VEGF$_{189}$ binds NRP1 and is sufficient for VEGF/NRP1-dependent neuronal patterning in the developing brain

Miguel Tillo$^{1}$, Lynda Erskine$^{2}$, Anna Cariboni$^{1,3}$, Alessandro Fantin$^{1}$, Andy Joyce$^{1}$, Laura Dent$^{1}$ and Christiana Ruhrberg$^{1,*}$

ABSTRACT
The vascular endothelial growth factor (VEGFA, VEGF) regulates neurovascular patterning. Alternative splicing of the Vegfa gene gives rise to three major isoforms termed VEGF$_{121}$, VEGF$_{165}$ and VEGF$_{189}$, and promotes the migration, survival and axon guidance of subsets of neurons, whereas VEGF$_{121}$ cannot activate NRP1-dependent neuronal responses. By contrast, the role of VEGF$_{189}$ in NRP1-mediated signalling pathways has not yet been examined. Here, we have combined expression studies and in situ ligand-binding assays with the analysis of genetically altered mice and in vitro models to demonstrate that VEGF$_{189}$ can bind NRP1 and promote NRP1-dependent neuronal responses.

KEY WORDS: Vascular endothelial growth factor (VEGF), VEGF189, Neuron, Neuropilin, Mouse

INTRODUCTION
Vascular endothelial growth factor A (VEGFA, VEGF) is a potent inducer of blood vessel growth, but also has essential roles in neurodevelopment (Mackenzie and Ruhrberg, 2012). In humans, VEGF is encoded by a single gene (VEGFA) of eight exons that is alternatively spliced into isoforms, the major ones containing 121, 165 and 189 amino acid residues and therefore termed VEGF$_{121}$, VEGF$_{165}$ and VEGF$_{189}$, respectively (Fig. 1A; Koch et al., 2011). The alternatively spliced exons 6 and 7 encode domains that enable extracellular matrix (ECM) binding and additionally mediate differential binding to VEGF receptors. All VEGF isoforms bind the receptor tyrosine kinases VEGFR1 (FLT1) and VEGFR2 (KDR, FLK1), whereas the non-catalytic receptors neuropilin 1 (NRP1) and NRP2 are VEGF isoform-specific receptors that preferentially bind VEGF$_{188}$ over VEGF$_{121}$ (Fig. 1A; Gluzman-Poltorak et al., 2000; Soker et al., 1998). Unexpectedly, recent studies showed that VEGF binding to NRP1 is largely dispensable for embryonic angiogenesis (Fantin et al., 2014). By contrast, VEGF signalling through NRP1 has multiple roles in neurodevelopment, including guiding migrating facial branchiomotor (FBM) neurons in the hindbrain (Schwarz et al., 2004), promoting the survival of migrating gonadotropin-releasing hormone (GnRH) neurons (Cariboni et al., 2011) and enhancing the contralateral projection of retinal ganglion cell (RGC) axons across the optic chiasm (Erskine et al., 2011).

To demonstrate roles for VEGF binding to NRP1 in neurons, prior studies used Vegfa$_{120/120}$ mice, which express VEGF$_{120}$, the murine equivalent of VEGF$_{121}$, but lack VEGF$_{164}$ and VEGF$_{188}$, corresponding to human VEGF$_{165}$ and VEGF$_{189}$, respectively (Carmeliet et al., 1999). Vegfa$_{120/120}$ mice phenocopy the defects in FBM neuron migration, GnRH neuron survival and RGC axon guidance (Cariboni et al., 2011; Erskine et al., 2011; Schwarz et al., 2004). In all three systems, VEGF signalling was attributed to the activity of VEGF$_{165}$ because it evokes appropriate neuronal responses in tissue culture models (Cariboni et al., 2011; Erskine et al., 2011; Schwarz et al., 2004), and because the ability of NRP1 to bind VEGF$_{165}$ is well established (Fantin et al., 2014; Soker et al., 1998). However, Vegfa$_{120/120}$ mutants lack VEGF$_{188}$ in addition to VEGF$_{164}$. Yet, it has never previously been examined whether VEGF$_{189}$ can also function as a NRP1 ligand in vivo. Moreover, it is not known whether VEGF$_{121}$ can bind NRP1 in a physiologically relevant context, even though it has been suggested that the exon 8 domain, which is present in all major VEGF isoforms, including VEGF$_{121}$, can mediate NRP1 binding in vitro (Jia et al., 2006; Pan et al., 2007; Parker et al., 2012).

