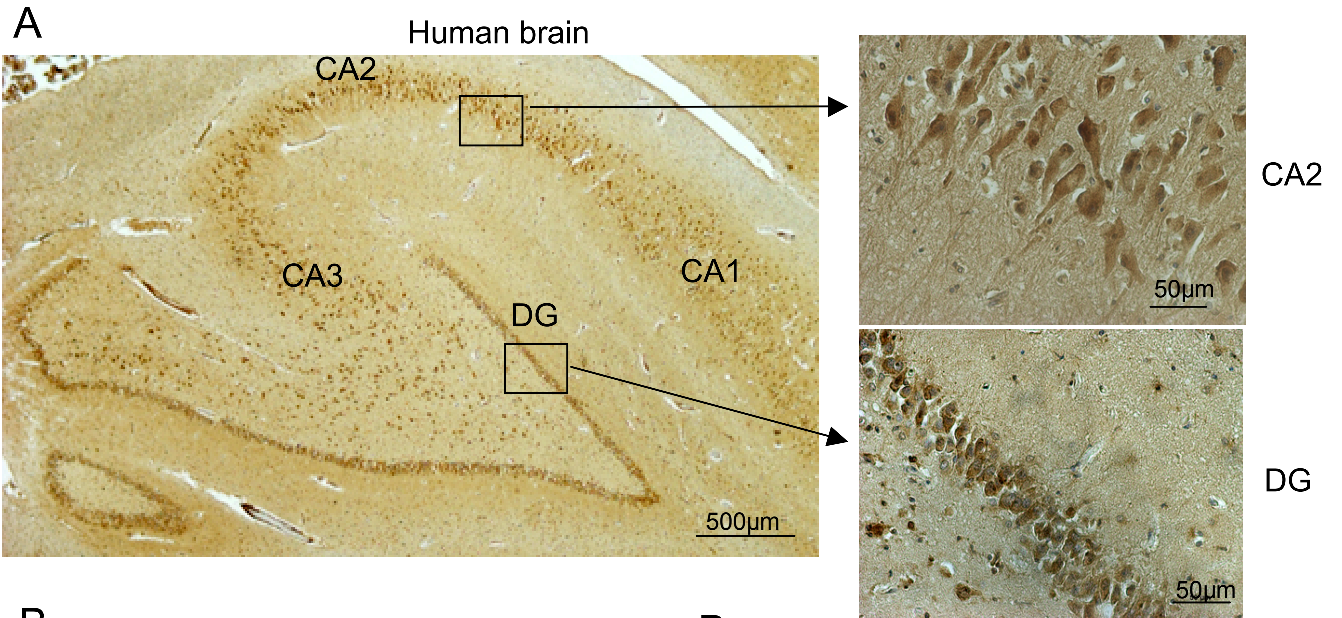
Whittles CE, Pocock TM, Wedge SR, Kendrew J, Hennequin LF, Harper SJ, Bates DO (2002) ZM323881, a novel inhibitor of vascular endothelial growth factor-receptor-2 tyrosine kinase activity. Microcirculation 9:513-522.

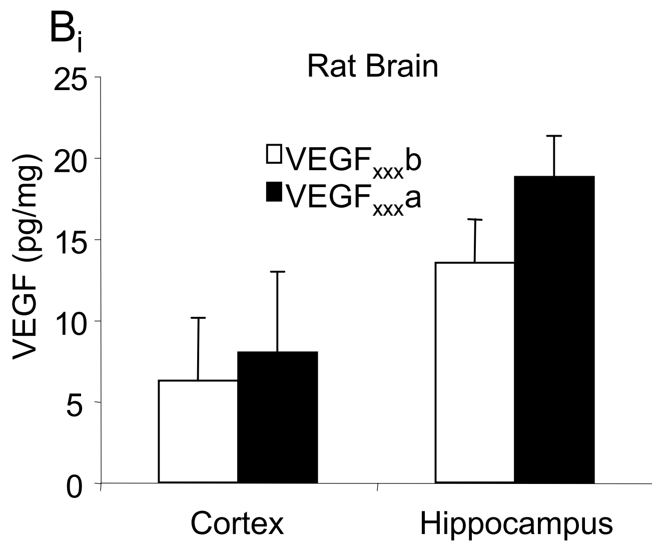
Wood JM et al. (2000) PTK787/ZK 222584, a novel and potent inhibitor of vascular endothelial growth factor receptor tyrosine kinases, impairs vascular endothelial growth factor-induced responses and tumor growth after oral administration. Cancer research 60:2178-2189.

