Direct and indirect roles of Fgf3 and Fgf10 in innervation and vascularisation of the vertebrate hypothalamic neurohypophysis

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SUMMARY

The neurohypophysis is a crucial component of the hypothalamo-pituitary axis, serving as the site of release of hypothalamic neurohormones into a plexus of hypophyseal capillaries. The growth of hypothalamic axons and capillaries to the forming neurohypophysis in embryogenesis is therefore crucial to future adult homeostasis. Using ex vivo analyses in chick and in vivo analyses in mutant and transgenic zebrafish, we show that Fgf10 and Fgf3 secreted from the forming neurohypophysis exert direct guidance effects on hypothalamic neurosecretory axons. Simultaneously, they promote hypophyseal vascularisation, exerting early direct effects on endothelial cells that are subsequently complemented by indirect effects. Together, our studies suggest a model for the integrated neurohedral wiring of the hypothalamo-neurohypophyseal axis.

KEY WORDS: FGF, Guidance, Hypothalamus, Zebrafish, Chick

INTRODUCTION

Neurosecretory hypothalano-neurohypophyseal (H-NH) neurons project axons to the neurohypophysis (NH), a richly vascularised protrusion of the ventral forebrain floor (Hofkelt and Fuxe, 1972; Gorbman et al., 1983; Markakis, 2002). Neurohormones and neurotransmitters are released from axon terminals into capillary networks and are transported, either locally to the anterior pituitary/adenohypophysis (AH) or to distant body targets. The close proximity of H-NH axons and capillary vessels in the NH thus underlies the future functioning of the H-NH axis and homeostatic balance.

NH architecture, and the projection patterns of hypothalamic axons have been well-described in many species (Cajal, 1894; Bargmann, 1949; Harris, 1955; Gorbman et al., 1983; Markakis, 2002). In amniotes, the NH is composed of the anterior median eminence (ME), the neural stalk (pituitary stalk/stem) and the posterior neural lobe (pars nervosa/posterior pituitary). Two major classes of H-NH neurons, parvocellular and magnocellular, project axons through the medial forebrain bundle, then exit and extend ventrally into the NH. H-NH axons do not cross the ventral midline. Instead, parvocellular axons project to the ME where they secrete neurohormones/neurotransmitters into portal vessels that transport them to a pituitary plexus (Green and Harris, 1947). By contrast, magnocellular axons traverse the ME and neural stalk to terminate on capillaries of the neural lobe, where they release oxytocin and vasopressin. The architecture of the NH in anamniotes is broadly similar. A neural lobe-like region contains nerve endings of magnocellular neurons, releasing Arginine-Vasopressin-like (Avpl) or Oxytocin-like (Oxtl) homologues. Anamniotes do not possess a well-defined ME and portal capillary system, but some species have an anterior neurohemal area (Nishioka and Bern, 1966). Thus, in anamniotes, parvocellular axons either synapse directly with cells in the AH or contact capillaries within the anterior neurohemal area.

Although structure-function analyses of the NH have been the focus of intense research for the last century (Bargmann, 1949; Harris, 1955; Gorbman et al., 1983), little is understood of the mechanisms that establish H-NH axonal tracts in embryogenesis. Chemoattractive cues that direct the growth of axons to the ventral midline throughout most of the forming CNS, notably Netrin and Shh (Charron et al., 2003; Sánchez-Camacho and Bovolenta, 2009) are not expressed at the hypothalamic ventral midline, and so are absent in the forming NH (infundibulum) (Pearson et al., 2011). Instead, previous studies have suggested a role for other cues, reporting that Fgf and Notch signalling can influence H-NH axonal projections (Giñ and Tsai, 2006; Auja et al., 2011). Once at the NH, however, H-NH axons play a crucial role in shaping NH vasculature. Studies in zebrafish show that the neurotransmitter Oxytocin-like (referred to as Oxytocin), released by neurohypophyseal nerve endings, can promote vessel growth (Gutnick et al., 2011). Nevertheless, it remains unclear whether other factors operate alongside Oxytocin, potentially contributing to the H-NH vascularisation profile.

The evolutionarily conserved fibroblast growth factors (FGFs) (Ornitz and Itoh, 2001; Beenen and Mohammadi, 2009) play multiple roles in embryogenesis, including diverse roles in hypothalamic development (Thissie and Thissie, 2005; Tsai et al., 2011). Fgf3 and Fgf10, the subject of this work, belong to the same subfamily, the FGF7 subfamily. Previous studies have demonstrated...
a role for members of other FGF subfamilies in fashioning axonal and vascular networks: Fgf8 selectively attracts spinal motor axons to the dermomyotome (Shirasaki et al., 2006), and Fgf2 exerts pro-angiogenic effects (Cross and Claesson-Welsh, 2001; Beeken and Mohammadi, 2009). However, it is unknown whether Fgf3 and Fgf10 play a direct role in axonal or vessel navigation, or can coordinate the concomitant innervation and vascularisation of neurohemal organs.

Here, we dissect the role of FGFs secreted from the forming NH in neurohypophyseal innervation and vascularisation in chick and zebrafish embryos. Ex vivo studies in chick and in vivo analyses of zebrafish fgf3 mutant and transgenic fish allowing temporally controlled, or cell type-specific, blockade of FGF signal reception reveal that Fgf3 in the zebrafish, and Fgf3 and Fgf10 in the chick, are crucial in directing NH innervation and vascularisation. Our studies describe an essential role for FGF signalling in the directed growth of early H-NH axons to the NH. We demonstrate a dose-dependent effect of Fgf10 on H-NH axons, its attractive function switching to repellant activity at high concentrations, suggesting a mechanism for the stalling of axons as they reach the NH. In contrast to its direct effects on NH innervation, FGF signalling operates in a largely indirect manner to govern H-NH vascularisation. Thus, although FGF signalling is transiently required for NH-evoked endothelial outgrowth in vitro, loss of FGF signal reception in endothelial cells in vivo leads to only minor perturbations. Together, these studies reveal multi-step roles for FGF7 subfamily members in the integrated wiring of the NH.

MATERIALS AND METHODS

Chick strains and fish lines

Bovan-Brown chicks and the zebrafish potential fgf3 null allele t24152 (Herzog et al., 2004) were used. Transgenic zebrafish lines used were: Tg(otipb:1EGFP)z49 (Gutnick et al., 2011), Tg(pomca:GFP)zj44 (Liu et al., 2003), Tg(otipr:RFP)zj44 (Liu et al., 2006), Tg(krd1:Ha.HRAS-mCherry)s7896 (Chi et al., 2008), Tg(hsp70:dnfgfr1-EGFP)pd1 (Lee et al., 2005), Tg(flipe:gai4)f4;h04b (Gutnick et al., 2011). See below for generation of the tga(vglf:KaITa4)fj31 line. Heatshock induction was performed by transferring embryos from 28°C to 39°C for 30 minutes.