Here, we have generated alkaline phosphatase (AP)-conjugated VEGF isoforms for in situ ligand-binding assays (Fantin et al., 2014) to examine whether VEGF$_{121}$ or VEGF$_{189}$ can bind NRP1 in vivo, as previously reported for VEGF$_{165}$. Our studies demonstrate that VEGF$_{189}$ binds NRP1-expressing axon tracts in intact hindbrain tissue, but that VEGF$_{121}$ is unable to do so. We further show that VEGF$_{189}$ is co-expressed with the other isoforms during VEGF/NRP1-dependent FBM migration, GnRH neuron survival and RGC axon guidance, and that VEGF$_{189}$ is sufficient to control all three processes, whereas VEGF$_{120}$ is not. We conclude that VEGF$_{189}$ effectively binds NRP1 and has the capacity to evoke NRP1-dependent signalling events, similar to VEGF$_{164}$. Considering that VEGF$_{189}$ has the highest affinity for ECM and therefore tissue retention amongst the VEGF isoforms, future research may therefore wish to consider the mechanistic contribution and therapeutic potential of this understudied VEGF isoform.

RESULTS AND DISCUSSION
VEGF$_{188}$ is co-expressed with VEGF$_{120}$ and VEGF$_{164}$ in developing hindbrain, nose and diencephalon, and binds axons in a NRP1-dependent fashion
Because prior studies implicated VEGF signalling through NRP1 in FBM neuron migration in the hindbrain, GnRH neuron survival in the nose and RGC axon guidance in the diencephalon (Cariboni et al., 2011; Erskine et al., 2011; Schwarz et al., 2004), we asked which Vegfa isoforms were expressed in these developmental contexts. For this experiment, we designed isoform-specific primers that can distinguish the Vegfa$_{120}$, Vegfa$_{164}$ and Vegfa$_{188}$ mRNA.
species by reverse transcription (RT)-PCR (Fig. 1A,B; supplementary material Fig. S1A). This analysis demonstrated that all three isoforms were co-expressed during relevant periods of VEGF/NRP1-dependent neurodevelopment in mice (Fig. 1C).

Because prior studies of VEGF binding to NRP1 have not examined whether VEGF189 or VEGF121 can bind NRP1 in vivo, we used the mouse hindbrain as a physiologically relevant model to compare the ability of the three major VEGF isoforms to bind NRP1 in a tissue context. We first performed immunostaining with a validated antibody for NRP1 (Fantin et al., 2010) to confirm that NRP1 localises to blood vessels in wild-type, but not NRP1 knockout, hindbrains (Fig. 1D; note unspecific staining of blood in the dilated vessels of mutants). Immunolabelling also confirmed NRP1 expression in TUJ1-positive dorsolateral axons on the pial side of wild-type, but not mutant, hindbrains (Fig. 1D; supplementary material Fig. S1B). Nrp1−/− hindbrains showed some defasciculation of these dorsolateral axons, but they were still clearly present in the mutant, suggesting that this is a suitable model to examine VEGFA isoform binding to NRP1.

To compare the binding properties of VEGF121, VEGF165 and VEGF189, we fused each isoform to AP and performed in situ ligand binding assays on E12.5 hindbrains. As expected, all three isoforms bound vessels (Fig. 1E), because they express the pan-VEGF receptor VEGFR2 (Lanahan et al., 2013). We next examined binding to dorsolateral axons, because they express NRP1, but lack VEGFR2 (Lanahan et al., 2013). Both VEGF165 and VEGF189 bound these axons, whereas VEGF121 did not (Fig. 1E). These observations indicate that all VEGF isoforms are capable of binding VEGFR2/NRP1-positive vessels. By contrast, only VEGF165 and VEGF189, but not VEGF121, bound NRP1-expressing axons lacking VEGFR2, consistent with the previously reported 10-fold lower affinity of VEGF121 for NRP1 in vitro (Parker et al., 2012). Thus, low-affinity binding of VEGF121 to NRP1, even though previously observed in vitro, is unlikely to be relevant in vivo, at least in a neuronal context.