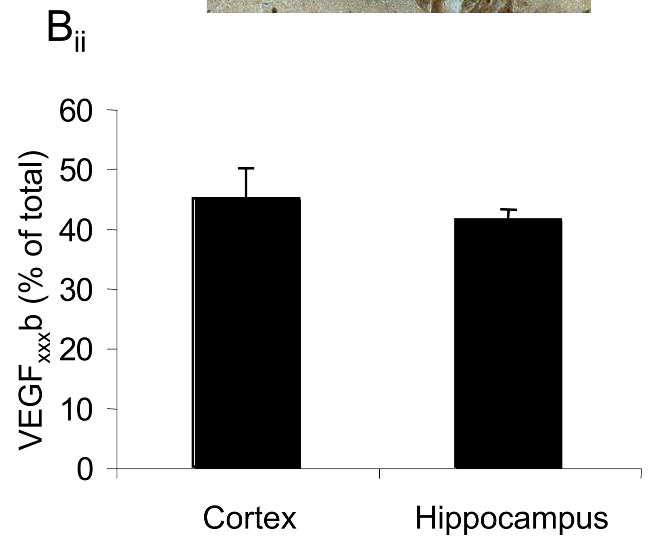
Woolard J, Wang WY, Bevan HS, Qiu Y, Morbidelli L, Pritchard-Jones RO, Cui TG, Sugiono M, Waine E, Perrin R, Foster R, Digby-Bell J, Shields JD, Whittles CE, Mushens RE, Gillatt DA, Ziche M, Harper SJ, Bates DO (2004) VEGF165b, an inhibitory vascular endothelial growth factor splice variant: mechanism of action, in vivo effect on angiogenesis and endogenous protein expression. Cancer research 64:7822-7835.

Yasuhara T, Shingo T, Muraoka K, Kameda M, Agari T, Wen Ji Y, Hayase H, Hamada H, Borlongan CV, Date I (2005) Neurorescue effects of VEGF on a rat model of Parkinson's disease. Brain Res 1053:10-18.

Zachary I (2005) Neuroprotective role of vascular endothelial growth factor: signalling mechanisms, biological function, and therapeutic potential. Neurosignals 14:207-221.


A
Human brain
CA2
CA3
CA1
DG
500µm
50µm
CA2
50µm
DG


B$_i$
Rat Brain
VEGF (pg/mg)
25
20
15
10
5
0
VEGF$_{xxx}$b
VEGF$_{xxx}$a
Cortex
Hippocampus