Constructs for transient zebrafish transgenesis and generation of stable tga(vglf:KaITa4) transgenic line

UAS:dnfgfr1-mCherry, UAS:dnfgfr1-egfp, UAS:egfp and UAS:mCherry constructs used for transient transgenesis were taken from (UAS:egfp) or generated (UAS:dnfgfr1-mCherry, UAS:dnfgfr1-egfp, UAS:mCherry) using the gateway-based Tol2 kit (Kwan et al., 2007). Following primer pairs were used to generate (1) UAS:dnfgfr1-mCherry, UAS:dnfgfr1-egfp: 5'-GGGACACATGTTGTACAAAAAGAGGCTCACCACATGATATGAGAAGACACACGCTG-3', 5'-GGGACACATGTTGTACAAAAAGAGGCTCACCACATGATATGAGAAGACACACGCTG-3'; (2) UAS:mCherry: 5'-GGGACACATGTTGTACAAAAAGAGGCTCACCACATGATATGAGAAGACACACGCTG-3', 5'-GGGACACATGTTGTACAAAAAGAGGCTCACCACATGATATGAGAAGACACACGCTG-3'; (3) Tg(aVpl:1EGFP)zc49 (Gutnick et al., 2011). See below for generation of the tga(vglf:KaITa4)fj31 line. Heatshock induction was performed by transferring embryos from 28°C to 39°C for 30 minutes.

Explant and grafting cultures

Embryonic day (E) 4 or E7 hypothalamic neuroectoderm and E5 CAM vessels were dispase-isolated (1 mg/ml, Roche) and cultured in matrigel or collagen. In co-cultures, explants, or explants and beads, were positioned 100-200 µm apart and cultured for up to 48 hours. In grafts, hypothalamic neurons from Roslin GFP embryos were transplanted to lateral regions of wild-type hypothalami. FGFs (R&D Systems) were pre-soaked on beads (Affigel, Pharmacia Biotech). SU5402 (Calbiochem; 20 µM) was added at the start of culture. Fgf10 and Fgf3 blocking antibodies (Santa Cruz Biotechnology; 50 ng/µl) were added to medium or pre-incubated with the EGFP cassette was removed by an In situ hybridisation analysis of embryos (n=15-20) and explants was performed according to standard whole-mount or cryostat sectioning techniques (Pearson et al., 2011; Nowak and Hammerschmidt, 2006). In chick work, antibodies used were: anti-TUJ1 (anti neuron-specific class III β-tubulin; Calbiochem); anti-Neurofilament (3A10; DSHB); anti-Sox2 (Abcam); anti-TH rabbit polyclonal (Chemicon); anti-Tie1 (Santa Cruz Biotechnology); anti-Vaspressin (Bachem, Torrance, CA, USA); anti-VE cadherin (Abcam). In zebrafish work, antibodies used were: rabbit anti-Prl (Kawachi et al., 1983); rabbit anti-(Arg8)-Vaspressin (Peninsula Laboratories) (Larson et al., 2006); anti-acetylated Tubulin (AcTub) (Sigma); rabbit anti-RFP (Abcam) and chicken anti-GFP (Molecular Probes). Secondary antibodies were conjugated to Fluorescein isothiocyanate (FITC; Jackson ImmunoResearch), Alexa Fluor 555 or Alexa Fluor 488 (Molecular Probes). Nuclear counterstaining was performed with DAPI (Sigma). Fixation prior to anti-AcTub immunofluorescence was carried out in 2% trichloroacetic acid in PBS for 3 hours at room temperature.

In situ and double fluorescent hybridisations were carried out as described (Nica et al., 2004; Clay and Ramakrishnan, 2005; Pearson et al., 2011). For double analyses with AcTub, specimens were treated with collagenase (Sigma) instead of proteinase K.

Imaging

Chick tissue was imaged on a Zeiss Apotome or Olympus BX60 with Spot RT software v3.2 (Diagnostic Instruments). Zebrafish sections were imaged...
on a single-photon Zeiss LSM510 confocal. Live transgenic zebrafish larvae were imaged on a Zeiss LSM710 confocal using Non-Descanned-Detectors after excitation with a two-photon Chameleon laser.

**Statistical analyses**

Axon and endothelial process numbers were recorded after 48 hours, and data analysed using GraphPad Prism. Statistical significance of differences in means between groups was determined using a two-tailed Student’s t-test. Standard error bars are shown.

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**RESULTS**

**Innervation of chick neurohypophysis**

In chick, the NH is first apparent at E4 as a button-like protrusion in the diencephalic ventral midline (Fig. 1A). By E7, it has grown, and projects ventrally above the adenohypophysis (Fig. 1E,F; supplementary material Fig. S1). Fate-mapping studies show that midline cells in the button-like protrusion give rise to the ventral NH (nh), whereas an overlapping/adjacent ‘collar region’ gives rise to the dorsal NH (Fig. 1A legend) (Pearson et al., 2011). A number of FGF genes are expressed in the developing NH. Fgf10 and Fgf19 are detected in the button-like cells at E4 (Fig. 1B,C) (Gimeno and Martinez, 2007). By E7, Fgf10 is detected throughout the NH (Fig. 1F,G) (Gimeno and Martinez, 2007; Pearson et al., 2011), with highest levels in the ventral NH (supplementary material Fig. S2A,B). Fgf3 and Fgf8 are detected in the collar region from E4 to E7 (Fig. 1D,H) (Pearson et al., 2011; Proszkowiec-Weglarz et al., 2011). Over this period, these FGF genes are not detected elsewhere in the chick diencephalon (Gimeno and Martinez, 2007; Pearson et al., 2011; Proszkowiec-Weglarz et al., 2011).

TUJ1+ pioneering axons project towards the prospective NH (prosp-NH) at ~E4.5 (Fig. 1I), project through the collar region by E6 (Fig. 1J), but turn before they reach the ventral NH, rather than projecting into/beneath it (Fig. 1K, arrowheads). Analysis of Tyrosine hydroxylase (TH), a marker of parvocellular dopaminergic neurons, and of Vasopressin (Vp), a marker of magnocellular neurons, confirms that H-NH neurosecretory cells differentiate some time after pioneers, then follow a similar trajectory (Fig. 1L; supplementary material Fig. S2C; data not shown). Thus, TH+ axons project to dorsal NH regions (the median eminence and collar) but do not initially project into/beneath the ventral-most NH (Fig. 1M,N). The first TUJ1+ pioneering axons project into/beneath the ventral NH only at E10 (Fig. 1O; supplementary material Fig. S2D,E). Retrograde Dil labelling reveals that in the subsequent 48 hours, many axons project deep into the NH (Fig. 1P).

Together, our results suggest that, in chick, the first H-NH axons respond to potential guidance cues from the prosp-NH at E4-E5, project to dorsal regions of the NH by E6, but extend into the ventral NH only at E10.

**Chemoattractive effects of the prosp-NH**

To investigate whether the ventral hypothalamus exerts a long-range guidance effect on H-NH axons, chick lateral [L] hypothalamic explants containing nascent H-NH neurons were cultured at a distance from E4 ventral hypothalamic explants in vitro (Fig. 2A,B). Long axons/fascicles emerge, almost exclusively from the proximal face of [L] explants (Fig. 2B, arrow; data not shown) and project to the ventral hypothalamic explant. Notably, axons project to the prosp-NH and not to retrochiasmatic or mamillary pouch regions (Fig. 2B). By contrast, few axons emerge from [L] explants cultured alone (Fig. 2C). These results indicate that the prosp-NH secretes a diffusible molecule(s) that exerts a long-range effect on H-NH axons.

To test this idea, Fgf3+ Fgf10+ E4 prosp-NH explants were cultured a short distance from [L] explants (Fig. 2D; data not shown). Robust axon outgrowth is elicited by the prosp-NH, with significantly more axons projecting from the proximal than the distal face of [L] explants (Fig. 2E,F; supplementary material Table S1).