We next confirmed that axonal VEGF189 binding is NRP1 dependent. The AP ligand-binding assay showed that VEGF189 bound vessels (Fig. 1F) in Nrp1-null mutant hindbrains with their characteristic vascular tufts (Fantin et al., 2013a). Strikingly, AP-VEGF189 failed to bind axons in Nrp1-null hindbrains, similar to AP-VEGF165 (Fig. 1F). VEGF189 can therefore bind axons in a
NRP1-dependent fashion. By contrast, loss of NRP2 (Giger et al., 2000) did not abolish VEGF189 binding (Fig. 1F). Taken together, the ligand binding assays of intact hindbrain tissue show that NRP1 serves as a neuronal receptor for VEGF165 and VEGF189, but not for VEGF121.

VEGF188 is sufficient for the NRP1-dependent migration of FBM neurons

Vegfa is a haploinsufficient gene for which deletion of just one allele results in early embryonic lethality due to a complete failure of blood vessel formation (Carmeliet et al., 1996; Ferrara et al., 1996). However, retention of any one of the major VEGF isoforms rescues this severe phenotype and instead gives rise to more subtle neuronal and vascular phenotypes (Ruhrberg et al., 2002; Stalmans et al., 2002). Understanding the receptor-binding properties of the VEGF isoforms has therefore become a priority in the field. We first examined if VEGF188 can substitute for VEGF164 in FBM neuron guidance with an established hindbrain explant assay in which implanted beads provide exogenous VEGF, and FBM neuron migration is visualised by immunolabelling with the motor neuron marker ISL1 (Schwarz et al., 2004; Tillo et al., 2014). Agreeing with previous observations, FBM neurons were attracted to VEGF164, but not to control beads lacking growth factors (Fig. 2B). VEGF188 beads also attracted FBM neurons (Fig. 2B). Quantification confirmed that FBM neuron migration was significantly enhanced on the hindbrain side containing a VEGF164- or VEGF188-soaked bead relative to the control side of the same hindbrain (Fig. 2C). VEGF188 can therefore promote NRP1-dependent neuronal migration similar to VEGF164.

We next examined FBM neuron migration in vivo by Isl1 in situ hybridisation. As previously shown (Schwarz et al., 2004), Vegfa120/120 hindbrains demonstrated abnormal streaming of FBM neurons on the ventricular side and dumbbell-shaped nuclei on the pial side (Fig. 2D). By contrast, Vegfa188/188 mice, which express only VEGF188, showed normal FBM neuron migration (Fig. 2D). Moreover, replacing one Vegfa120 allele in Vegfa120/120 mutants with the Vegfa188 allele was sufficient to prevent FBM neuron defects (Fig. 2D). Unlike VEGF120, VEGF188 is therefore sufficient to direct NRP1-dependent neuronal migration.

VEGF188 is sufficient to guide NRP1-dependent axon crossing at the optic chiasm

We next investigated whether VEGF188 can evoke neuronal responses similar to VEGF164 in the developing visual system. To establish binocular vision, RGC axons project through the optic chiasm to both the ipsilateral and contralateral brain hemispheres (Erskine and Herrera, 2007). VEGF164, but not VEGF120, promotes RGC axon guidance in a NRP1-dependent fashion in vitro, and Vegfa120/120 mice therefore develop an abnormal chiasm (Erskine et al., 2011). To examine whether VEGF188 can also promote RGC axon guidance, we performed DiI labelling in VEGF isoform mutants. Anterograde labelling of RGC axons from one eye at E14.5 demonstrated that VEGF188 was sufficient for NRP1-mediated chiasm patterning (Fig. 3A). Thus, Vegfa120/120 mice had a significantly increased ipsilateral projection index as well as defasciculation of the ipsilateral and contralateral optic tracts (Erskine et al., 2011), but the ipsilateral index and shape of the optic chiasm appeared unaffected in Vegfa188/188 mice (Fig. 3B,C). Moreover, replacing one Vegfa120 with the Vegfa188 allele was sufficient to prevent chiasm defects in Vegfa120/120 mutants (Fig. 3B,C).