B$_{ii}$
VEGF$_{xxx}$b (% of total)
60
50
40
30
20
10
0
Cortex
Hippocampus

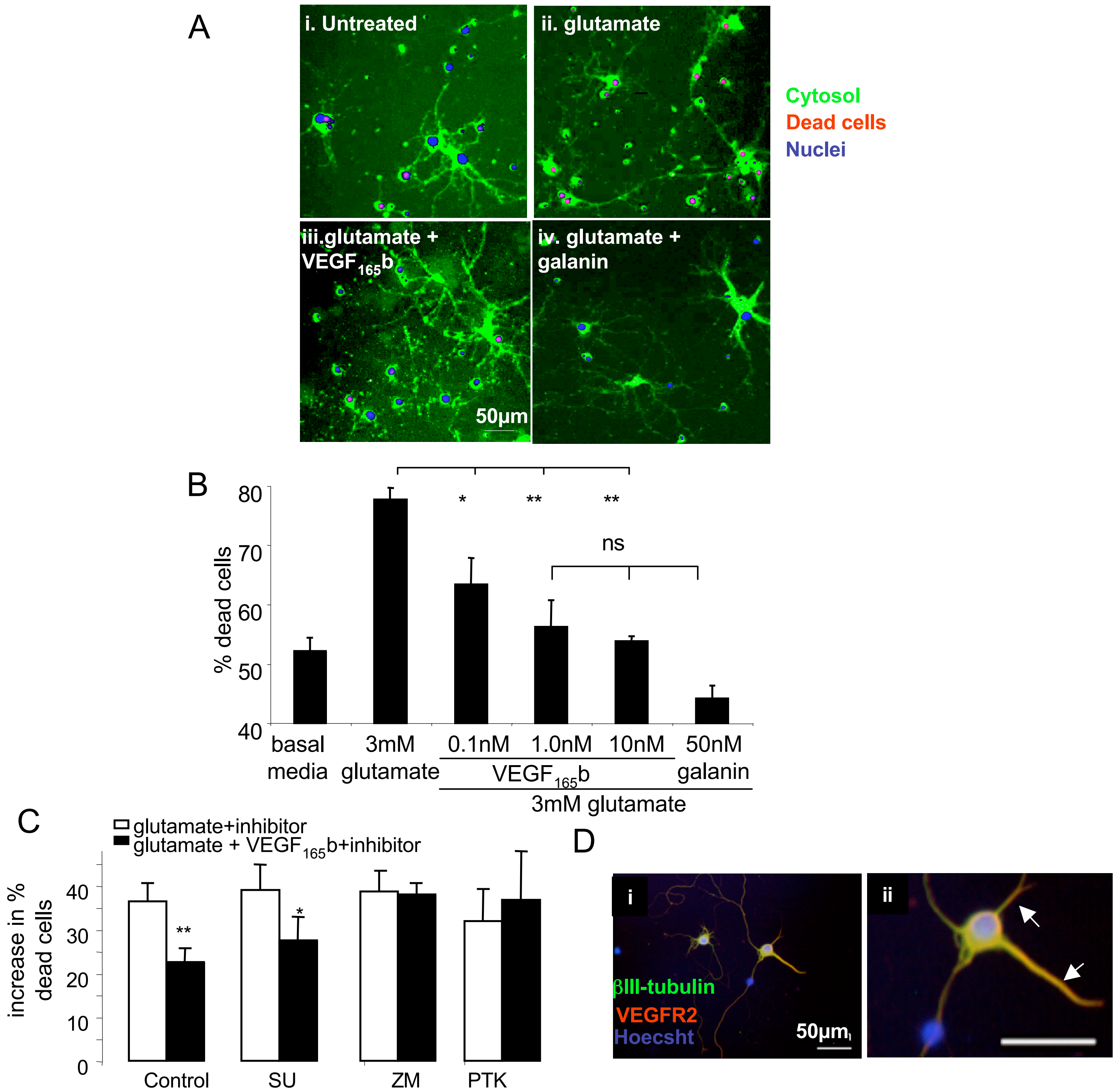


Figure 2

A

Cytosol Dead nuclei All nuclei