The prosp-NH could, in principle, promote axon extension without influencing direction. To seek evidence that it can govern axonal trajectory, [L] explants from GFP-transgenic chicks (McGrew et al., 2004) were grafted to E4 wild-type hypothalami (n=7; Fig. 2G, schematic) and cultured ex vivo. The NH develops in...
the first 48 hours (Fig. 2G, schematic) and promotes extensive directed outgrowth from grafted [L] explants. GFP+ axons emerging from the graft show oriented growth, extending down the side of the host hypothalamus, towards the developing NH. As they reach the NH, axons further re-orient their trajectories, growing towards it (Fig. 2H,I). As in vivo, axons project to the Fgf10+ NH but do not invade deep into it (Fig. 2I,J; supplementary material Fig. S2C,D). This suggests that the developing NH exerts a long-range chemoattractive effect on hypothalamic axons, but exerts a local stalling/halting effect once axons reach it.

NH-derived FGFs direct H-NH axons

FGFs can direct motor axons (Shirasaki et al., 2006) and show restricted expression within the prosp-NH. Given this, we examined whether FGF-soaked beads mimic the chemoattractive effect of the NH. Fgf3- and Fgf10-, but not PBS- or Fgf8-soaked beads elicit axon outgrowth from chick E4-E5 [L] explants, with the vast majority of axons projecting from the face of the explant proximal to the FGF source (Fig. 3A,A'H11032,D,D'H11032; supplementary material Fig. S3A,B and Table S1). Immunolabelling shows that many axons grow within [L] explants, but only project out in the presence of FGFs (supplementary material Fig. S3C,D). Both parvocellular (TH+) and magnocellular (Vp+) axons respond to FGFs (Fig. 3B,C,E,F). An inherent bias in axon growth in [L] explants (supplementary material Fig. S3C; F.L. and M.P., unpublished) enables FGF-soaked beads to be positioned orthogonal to the normal axis of axon growth. In this configuration, axons re-orient to grow towards the bead (supplementary material Fig. S3B,D), suggesting an axon-guiding effect of FGFs. Fgf3 and Fgf10 do not alter the numbers of Nkx2.1+ progenitors or TH+ neurons (supplementary material Fig. S3E-L; data not shown), consistent with findings that the ventral hypothalamus does not exert neurogenic or trophic effects on hypothalamic neurons at these stages (Rogers et al., 1997; Gibson et al., 2000).

To address whether the chemoattractive effect of prosp-NH explants involves FGF signalling, explants were exposed to SU5402, a small molecule inhibitor that suppresses tyrosine kinase activity of FGF and vascular endothelial growth factor (VEGF) receptors (Mohammadi et al., 1997). SU5402 does not provoke outgrowth of axons from [L] explants cultured alone (supplementary material Fig. S3M), and does not alter inherent axonal growth within [L] explants, or Nkx2.1’ and TH’ cell numbers (supplementary material Fig. S3H-L-P). However, SU5402 eliminates Fgf10- and Fgf3-evoked axon extension (Fig. 3G-G'H11033; supplementary material Table S1). Furthermore, SU5402 significantly reduces extension evoked by the prosp-NH; the few axon fascicles that do emerge are significantly shorter than normal (Fig. 3H,I).

To address the specific requirements for Fgf3 and Fgf10, we analysed the effects of Fgf10- or Fgf3-blocking antibodies on [L]-prosp-NH co-cultures, and on [L] hypothalamic grafts. In vitro, blockade of either Fgf10 or Fgf3 results in a marked decrease in axon outgrowth evoked by the prosp-NH, and this effect is...
potentiated by blockade of both (Fig. 3J-L; supplementary material Table S1). Similarly, blockade of Fgf3 and Fgf10 eliminates the chemotactic effect of the prosp-NH ex vivo (supplementary material Fig. S2H). Therefore, both Fgf3 and Fgf10 secreted by the forming NH might act as long-range chemotactants for H-NH axons.

In the collagen culture system, we noted that as axons approach Fgf10-soaked beads, they frequently re-orient their growth, turning away from the beads (Fig. 3A,M, arrows). To test whether this is a direct effect of high concentrations of Fgf10, [L] explants were challenged with beads soaked in high concentrations of Fgf10 (supplementary material Table S1). Under these conditions, the chemotactic effect of Fgf10 is eliminated or reversed (Fig. 3N,N’). Only a few short axons emerge from the proximal side of the [L] explant (Fig. 3N, white arrow; data not shown); instead, many axons now emerge from the distal side (Fig. 3N, yellow arrows; supplementary material Table S1). This effect was not observed with Fgf3 (supplementary material Table S1). Thus, Fgf10 might exert a graded action, stimulating the outgrowth of H-NH axons at lower concentrations, but stalling/repelling them at higher concentrations.

An early role for the prosp-H-NH in chick endothelial growth

The functioning of the NH depends on the intimate association of axonal terminals with vascular capillaries. In chick, calponin+ endothelial cells are first detected close to the forming NH at E4 (Fig. 4A,B) and by E5, Tie1+ VE-cadherin+ cells are aggregating to vessel-like structures that lie between the NH and underlying adenohypophysis (Fig. 4C). Analysis of serial adjacent sections at E6 reveals that endothelial cells project into/under the NH before pioneering axons (Fig. 4D,E), but are closely associated with, and just ventral to, H-NH neurons/axons (Fig. 4F) and maintain this close association at E10, when H-NH pioneers project into the ventral NH (Fig. 4G).

Given the role of FGFs in angiogenesis and vasculogenesis (Cross and Claesson-Welsh, 2001; Beenken and Mohammadi, 2009), we speculated that NH-derived Fgf3 and Fgf10 might simultaneously stimulate NH innervation and vascularisation. To test this, we adapted the in vitro culture system, challenging enzymatically isolated chorioallantoic membrane endothelial capillary vessel [CAMv] explants with prosp-NH explants alone (H-H’) or with inhibitors (H-L’). (M) Local chemorepulsion (arrow) in proximity to an Fgf10-soaked bead (N,N’) [L] explants cultured with high concentrations of FGF. In high powered views (N) white arrow points to short axons that emerge from proximal face, yellow arrows point to axons emerging from distal face. Error bars represent s.e.m. ***P<0.005. d, distal; p, proximal. Scale bars: 100 μm in A,D,G,H-L,M,N; 50 μm in B,C,E,F.
Exposure of [CAMv] explants to a point-source of FGFs results in the appearance of long (>90 μm) projections, preferentially on the side of the [CAMv] explant facing the bead (Fig. 4O-Q; supplementary material Table S2). Section analyses reveal these are VE-cadherin+Tie1+ endothelial cellular assemblies (Fig. 4R,R/H11032).

When presented uniformly, FGFs similarly lead to a significant lengthening of Tie1+ process outgrowth (Fig. 4S) without altering the number of DAPI+ cells, or M-phase cells within [CAMv] explants (Fig. 4T,U; supplementary material Table S2). To investigate whether prosp-NH-evoked outgrowth requires FGFs, E4 prosp-NH explants were pre-soaked in blocking antibodies to Fgf3 and Fgf10, and then co-cultured with [CAMv] explants. Pre-treatment with anti-Fgf3 or anti-Fgf10 does not alter the character of the prosp-NH explants (they continue to express characteristic markers, including Fgf10 and Tbx2; Fig. 4V; data not shown) but eliminates their ability to promote endothelial outgrowth. Thus, although short radial outgrowths occur from [CAMv] explants, no long cellular projections are detected (Fig. 4W, compare with 4L; supplementary material Table S2).