Fig. 2. VEGF188 is sufficient for FBM neuron migration. (A) Schematic representation of FBM neuron migration in the mouse. (B) ISL1 staining of E12.5 hindbrain explants containing implanted heparin beads soaked in PBS (n=10) or PBS containing VEGF164 (n=10) or VEGF188 (n=6). Red dotted circles indicate the position of heparin beads; white arrowheads indicate normal migration; red arrows indicate migration towards heparin beads; asterisks indicate the midline. Scale bar: 200 µm. (C) Distance migrated by FBM neurons. Migration distance was quantified as migration away from r5 territory on the hindbrain side with a bead relative to the control half of the same hindbrain; mean ± s.e.m. control 1±0.09 versus VEGF164 bead 1.39±0.05; control 1±0.11 versus VEGF188 bead 2.04±0.17; **P<0.01, VEGF compared with control (t-test). (D) Whole-mount Isl1 in situ hybridisation of E12.5 hindbrains of the indicated genotypes detects migrating FBM neurons (VIIm) (control, n=10; Vegfa120/120, n=6; Vegfa188/188, n=4; Vegfa120/188, n=5). Brackets indicate the width of the neuronal stream on the ventricular side; red arrowheads indicate dumbbell-shaped nuclei on the pial side; asterisks indicate the midline. Scale bar: 25 µm.
We next performed retrograde DiI labelling of RGC axons from the dorsal thalamus in VEGF isoform mice and compared the number of labelled RGCs in flatmounted ipsilateral and contralateral retina (Fig. 3D). Quantitation showed that the proportion of DiI-labelled ipsilateral RGCs was significantly increased in Vegfa120/120 compared with control mice, but was normal in Vegfa188/188 and Vegfa120/188 mice (Fig. 3E). Flat-mount images also revealed the preferential origin of ipsilaterally projecting neurons from the ventrotemporal retina in wild types (Fig. 3F). Their distribution is affected in Vegfa120/120 mice, which contain ipsilaterally projecting RGCs throughout the nasal retina (Erskine et al., 2011), but this defect was rescued by the introduction of a single Vegfa188 allele (Fig. 3F). VEGF188 is therefore sufficient to promote NRP1-dependent aspects of optic chiasm development.

**VEGF188 is sufficient to ensure normal GnRH neuron survival**

As a third model to study VEGF188 in neurodevelopment, we investigated GnRH neuron survival. GnRH neurons are born in the nasal placode and travel along nasal axons to reach the forebrain (Fig. 4A; Cariboni et al., 2007). We have previously shown that Vegfa120/120 mice have significantly fewer migrating GnRH neurons and demonstrated that VEGF164 signals through NRP1 to promote the survival of GN11 cells, which recapitulate many features of migratory GnRH neurons (Cariboni et al., 2011). We therefore examined whether VEGF188 promotes GN11 survival, similar to VEGF164. Whereas 72 h of serum withdrawal caused the death of over half of the GN11 cells, the inclusion of serum, VEGF164 or VEGF188 for the last 12 h of culture significantly reduced cell death, and VEGF188 was as effective as VEGF164 in preventing cell death; by contrast, and as expected, VEGF120 did not promote survival (Fig. 4B; percentage of propidium iodide-positive cells, mean±s.e.m.: control, 44±3%; serum, 2±1%; VEGF120, 37±3; VEGF164, 11±2%; VEGF188, 11±2%). These observations suggest that VEGF188, similar to VEGF164, can promote GnRH neuron survival. The ineffectiveness of VEGF120 agreed with the previously observed NRP1-dependent neuroprotection of GN11 cells and the...
fact that Vegfa<sup>120/120</sup> mice have fewer GnRH neurons (Cariboni et al., 2011). Also in agreement with the in vitro findings, the GnRH neuron number was normal in Vegfa<sup>188/188</sup> mice that express VEGF<sub>188</sub> but lack VEGF<sub>164</sub> (Fig. 4C,D). Moreover, replacing one Vegfa<sup>120</sup> allele in Vegfa<sup>120/120</sup> mutants with the Vegfa<sup>188</sup> allele was sufficient to prevent their GnRH neuron survival defect (Fig. 4C,D). Together, these data show that VEGF<sub>188</sub> is sufficient to promote NRP1-dependent neuronal survival.