Neurobasal media | L-glutamic acid | L-glutamic acid +rhVEGF$_{165}$b

Vehicle

+ PD098059

+ LY294002

+ SB203580

Scale bar: 25μm

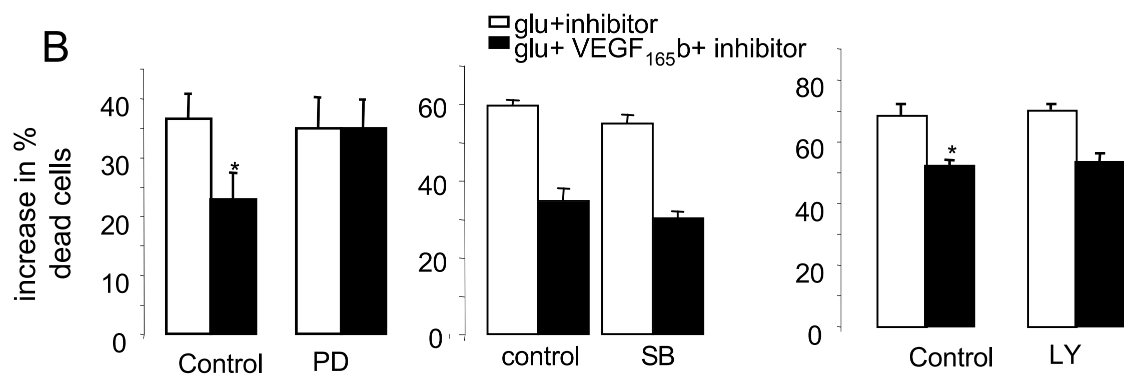


Figure 3