To gain insights into the temporal requirements of FGFs for NH vascularisation, we analysed the endothelial outgrowth-promoting activity of E6 NH explants (in which innervation has begun). E6 explants show a diminished capacity to promote endothelial outgrowth, compared with E4 explants (supplementary material Fig. S4A, compare with Fig. 4L; supplementary material Table S2). Furthermore, in contrast to E4 explants, E6 NH-stimulated outgrowth cannot be completely abolished upon pre-treatment with Fgf3 or Fgf10 antibodies (supplementary material Fig. S5B, compare with Fig. 4W; supplementary material Table S2).

Fig. 4. Prosp-NH-derived FGFs stimulate outgrowth of endothelial processes. (A-G) Transverse (B-F) and sagittal (G) sections through chick NH at planes indicated in A. D,E show serial adjacent sections. Arrowheads and arrows point to endothelial processes/axons outside (arrowheads) or within/beneath (arrow) ventral NH. (H-I) [CAMv] isolation from wild-type or Roslin-GFP embryos. (J-K) Short radial projections emerge from [CAMv] explants cultured alone for 48 hours. Whole-mount immunolabelling shows that these are Tie1+ (J'). Dotted circles in J' outline explant and ring of short processes. (L-L') [CAMv]-E4 prosp-NH co-culture after 24 (L) and 40 hours (L'). Arrows indicate long endothelial processes. (L') Quantitative analysis of long processes from proximal versus distal faces of [CAMv] explants. Error bars represent s.e.m. ***P<0.001. d, distal; p, proximal. (M,M') High-power views, after sectioning; extending processes are DAPI+VE-cadherin+Tie1+ (M'). (N-R) GFP [CAMv] explants cultured with PBS- (N), Fgf10- (O,P) or Fgf3- (Q) soaked beads for 42 hours. FGF-beads promote significant numbers of long projections from proximal faces of [CAMv] explants. Analyses of wild-type co-cultures reveals that endothelial projections are DAPI+VE-cadherin+Tie1+ (R,R'). (S,S') Wild-type [CAMv] explant cultured with Fgf10 for 40 hours. Whole-mount immunohistochemical analyses show many long (>90 μm) Tie1+ projections. Dotted circles show explant outline and reference for control process lengths (see J'). (T,U) Transverse sections through [CAMv] explants cultured with/without Fgf10. No significance difference is detected in DAPI+ or pH3+ cells. (V,V') Transverse sections through prosp-NH explants pre-soaked in anti-Fgf3 and anti-Fgf10 antibodies. Fgf10 and Tbx2 are expressed normally (compare with Fig. 2D). (W) Prosp-NH explant pre-soaked in anti-Fgf3 and anti-Fgf10 fails to provoke long process outgrowth from GFP [CAMv] explants, which appear similar to controls (compare with K). Righthand panel shows quantitative analysis. Scale bars: 70 μm in A-E,G-L',J',N-Q,S,S',W; 45 μm in T,U; 25 μm in MM',RR'; 20 μm in F.
Together, these analyses indicate that FGFs deriving from the prosp-NH play a direct role in promoting endothelial growth to the chick NH, but suggest that this role might be transient and partly complemented by other factors during later stages of hypothalamo-hypophyseal development.

Simultaneous innervation and vascularisation of the zebrafish neurohypophysis

To test whether the described roles of FGFs are conserved among vertebrates and to investigate their in vivo relevance, we analysed FGF gene expression profiles and neurohypophyseal innervation and vascularisation in zebrafish. fgf10 is not expressed in the developing hypothalamo-hypophyseal system of the zebrafish, but functions elsewhere (Nechiporuk and Raible, 2008). However, from 18 hours post-fertilisation (hpf), fgf3 is expressed in the ventral hypothalamic floor, extending anteriorly to the prosp-NH (Toro et al., 2009), directly dorsal to AH precursors (Hertzog et al., 2004). At 32 and 33 hpf, fgf3 expression persists in posterior-most cells of the prosp-NH, characterised by co-expression of crabp1a (Liu et al., 2005), and in a stripe of ventral hypothalamic cells just posterior to the prosp-NH (Fig. 5A,D). fgf3 expression becomes restricted to regions posterior to the prosp-NH at 36 hpf and later (Fig. 5B,C,E), indicating a progressive posterior shift of the fgf3 expression domain.

Innervation of the NH starts at 36 hpf, when anti-acetylated Tubulin (AcTub) labels axons that have entered the anterior half of the NH anlage (Fig. 5I,L, compare with 5F for position of NH). By 48 hpf, axons have started to grow posteriorly within the NH (Fig. 5G,J,M), and are present along its entire length by 72 hpf (Fig. 5H,K,N).

NH vascularisation is initiated concomitantly with its innervation. At 72 hpf, Tg(kdrl:Has.HRAS-mCherry) Tg(pomca:GFP) double transgenic fish display a circular blood vessel system at the interface of the AH and the NH. The hypophyseal artery (hya) runs along the midline from anterior to posterior regions of the hypothalamus and splits into two capillary branches (hyc) at the anterior aspect of the pituitary to bilaterally encompass the gland. Directly posterior to the pituitary, the two capillaries fuse with the hypophyseal vein (hyv), which in turn is connected to the primary head sinus (Fig. 5Q; supplementary material Fig. S5) (Isogai et al., 2001). Endothelial cells are already apparent close to anterior regions of the pituitary at 36 hpf (Fig. 5O, arrowhead) and have started to form the hypophyseal artery and bilateral capillaries by 48 hpf (Fig. 5P, arrowheads). In addition, thin endothelial structures, probably contributing to the hypophyseal vein, are apparent close to posterior regions of the pituitary at 48 hpf (Fig. 5P, arrows).

Zebrafish fgf3 mutants lack NH innervation and vascularisation

As a first step to investigate whether Fgf3 signalling is required for zebrafish NH innervation and vascularisation in vivo, we studied fgf3 loss-of-function mutants (Hertzog et al., 2004). In these mutants, most adenohypophyseal cells undergo apoptosis between 28 and 32 hpf (Hertzog et al., 2004), shortly before hypothalamic axons normally reach and enter the NH. In comparison, neurohypophyseal cells are affected much later, as judged by expression of crabp1a. At 33 hpf, fgf3 mutants display normal crabp1a expression in the NH anlage, and crabp1a expression is only moderately reduced at 48 hpf (Fig. 6C,D). Severe reduction/spatial disorganisation of crabp1a expression is observed later, at 72 hpf and 120 hpf (Fig. 6E-H). This suggests that, as in chick (Pearson et al., 2011), the zebrafish NH initially develops independently of Fgf3, but requires it for later steps of its development and/or maintenance (see Discussion).