Conclusions

Our study has demonstrated that human VEGF<sub>189</sub>, but not VEGF<sub>121</sub>, binds NRP1 in a tissue context, that mouse VEGF<sub>189</sub> is co-expressed with VEGF<sub>164</sub> in a neuronal context, and that mouse VEGF<sub>189</sub> expressed from the endogenous Vegfa locus can evoke NRP1-dependent neuronal responses in vitro and in vivo, similar to VEGF<sub>164</sub> and unlike VEGF<sub>121</sub>. Future work on the role of VEGF signalling through NRP1, especially studies using Vegfa<sup>120/120</sup> or tissue-specific Vegfa<sup>null</sup> alleles, should therefore consider the possibility that VEGF<sub>189</sub> similar to VEGF<sub>164</sub>, can regulate the process under investigation. This consideration would be relevant for both neural and vascular studies, or indeed any context in which VEGF signalling through NRP1 is implicated. The finding that the relatively understudied VEGF<sub>189</sub> is capable of evoking VEGF isoform-specific signalling events may have broad implications for the therapeutic use of VEGF. Thus, VEGF application has been considered in many studies for pro-angiogenic, pro-neurogenic and neuroprotective therapies, e.g. the treatment of amyotrophic lateral sclerosis (reviewed by Storkebaum et al., 2011). Most prior studies have used VEGF<sub>165</sub> to ensure comprehensive receptor targeting; however, the retention of VEGF<sub>165</sub> in tissues is inferior to that of VEGF<sub>189</sub> due to the presence of only one instead of two heparin/matrix-binding domains. Our work demonstrating that VEGF<sub>189</sub> is fully capable of engaging NRP1, in addition to its known ability to bind VEGFR1 and VEGFR2, therefore suggests that VEGF<sub>189</sub> may be better suited than VEGF<sub>165</sub> to induce localised tissue effects in therapeutic applications.

**Fig. 4. VEGF<sub>188</sub> is sufficient to promote GnRH neuron survival.** (A) GnRH neuron migration (blue dots). The neurons are born in the nasal placodes that give rise to the olfactory and vomeronasal epithelia (OE, VNO) and migrate along olfactory and vomeronasal axons (purple, Oll/VFN) through the nasal compartment (NC) to reach the forebrain (FB). (B) Serum-starved GN11 cells were treated with DMEM or DMEM-containing serum, VEGF<sub>120</sub>, VEGF<sub>164</sub> or VEGF<sub>188</sub>; cell death was visualised by propidium iodide staining (red); Hoechst staining (blue) identified the total number of cells. Scale bar: 25 µm. (C) Sagittal sections of E14.5 mouse heads of the indicated genotypes, immunolabelled for GnRH. Arrows indicate migrating neurons; open triangles indicate the absence of migrating neurons; dotted lines indicate the FB boundary. OB, olfactory bulb. Scale bar: 100 µm. (D) GnRH neuron number in E14.5 heads of the indicated genotypes (mean±s.e.m.): control, 1246±46, n=6; Vegfa<sup>120/120</sup>, 854±21, n=5; Vegfa<sup>188/188</sup>, 1335±63, n=3; Vegfa<sup>120/188</sup>, 1314±56, n=3; t-test; ***P<0.001 compared with control.

**MATERIALS AND METHODS**

**Animals**

Animal procedures were performed in accordance with institutional and UK Home Office guidelines. The Vegfa<sup>120</sup> and Vegfa<sup>188</sup> isoforms were amplified by PCR using MegaMix (Microzone) and the following oligonucleotide pairs: 120-F 5′-GTAACGATGAAGCGCCGCTG-GAG-3′ and 120-R 5′-CCCTGTGATGACTCTTCATTTTTC-3′; 188-F 5′-AGGAGAGCCGAGATGTT-3′ and 188-R 5′-GCCCTGCTCTTGATCATCT-3′; 164-F 5′-AGTTCAAGAGGAAGGGAAGG-3′ and 164-R 5′-GCCTTGCTGCTCCTGAC-3′.

**RT-PCR and sequencing**

Total RNA was reverse transcribed using Superscript III (Life Technologies) and Vegfa isoforms amplified by PCR using MegaMix (Microzone) and the following oligonucleotide pairs: 120-F 5′-GTAACGATGAAGCGCCGCTG-GAG-3′ and 120-R 5′-CCCTGTGATGACTCTTCATTTTTC-3′; 188-F 5′-AGGAGAGCCGAGATGTT-3′ and 188-R 5′-GCCCTGCTCTTGATCATCT-3′. The PCR products were cloned into pAG-AP containing an upstream in-frame AP cassette. Binding assay were performed as described previously (Fanti et al., 2013b).