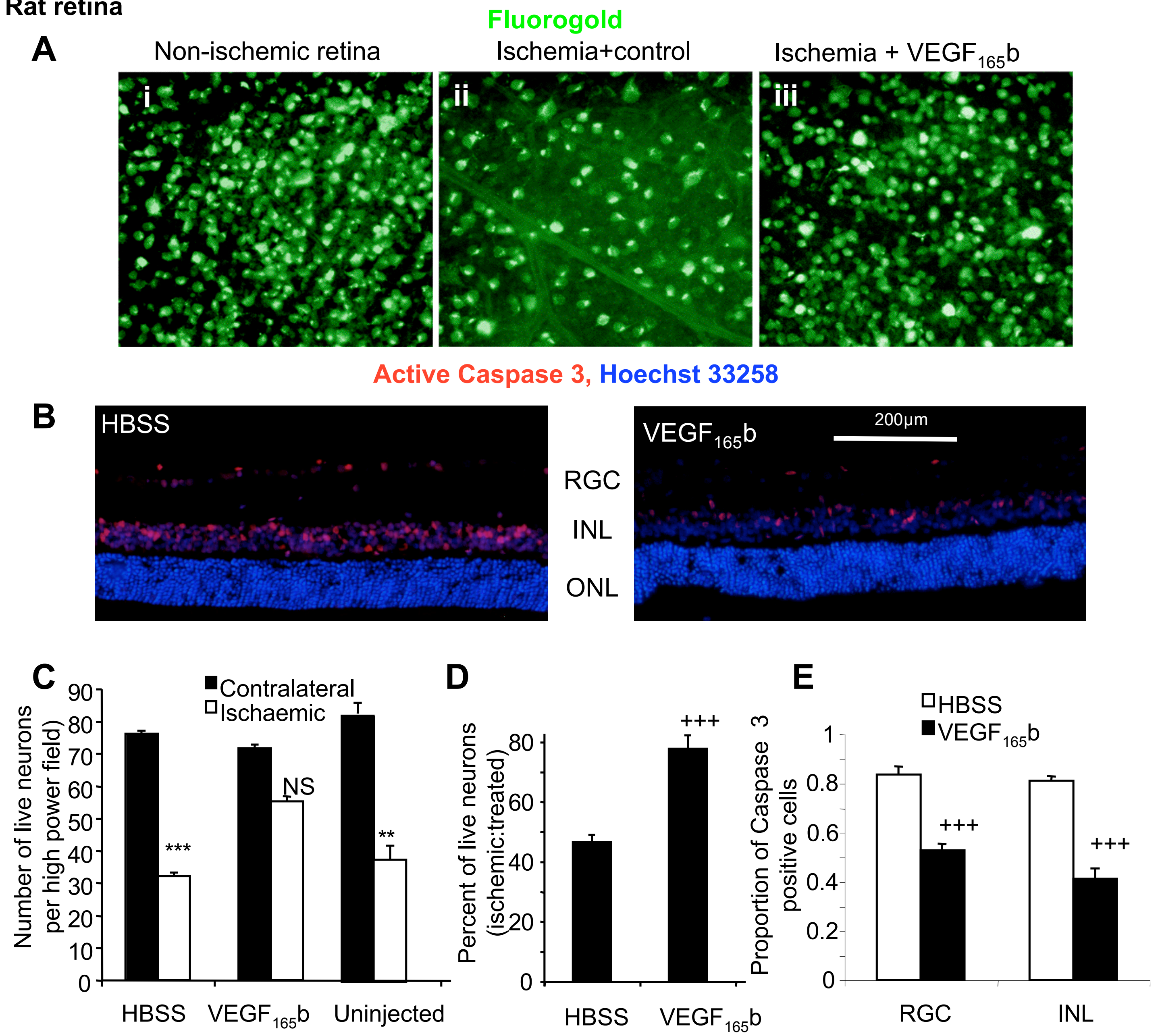


Figure 4

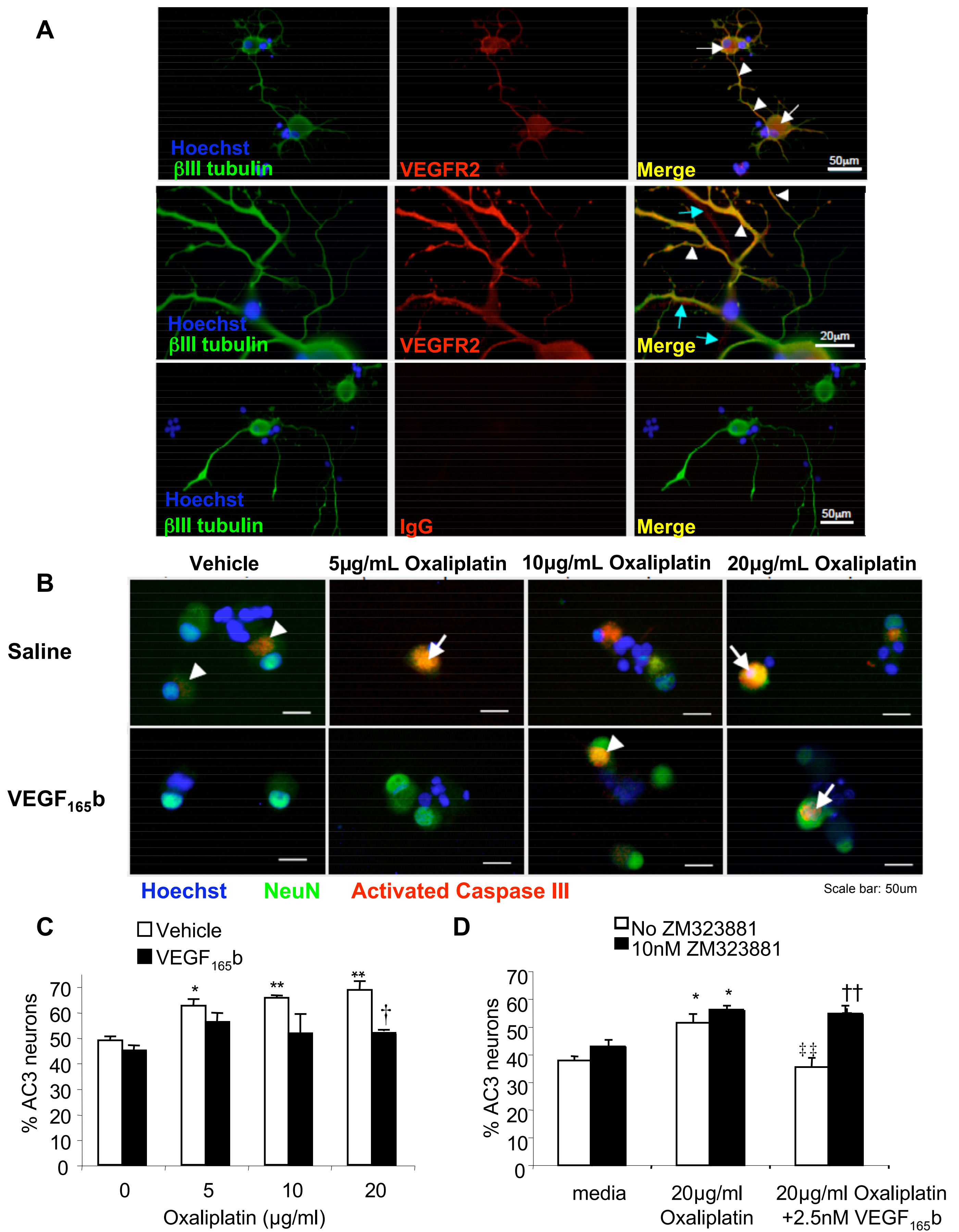
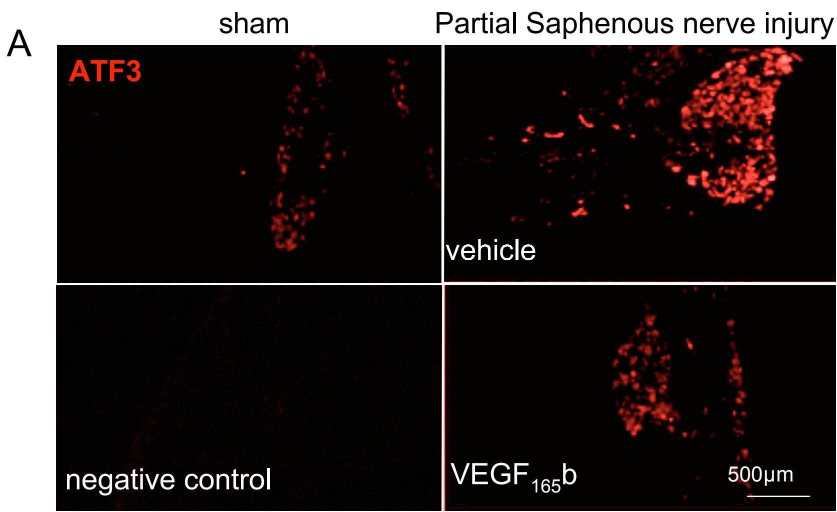
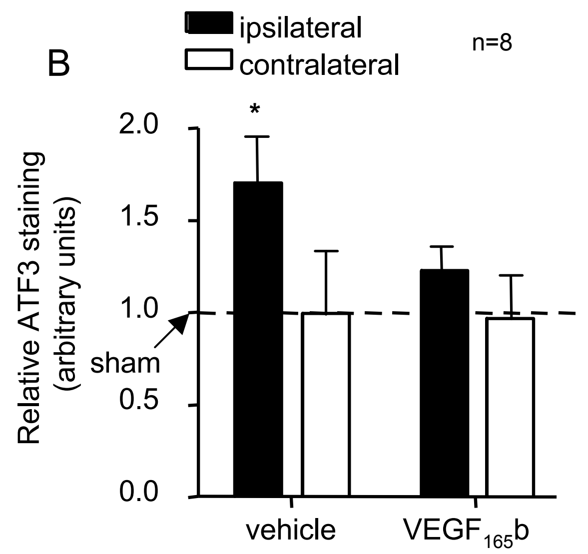


Figure 5


A
sham
Partial Saphenous nerve injury
ATF3
vehicle
negative control
VEGF165b
500μm


B
ipsilateral
contralateral
n=8
Relative ATF3 staining (arbitrary units)
2.0
1.5
1.0
0.5
0.0
*
sham
vehicle
VEGF165b