Despite the presence of crabp1a+ cells, hypothalamic axons fail to enter the NH of fgf3 mutants, but remain in the anterior/dorsal hypothalamus both at 48 hpf (Fig. 6J) and later (Fig. 6K,L). Both TH+ parvocellular and Arginine-vasopressin-like (Avpl) peptide+ magnocellular axons are affected. At 120 hpf, neither TH nor Avpl
peptide is visible in the region containing the NH-remnant (Fig. 6N,P), whereas in wild-type siblings they can be readily detected (Fig. 6M,O). Consistent results were obtained with transient transgenic zebrafish that express eGPF under the control of cis-regulatory elements of the avpl gene (avpl: eGFP). Whereas GFP+ axons project into the NH in wild-type zebrafish (Fig. 6S), such axons are absent in fgf3 mutants (Fig. 6T), although mutants and wild types contain indistinguishable numbers of GFP-positive avpl neurons in the hypothalamus (Fig. 6Q,R). Together, these data indicate that both parvocellular and magnocellular neurosecretory axons require Fgf3 to project to the NH. In addition, fgf3 mutants lack proper vascularisation of the NH. The hypophyseal artery and bilateral capillaries do not form, although the hypophyseal vein remains present (Fig. 6T). This indicates that FGF signalling is required both for the early projection of hypothalamic axons towards the NH and for later extension of axons within the NH. Furthermore, heatshock application at 33 hpf, shortly after the requirement of Fgf3 during AH development, but before the onset of NH innervation (see Fig. 5I), yields embryos with normal Prolactin expression in the AH at 72 hpf, but with much-reduced NH innervation (Fig. 7A,B), comparable to untreated fish at 36 hpf (Fig. 5I). Similarly, application of the heatshock at 42 hpf, after pioneering hypothalamic axons have reached the NH, causes stalling of hypothalamic axons in intermediate positions of the NH at 120 hpf (Fig. 7C,D), resembling the situation in untreated fish at 48 hpf (Fig. 5J). This indicates that FGF signalling is required both for the early projection of hypothalamic axons towards the NH and for later extension of axons within the NH.

The role of zebrafish FGF signalling in NH innervation and vascularisation is independent of its role in AH development

To dissect temporally the effect of Fgf3 on NH innervation and vascularisation from its earlier effect on AH development, we used the transgenic line Tg(hsp70l: dnfgfr1-EGFP), in which a dominant-negative, C-terminally truncated FGF receptor fused to GFP is expressed under the control of the heat-inducible hsp70 promoter, allowing temporal control of transgene activation (Lee et al., 2005). Heatshock application at 33 hpf, shortly after the requirement of Fgf3 during AH development, but before the onset of NH innervation (see Fig. 5I), yields embryos with normal Prolactin expression in the AH at 72 hpf, but with much-reduced NH innervation (Fig. 7A,B), comparable to untreated fish at 36 hpf (Fig. 5I). Similarly, application of the heatshock at 42 hpf, after pioneering hypothalamic axons have reached the NH, causes stalling of hypothalamic axons in intermediate positions of the NH at 120 hpf (Fig. 7C,D), resembling the situation in untreated fish at 48 hpf (Fig. 5J). This indicates that FGF signalling is required both for the early projection of hypothalamic axons towards the NH and for later extension of axons within the NH.
that FGF signalling promoting NH vascularisation is required between 33 and 48 hpf.

Together, these results point to a concomitant role of FGF signalling in NH innervation and vascularisation that is independent of its earlier role in AH development.

**Cell type-specific blockade of FGF signal reception affects both NH innervation and vascularisation**

To determine whether FGF signalling exerts a direct effect, both on hypothalamic neurons projecting to the NH and on endothelial cells contributing to the hypophyseal vascular system, we employed Gal4-UAS transgenesis for specific expression of the dominant-negative Fgfr1-mCherry responder DNA in hypothalamic Avpl neurons, UAS:dnfgr1-mCherry responder DNA was co-injected into tg(avpl:KaIT4A) (attenuated Gal4); tg(otpb::gfp) double-transgenic embryos; for expression in endothelial cells, UAS:dnfgr1-gfp DNA was injected into

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**Fig. 7. FGF signalling affects NH innervation and vascularisation independently of its effect on AH development and via direct effects on hypothalamic neurons and endothelial cells.** Whole-mount immunolabelling (A-D) and confocal in vivo images (E-P) of wild-type (wt) or transgenic fish, as indicated. (A-D) Double-labelled specimens for AcTub and Prolactin. (A,B) Maximal confocal projections at 72 hpf (A,B) or 120 hpf (C,D) in wt or after heatshock-activated dnFgr1 expression at 33 hpf (B) or 42 hpf (D). Prolactin expression in the AH is normal, whereas NH axons appear to stall (compare B with Fig. 5I, and D with Fig. 5J). (E-I) Maximal projections of kdrl-driven mCherry fluorescence in endothelial cells at 72 hpf (E-G) or 75 hpf (H-I). Heatshock-activated dnFgr1 expression at 33 and 48 hpf leads to loss of hypophyseal artery and capillaries, but the hypophyseal vein remains present (compare panel G with E and with Fig. 6T). Single heatshock application at 33 hpf leads to an intermediate vessel phenotype (F), whereas heatshock applications at 48 hpf and 60 hpf leave hypophyseal vascularisation unaffected (compare panel I with H; inset in I shows strong and widespread expression of dnFgr1-GFP). (J-N) Injection of UAS:dnfgr1-mCherry plasmid (JKM) or UAS:mCherry plasmid (L,N) into tg(otpb:egfp)tg(avpl:gal4) double transgenic animals leads to mosaic expression of dominant-negative Fgr1-mCherry fusion protein (UK) or mCherry protein (L) in a subset of pre-optic hypothalamic cells (marked by otpb-driven EGFP expression, green). Avpl+ neurons expressing dnFgr1-mCherry (arrowheads in JK) fail to project axons and to innervate the NH (M), whereas neurons lacking dnFgr1-mCherry display normal axogenesis (JK). Axogenesis (L) and NH innervation (N) are also normal in Avpl+ neurons of control transgenics expressing mCherry (arrows in L). (O-P) Injection of UAS:dnfgr1-egfp plasmid into gll(fli1:gal4),tg(kdrl:mcherry) double transgenic animals leads to mosaic expression of dominant-negative Fgr1-EGFP fusion protein (green) in a subset of endothelial cells (marked by kdrl-driven mCherry expression, red). Contribution of cells expressing dnFgr1 to one of the hypophyseal capillaries abrogates its fusion with the hypophyseal vein (O; arrowhead), whereas the fusion is not affected by cells carrying a UAS:egfp control plasmid (instead of UAS:dnfgr1-egfp) (P; arrows). Arrow in O indicates normally wired non-transgenic endothelial cells. cadi, caudal division of internal carotid artery; hya, hypophyseal artery; hyc, hypophyseal capillary; hyv, hypophyseal vein; nh, neurohypophysis; phs, primary head sinus. Scale bars: 50 μm in A-I,M-P; 15 μm in J-L.

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Hypothalamic (otpb-positive) neurons carrying the UAS:dnfgr1-mCherry transgene fail to extend axons at 36 and 46 hpf (Fig. 7J,K) and to innervate the NH (Fig. 7M) (n=9/9). By contrast, axon formation and NH innervation is normal in otpb neurons carrying a UAS:egfp control transgene (Fig. 7L,N; n=6/6), suggesting that they need to receive FGF signals directly to project towards the NH. Mosaic expression of the UAS:dnfgr1-gfp transgene in endothelial cells results in a reduced contribution of transgenic cells to the hypophyseal blood vessel system (in 12/30 embryos: 40%), compared with cells carrying a UAS:egfp control transgene (in 25/32 embryos: 78%), despite similar contribution rates to other cephalic vessels (supplementary material Fig. S6A,B). Furthermore, when UAS:dnfgr1-gfp cells contribute to one of the two hypophyseal capillaries, this branch fails to fuse with the posteriorly...
located hypophyseal vein, but instead fuses with the opposing capillary (Fig. 7O; n=8/8). Capillaries lacking transgenic cells, or capillaries containing UAS:egfp control cells (n=13/13) fuse in the normal pattern (Fig. 7P). The hypophyseal vein itself, which forms normally in fgf3 mutants (see above; Fig. 6T) is unaffected by incorporated UAS:dnfgfr1-gfp cells (supplementary material Fig. S6C,D). Together, this suggests that direct reception of FGF signals by endothelial cells has an impact on their recruitment to the NH vasculature and on the wiring of the system. However, the direct effect on endothelial cell recruitment is not absolutely essential, but is complemented by indirect mechanisms and other (Fgf3-dependent) factors (see Discussion).