**AP-fusion protein binding assays**

Open reading frames for the VEGF isoforms were amplified by PCR with the oligonucleotides 5′-AAATAATGGATCCGCCCATGGGCAAGGAG-AG-3′ and 5′-TATATGTCCGAGCTACCCGCTTGGCTGTC-3′. The PCR products were cloned into pAG-AP containing an upstream in-frame AP cassette. Binding assay were performed as described previously (Fanti et al., 2013b).

**Immunolabelling and in situ hybridisation**

Primary antibodies used were: rabbit anti-mouse GnRH (Immunostar, 20007S, 1:1000), goat anti-rat NRP1 (R&D Systems, AF566, 1:100), rabbit anti-mouse TUJ1 (Covance, MRB-435p, 1:250) and mouse anti-rat ISL1 (DSHB, 39.405, 1:100). Secondary antibodies used were: Alexa594-conjugated rabbit anti-goat Fab (Jackson ImmunoResearch, 240-594-162, 1:200). To detect blood vessels, we used biotinylated IB4 (Sigma) conjugated rabbit anti-goat Fab (Jackson ImmunoResearch, 305-587-003, 1:100), goat anti-mouse (Life Technologies, A-110011, 1:200) and Alexa488-conjugated goat anti-mouse (Life Technologies, A-110011, 1:200) and biotinylated goat anti-rabbit (Vector Laboratories, BA-1000, 1:200). To detect blood vessels, we used biotinylated IB4 (Sigma) followed by Alexa633-conjugated streptavidin (Life Technologies). For in situ hybridisation, we used a digoxigenin-labelled Isl1 probe (Schwarz et al., 2004).
Hindbrain explant culture
Hindbrain explants were cultured as previously described (Schwarz et al., 2004; Tillo et al., 2014). Affi-Gel heparin beads (Bio-Rad) were soaked overnight in 100 ng/ml of VEGF164 in PBS (Preprehct) or VEGF188 (Reliatech). FBM neuron migration was measured with ImageJ (NIH) as the distance travelled from r5 to the leading group of cells in r6 in each hindbrain and normalised to the control side of each hindbrain.

Dil labelling
Dil labelling was performed with fixed tissues as described previously (Erskine et al., 2011). Briefly, a Dil crystal (Life Technologies) was placed over the optic disc of one eye for anterograde labelling. After 3 days at 37°C, dissected brains were imaged ventral side upwards. ImageJ was used to determine the pixel intensity in defined areas of the ipsilateral and contralateral optic tracts, and the ipsilateral index calculated as the ratio of fluorescent intensity in the ipsilateral relative to the ipsilateral plus contralateral tracts. For retrograde labelling, the cortex was removed unilaterally and Dil crystals placed in a row over the dorsal thalamus for 15 minutes at room temperature; we imaged flatmounted retinas as above and determined the percentage of labelled ipsilateral RGCs relative to the ipsilateral plus contralateral RGCs.

GnRH neuron analysis and survival assays
Immunolabelled GnRH-positive cells were quantitated and GN11 survival assays performed as described previously (Cariboni et al., 2011). For survival assays, cells were serum starved for 72 h and treated for 12 h with media containing 10% FBS, 10 ng/ml VEGF120, VEGF164 or VEGF188.

Acknowledgements
We thank Dr. Jonathan Raper for the pAG3-AP plasmid and the staff of the Biological Resources Unit at the UCL Institute of Ophthalmology for help with mouse husbandry.

Competing interests
The authors declare no competing financial interests.

Author contributions
C.R. and M.T. planned the experiments and wrote the manuscript. M.T., L.E., A.C., A.F., A.J., L.D. and C.R. performed the experiments. All authors have read, commented on and approved the manuscript.

Funding
This research was funded by a Wellcome Trust PhD fellowship to M.T. [092839/Z/10/Z] and a BBSRC project grant to C.R. and L.E. [BB/J00930X/1]. Deposited in PMC for immediate release.

Supplementary material
Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.115998/-/DC1

References