**DISCUSSION**

The functioning of neurohemal organs depends on the intimate association of axonal terminals with vascular capillaries. Many studies have pointed to the common usage of guidance cues in the directed growth of axons and blood vessels (Carmeliet and Tessier-Lavigne, 2005; Adams and Eichmann, 2010). Here, we investigated whether Fgf3 and Fgf10 might display such a dual role during the formation of the neurohypophyseal neurohemal system to ensure proper convergence of hypothalamic nerves and capillaries. Our studies reveal a direct effect of FGFs in H-NH axonal guidance. Fgf3 and Fgf10 exert a chemotropic effect on hypothalamic axons in chick explant cultures, and analyses of zebrafish fgf3 mutants and transgenic fish after temporally controlled or cell type-specific blockade of FGF signalling reveal a direct and indispensible effect of FGFs on hypothalamic axons in vivo. By contrast, our studies suggest that, although FGFs can exert a direct effect on endothelial cells, this role is not absolutely essential to the formation of a H-NH vasculature. Thus, FGFs secreted from the chick NH can promote endothelial cellular outgrowth in vitro, but this effect is weak. Consistent with this, in zebrafish, endothelial cells expressing a dominant-negative FGF receptor display a reduced, but not completely abrogated, contribution to the hypophyseal blood vessels that are missing in fgf3 mutants. These results, together with the demonstration that Otx1 released by neurohypophyseal nerve endings is required for proper hypophyseal vascularisation (Guttick et al., 2011), suggest a multi-step model in which FGFs have direct effects both on NH innervation and on NH vascularisation, but in which vascularisation involves the concerted action of FGFs and hormones from (FGF-dependent) neurohypophyseal axon terminals, thereby ensuring proper integration of the neuro-vascular wiring of this neurohemal organ.

**NH-derived FGFs mediate a direct chemotrophic effect on developing H-NH axons**

Molecular gradients play a pivotal role in directing axonal growth (Charron and Tessier-Lavigne, 2007; Sánchez-Camacho and Bovolenta, 2009; Chédotal and Richards, 2010). Although not as well-studied as other gradient-forming ligands, FGFs may operate as a gradient cue (Irving et al., 2002; Shirasaki et al., 2006) and can govern axonal extension (McFarlane et al., 1995; Szebenyi et al., 2001; Gill et al., 2004; Webber et al., 2005; Shirasaki et al., 2006). Early reports, moreover, suggest a role for FGF signalling in H-NH pathfinding, with compromised FGF signal transduction leading to defects in GnRH axonal extension (Gill and Tsai, 2004).

Our studies support and extend these observations, suggesting that an FGF ligand gradient forms in the hypothalamus that directs nascent H-NH axons towards their target area, the forming NH. In the chick, the FGF7 subfamily members Fgf3 and Fgf10 are expressed appropriately, confined to the forming NH when pioneering H-NH axons extend. In vitro, point-sources of Fgf3 or Fgf10 exert a chemotrophic effect on chick H-NH axons, and can stimulate and re-orient their growth. In vivo, the NH exerts a chemotrophic effect on H-NH axons. Temporal blockade of NH-derived Fgf3 or Fgf10 results in a significant decrease in the directed growth of H-NH axons; this effect is potentiated by simultaneous blockade of Fgf3 and Fgf10. Abrogation of FGF signalling does not lead to a simultaneous decline in progenitor cells or differentiated neurons. Thus, although we cannot exclude some involvement of other factors (as small numbers of short fascicles continue to emerge in the presence of Fgf3- and Fgf10-blocking antibodies), our studies suggest that Fgf3 and Fgf10 from the forming NH play direct and indispensable roles in attracting H-NH axons towards the midline.

Our in vivo work in the zebrafish substantiates these results and suggests a conserved role for FGFs in guiding H-NH axons. Zebrafish fgf3 is expressed in the ventral hypothalamus, its profile similar to that of its close relative Fgf10 in the chick and mouse. Vp neurons differentiate in fgf3 zebrafish mutants, but Vp axons fail to project to the NH. Similar results were obtained for Oxytocin+ magnocellular neurons (Guttick et al., 2011) (H-M.P. and M.H., unpublished data) and for TH+ parvocellular cells. The innervation defects in fgf3 mutants are apparent at a time when the NH itself appears unaltered, making it unlikely that these are a secondary consequence of compromised NH development. Rather, the NH defects seen in later-stage fgf3 mutants might be secondary consequences of a late requirement for FGF signalling in NH maintenance (Pearson et al., 2011) and/or a consequence of the failed innervation and/or neurohormone release, consistent with data reporting effects of Vp release on pituitary morphology (George et al., 1987; Rosso et al., 2004). Using heatshock-inducible temporally controlled transgenic expression of a truncated, dominant-negative version of Fgfr1, which cross-reacts with all FGF receptor subtypes (Lee et al., 2005), we further show that the innervation and vascularisation defects of the NH are independent of the earlier role of Fgf3 in AH specification (Herzog et al., 2004). Finally, lack of NH innervation specifically was also seen in mosaic transgenic embryos for hypothalamic neurons expressing the dominant-negative FGF receptor under the control of a Vp-specific promoter. In sum, these in vitro and in vivo data strongly suggest that the chemotrophic effect of Fgf3 and Fgf10 on magnocellular axons is direct.

**A dose-dependent dual function for FGFs in NH innervation**

Our studies in chick suggest a dual role for FGFs in regulating H-NH axonal projections, attracting and facilitating growth at lower concentrations, but stalling repelling growth at high concentrations. Chick Fgf10 and zebrafish fgf3 are expressed in a graded fashion, with highest levels in the posterior-ventral NH, anticipating the anterior-to-posterior innervation of the NH. Strikingly, axons initially avoid regions that display the highest and most persistent fgf3 and Fgf10 expression levels, in line with a dual role. We speculate that this provides a mechanism to ensure that H-NH axons project to, but do not cross, the ventral midline; uniquely in this region of the CNS, axons are non-commissural (see Chédotal, 2011). Our studies raise the possibility that H-NH growth cones integrate FGF signalling over space or time (Dessaud et al., 2008), with low levels of FGF signalling attracting them towards the NH, and higher levels stalling their growth.

**FGFs regulate NH vascularisation in partial functional redundancy with other factors**

Early in development, both the chick and the zebrafish prosop-NH are surrounded by isolated endothelial cells that subsequently
assemble to capillary-like structures. This suggests that rather than, or in addition to, angiogenesis (vessel formation via sprouting of pre-existing blood vessels), NH vascularisation is driven by vasculogenesis (the de novo formation of vessels via the assembly of endothelial cells, which subsequently connect to the existing vascular system).

Our studies reveal both a direct and an indirect contribution of FGFRs to NH vascularisation. Chick [CAMv] explants respond to the NH with enhanced outgrowth of endothelial cell projections. This effect is abrogated by pre-exposure of the prosp-NH to Fgf3- and Fgf10-blocking antibodies; conversely, Fgf3 and Fgf10 mimic the ability of the prosp-NH to promote endothelial outgrowth. However, blockade of Fgf3 and Fgf10 only eliminates E4 prosp-NH-stimulated endothelial outgrowth. By contrast, blockade is incomplete for E6 NH explants, when the NH is already innervated, indicating a transient FGFR requirement and the presence of other vasculatisation-promoting factors in the innervated NH. In zebrafish, fgf3 mutants lack both hypothalamic axonal projections and the hypophyseal artery and capillaries. However, transgenic endothelial cells expressing the dominant-negative FGFR receptor driven by the cell type-specific flila promoter do contribute to pituitary vessel formation, albeit with lower frequencies than do cells carrying a control transgene. This indicates that direct reception of FGFR signalling by endothelial cells has an impact on their recruitment to the pituitary vessel system, but is not essential. FGFR signal reception by endothelial cells contributing to the hypophyseal capillaries seems to be necessary for proper wiring of the hypophyseal capillaries to the hypophyseal vein, possibly pointing to a crucial role of FGFR signalling during the remodelling of the initial capillary plexus to a properly connected and closed vessel system.

Together, our in vitro and in vivo data indicate that although direct FGFR signalling does stimulate NH vascularisation, this function is partly redundant with other vasculatisation-stimulating factors, possibly neuropeptides released from H-NH axonal termini, as recently revealed for Oxl in the zebrafish (Gutnick et al., 2011) (see above).

Acknowledgements
We thank P. Ellis and I. Riedl-Quinkertz for technical help; Marc Tessier-Lavigne for helpful discussions; Markus Affolter, Wiebke Herzog, Shlomo Melmed, Joshua Bonkowsky and Kenneth Poss for transgenic zebrafish lines; Chi-Bin Chien for the Tol2kit plasmid vectors; R. Köster for the pCSKalTA4GI plasmid; and K. Kawakami for the iTol2-Amp construct.

Funding
This work was supported by the UK Medical Research Council [GO401310, G0501618 to M.P.]; the Wellcome Trust [077544 to M.P.]; and the German Research Foundation (DFG) [SFB 572; to M.H.]; Deposited in PMC for immediate release.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at http://dev.biologists.org具体内容被省略。


Fig. S1. Nissl staining of the E7 chick hypothalamus. (A-X) Nissl staining of serial transverse sections (15 μm) through the chick hypothalamus at E7 from anterior (A) to posterior (X). Anterior-most sections are from optic chiasm regions (oc, A). The anterior pituitary/adenohypophysis (ap/ah) can be identified beneath the ventral midline of the anterior hypothalamus (B-I), terminating close to the ME (me, I). The evagination of the NH can be identified (black arrows, K-M). Posterior to the NH, the recess of the third ventricle widens (N-T) then narrows (V-X). Scale bar: 200 μm.
Fig. S2. Axons project to the Fgf10+ NH, but do not initially project deep into it. (A,D) Scanning electron micrograph of E7 (A) or E10 (D) chick NH to indicate plane of section in B and view in E. In A, anterior is to the left; dorsal, up. In D, green arrow points to ventral side of NH. (B) In situ hybridisation analysis shows that Fgf10 is expressed throughout the NH, with highest levels in posterior-ventral regions. (C) The first Vp+ neurons differentiate at E5.5-E6. (E) Ventral view of isolated hypothalamus: at E10, TUJ1+ axons project beneath/into the ventral-most NH. (F,G) High-powered view of an E4 GFP-transplanted [L] graft (see Fig. 2G) analysed after 2 (F) and 3 (G) days of culture. Axons grow rapidly towards the NH (E6), turning towards it, but slow/stall as they enter, and do not initially project rapidly deeper into the NH (asterisk). Dotted outline marks NH (nh). (H) E4 GFP-transplanted [L] graft (see Fig. 2G) cultured for 2 days in the presence of Fgf3 and Fgf10 blocking antibodies. No axons project from the graft. Scale bar: 150 μm in A,B,F-H; 70 μm in C-E.
Fig. S3. FGF signalling promotes axonal re-orientation and growth without affecting cell numbers. Effects of FGFs and FGF inhibitors on chick lateral neuronal [L] explants. (A,B) Axon outgrowth is promoted by an Fgf10- but not a PBS-soaked bead. (C) The neurofilament marker 3A10 is detected in [L] explants cultured with a PBS-soaked bead (n=5); all axons remain confined within the explant. (D) 3A10 is similarly detected within [L] explants cultured with an Fgf10 bead but is also detected on axons that have emerged and extended towards the bead (n=5). Quantitative analysis reveals no apparent difference in the numbers of 3A10+ cells (137.6±12.75 with PBS beads versus 116.4±11.50 with Fgf10-beads). Arrows in C and D point to region of [L] explant with little neurofilament labelling: consistently, axons initially extend away from this region. (E-H,O) Transverse section (E) through E4 medial hypothalamus, showing expression of the transcription factor Nkx2.1 in the ventral and lateral medial hypothalamus (co-expressed with Shh in lateral regions). [L] explants cultured alone (F), with Fgf10 (G) or with SU5402 (O) show statistically similar numbers of Nkx2.1+ progenitor cells (H). (I-L,P) Transverse section (I) through E5 medial hypothalamus, showing expression of TH in lateral regions. [L] explants cultured alone (J), with Fgf10 (K) or with SU5402 (P) show similar numbers of TH+ cells. (M,N) SU5402 has no effect on axon outgrowth (M) or on 3A10 expression within [L] explants (N). Error bars represent s.e.m. Scale bars: 100 μm in A-D,I,M,N; 50 μm in F,G,J,K,O,P.
Fig. S4. E6 prosp-NH stimulates weak outgrowth in an FGF-independent manner in chick. (A,B) GFP [CAMv] explants co-cultured for 40 hours with E6 prosp-NH either untreated (A) or pre-soaked in anti-Fgf3 and anti-Fgf10 (B). A few long endothelial processes emerge from the [CAMv] explant in response to E6 prosp-NH; however, numbers are lower than upon co-culturing with E4 prosp-NH (compare Fig. 4L). Pre-treatment of E6 prosp-NH with anti-Fgf3 and anti-Fgf10 leads to a reduction of endothelial outgrowth (compare A and B), which, however, is not completely blocked, in contrast to the effect caused by anti-Fgf3 and anti-Fgf10 on E4 prosp-NH (compare B with Fig. 4W). Error bars represent s.e.m. **P<0.005; ***P<0.001. d, distal; p, proximal. (C) Immunohistochemical analysis post-culture reveal that E6 prosp-NH explants contains some TH⁺ axon termini. Scale bars: 100 μm in A,B; 50 μm in C.
Fig. S5. Schematic summarizing microangiography analyses of zebrafish ventral brain vessels at 84 hpf. Ventral view; modified from Isogai et al. (Isogai et al., 2001). Inset shows magnified view of hypophyseal region. At 84 hpf, the basal communicating artery shown in Fig. 5O,P at 36 and 48 hpf is positioned more dorsally and not included in this scheme. cadi, caudal division of internal carotid artery; hya, hypophyseal artery; hyc, hypophyseal capillary; hyv, hypophyseal vein; phs, primary head sinus.

Fig. S6. Endothelial cells expressing the dominant-negative Fgfr1 receptor contribute normally to other cephalic blood vessels and do not affect morphogenesis of the hypophyseal vein. (A-D) Confocal in vivo images of different transgenic fish, as indicated; 100 hpf; ventral views, anterior to the left. Injection of the UAS:egfp control (A) or the UAS:dnfgfr1-egfp plasmid (B,D) into tg(fli1a:gal4); tg(kdrl:mcherry) double transgenic animals leads to mosaic expression of EGFP (A) or the truncated Fgfr1 receptor fused to EGFP in endothelial cells. For simplicity, the red channel is omitted in A and B, and the green channel omitted in C. (A,B) Endothelial cells with activation of the UAS-egfp transgene (A) or the UAS:dnfgfr1-egfp transgene (B) display comparable contributions to cephalic blood vessels. Nevertheless, their contributions to the hypophyseal artery and the two branches encompassing the pituitary differ (80% versus 40%; data not shown). (C,D) Morphogenesis of the hypophyseal vein is unaffected by endothelial cells expressing the UAS:dnfgfr1-egfp transgene. cadi, caudal division of internal carotid artery; e, eye; hyv, hypophyseal vein; phs, primary head sinus. Scale bars: 50 μm.
Table S1. Quantitative analyses of lateral neuronal [L] vessel explants cultured in a 3-days matrix with hypothalamic tissue, or protein-soaked beads, alone, or with FGF inhibitors

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Number of axon fascicles:</th>
<th>Number of axon fascicles:</th>
</tr>
</thead>
<tbody>
<tr>
<td>(neuronal explants)</td>
<td></td>
<td>Proximal face (i)</td>
<td>Distal face (ii)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L alone</td>
<td>24</td>
<td>2.0 ± 0.25</td>
<td>1.7 ± 0.29</td>
</tr>
<tr>
<td>L + prosp-NH</td>
<td>20</td>
<td>27.4 ± 1.2</td>
<td>3.0 ± 0.33</td>
</tr>
<tr>
<td>L + Fgf10 bead</td>
<td>15</td>
<td>31.6 ± 3.3</td>
<td>2.8 ± 0.35</td>
</tr>
<tr>
<td>L + Fgf3 bead</td>
<td>12</td>
<td>27.0 ± 3.2</td>
<td>2.2 ± 0.23</td>
</tr>
<tr>
<td>L + Fgf8 bead</td>
<td>10</td>
<td>2.8 ± 0.38</td>
<td>2.3 ± 0.34</td>
</tr>
<tr>
<td>L + PBS bead</td>
<td>20</td>
<td>2.4 ± 0.27</td>
<td>2.2 ± 0.47</td>
</tr>
<tr>
<td>L + Fgf10 bead + SU5402</td>
<td>10</td>
<td>3.1 ± 0.29</td>
<td>2.5 ± 0.35</td>
</tr>
<tr>
<td>L + Fgf3 bead + SU5402</td>
<td>12</td>
<td>2.0 ± 0.48</td>
<td>2.1 ± 0.46</td>
</tr>
<tr>
<td>L + prosp-NH + SU5402</td>
<td>15</td>
<td>8.3 ± 0.36</td>
<td>2.9 ± 0.17</td>
</tr>
<tr>
<td>L + prosp-NH + anti-Fgf3</td>
<td>10</td>
<td>18.9 ± 1.2</td>
<td>1.8 ± 0.39</td>
</tr>
<tr>
<td>L + prosp-NH + anti-Fgf10</td>
<td>10</td>
<td>15.6 ± 3.1</td>
<td>2.0 ± 0.46</td>
</tr>
<tr>
<td>L + prosp-NH + anti-Fgf3/10</td>
<td>10</td>
<td>7.2 ± 2.8</td>
<td>3.1 ± 0.65</td>
</tr>
<tr>
<td>L + Fgf10 bead (high)</td>
<td>5</td>
<td>6.6 ± 0.9</td>
<td>23.0 ± 3.0</td>
</tr>
</tbody>
</table>

The mean number of axon fascicles (±s.e.m.) emerging from proximal and distal faces is shown. Maximal outgrowth, quantitatively similar to that evoked by prosp-NH explants, is promoted by beads soaked in 50-100 ng/μl Fgf10 and 100-200 ng/ml Fgf3. Repulsive effects occur in response to three- to fivefold higher levels of Fgf10 (300-500 ng/μl), but are not detected in response to Fgf3 (300-1000 ng/μl). n, number of explants analysed after 48 hours.
### Table S2. Quantitative analyses of [CAMv] explants cultured in a 3-days matrix alone, with FGF proteins or with FGF inhibitors

<table>
<thead>
<tr>
<th>Groups (vessel explants)</th>
<th>n</th>
<th>Mean number of processes Mean length of processes</th>
<th>Mean number of processes Distal face</th>
</tr>
</thead>
<tbody>
<tr>
<td>[CAMv] alone</td>
<td>20</td>
<td>40.2 ± 4.5</td>
<td>38.7 ± 5.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>68.4 ± 4.2 (0%)</td>
<td>69.2 ± 5.6 (0%)</td>
</tr>
<tr>
<td>[CAMv] + E4 prosp-NH</td>
<td>6</td>
<td>45.6 ± 3.2</td>
<td>40.1 ± 3.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>148 ± 5.6 (26%)</td>
<td>70.5 ± 4.3 (0%)</td>
</tr>
<tr>
<td>[CAMv] + E4 prosp-NH</td>
<td>6</td>
<td>38.2 ± 3.5;</td>
<td>41.7 ± 4.4</td>
</tr>
<tr>
<td>(anti-Fgf3/anti-Fgf10)</td>
<td></td>
<td>78.4 ± 4.1 (0%)</td>
<td>89.2 ± 5.2 (0%)</td>
</tr>
<tr>
<td>[CAMv] + E6 prosp-NH</td>
<td>6</td>
<td>66.7 ± 4.2</td>
<td>60.1 ± 3.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>121.5 ± 75.6 (9%)</td>
<td>75.8 ± 5.3 (0%)</td>
</tr>
<tr>
<td>[CAMv] + E6 prosp-NH</td>
<td>6</td>
<td>58.2 ± 6.5;</td>
<td>51.7 ± 5.4</td>
</tr>
<tr>
<td>(anti-Fgf3/anti-Fgf10)</td>
<td></td>
<td>72.4 ± 3.1 (7%)</td>
<td>77.2 ± 6.2 (0%)</td>
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<tr>
<td>[CAMv] + Fgf10</td>
<td>15</td>
<td>46.2 ± 5.2</td>
<td>42.1 ± 3.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>186.2 ± 6.7 (62%)</td>
<td>188.6 ± 7.1 (68%)</td>
</tr>
<tr>
<td>[CAMv] + PBS bead</td>
<td>15</td>
<td>47.1 ± 3.5</td>
<td>45.6 ± 4.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>73.5 ± 5.6 (0%)</td>
<td>72.5 ± 4.3 (0%)</td>
</tr>
<tr>
<td>[CAMv] + Fgf10 bead</td>
<td>15</td>
<td>51.2 ± 6.3</td>
<td>39.8 ± 4.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>142.4 ± 7.8 (65%)</td>
<td>68.3 ± 4.3 (0%)</td>
</tr>
<tr>
<td>[CAMv] + Fgf3 bead</td>
<td>15</td>
<td>45.8 ± 3.2</td>
<td>37.2 ± 6.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>136.3 ± 6.3 (61%)</td>
<td>43.3 ± 3.2 (0%)</td>
</tr>
<tr>
<td>[CAMv] + Fgf10 bead (300-500 ng/µl)</td>
<td>15</td>
<td>43.9 ± 3.7</td>
<td>47.2 ± 3.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>141 ± 8.2 (59%)</td>
<td>40.2 ± 4.1 (0%)</td>
</tr>
</tbody>
</table>

The mean number of processes emerging from proximal and distal faces is indicated, followed by the mean process length; parentheses indicate percentage of processes with length >90 μm. Beads soaked in 50-100 ng/ml Fgf10 or in 300-500 ng/ml Fgf10 evoked a similar response. n, number of explants analysed after 48 hours.