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VEGF signalling controls GnRH neuron survival via NRP1 independently of KDR and blood vessels

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SUMMARY

Gonadotropin-releasing hormone (GnRH) neurons are neuroendocrine cells that are born in the nasal placode during embryonic development and migrate through the nose and forebrain to the hypothalamus, where they regulate reproduction. Many molecular pathways that guide their migration have been identified, but little is known about the factors that control the survival of the migrating GnRH neurons as they negotiate different environments. We previously reported that the class 3 semaphorin SEMA3A signals through its neuropilin receptors, NRP1 and NRP2, to organise the axons that guide migrating GnRH neurons from their birthplace into the brain. By combining analysis of genetically altered mice with in vitro models, we show here that the alternative neuropilin ligand VEGF164 promotes the survival of migrating GnRH neurons by co-activating the ERK and AKT signalling pathways through NRP1. We also demonstrate that survival signalling relies on neuronal, but not endothelial, NRP1 expression and that it occurs independently of KDR, the main VEGF receptor in blood vessels. Therefore, VEGF164 provides survival signals directly to developing GnRH neurons, independently of its role in blood vessels. Finally, we show that the VEGF164-mediated neuronal survival and SEMA3A-mediated axon guidance cooperate to ensure that migrating GnRH neurons reach the brain. Thus, the loss of both neuropilin ligands leads to an almost complete failure to establish the GnRH neuron system.

KEY WORDS: GnRH neuron, Neuronal survival, VEGF, KDR, VEGFR2, FLK1, Neuropilin, Mouse

INTRODUCTION

Gonadotropin-releasing hormone (GnRH) neurons are neuroendocrine cells that are generated in the nasal placode from embryonic day (E) 10.5 onwards in mice, migrate along olfactory and vomeronasal nerves into the forebrain and then follow the caudal branch of the vomeronasal nerve to reach the hypothalamus by the time of birth (Wray et al., 1989). These cells regulate sexual reproduction by projecting to the median eminence, where they secrete the decapeptide GnRH into the pituitary portal vessels to induce the release of gonadotropins into the general circulation (Merchenthaler et al., 1984). In humans, failure of GnRH neurons and vomeronasal axons to reach the hypothalamus results in infertility and causes Kallmann syndrome (KS), a genetic disease characterised by hypogonadotropic hypogonadism (HH) and anosmia (Hardelin, 2001). Mutations in five genes have so far been identified in patients with KS, but they account for only ~30% of clinical cases (Hardelin and Dode, 2008), implying that additional, unknown genes regulate GnRH neuron development and function in humans.

In mice, most of the molecules reported to play essential roles in GnRH neuron development regulate their migration (Cariboni et al., 2011; Giacobini et al., 2008) (for a review, see Cariboni et al., 2007b). By contrast, there is a paucity of information about the factors that ensure their survival in the different tissue environments encountered during their migration. Thus, mice lacking a naturally truncated form of the p73 transcription factor, which regulates apoptosis of several neuronal cell types, contain only a few GnRH neurons in the hypothalamus (Tissir et al., 2009). However, it is not known whether this absence of GnRH neurons is due to cell-autonomous dependence on p73, or whether abnormal migration of these cells along mispatterned vomeronasal axons is responsible for their failure to reach the hypothalamus (Tissir et al., 2009). The combined loss of the related receptor tyrosine kinases AXL and TYRO3 reduces the number of GnRH neurons in the hypothalamus of adult mice by 10%, but this mild phenotype is likely to be caused by a combination of reduced neuronal survival and faulty migration during embryogenesis (Pierce et al., 2008).

We have shown previously that primary GnRH neurons express NRP1 (Cariboni et al., 2007a), a shared receptor for two distinct types of secreted proteins: the class 3 semaphorin SEMA3A, a classical axon guidance molecule, and the VEGF164 isoform of vascular endothelial growth factor (VEGF), a potent inducer of blood vessel growth (for a review, see Ruhrberg, 2003). We also reported that SEMA3A signals through NRP1 or NRP2 to guide vomeronasal axons and, therefore, migrating GnRH neurons into the developing brain (Cariboni et al., 2011). By contrast, the physiological role of VEGF164 in GnRH neuron development has not yet been explored.

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Even though VEGF is best known for its ability to induce the growth of blood vessels to support the metabolic demands of both developing and adult organs, recent studies demonstrated that VEGF also promotes neuronal migration in the embryonic brain stem (Schwarz et al., 2004) and cerebellum (Ruiz de Almodovar et al., 2010), as well as commissural axon guidance at the optic chiasm (Erskine et al., 2011) and in the spinal cord (Ruiz de Almodovar et al., 2011). Moreover, in vitro experiments raised the possibility that VEGF promotes neurogenesis and neuronal survival by signalling through the VEGF receptor KDR (also known as FLK1 or VEGFR2) (for reviews, see Rosenstein et al., 2010; Ruiz de Almodovar et al., 2009). Yet, the ablation of KDR in the neural progenitors that give rise to CNS neurons and glia did not obviously impair brain growth (Haigh et al., 2003). Further in vivo experiments are therefore required to establish whether VEGF affects neurogenesis and neuronal survival by directly acting on neuronal VEGF receptors or if, instead, VEGF-stimulated blood vessels release neurotrophic factors.

Here, we have investigated the physiological role of VEGF164 signalling in GnRH neuron development and found that it plays an important role in their survival. By combining the analysis of genetic mouse models deficient in VEGF164 or its receptors with cell biological and biochemical methods, we found that VEGF164/NRP1 signalling promotes GnRH neuron survival cell-autonomously, independently of its role in blood vessels and by co-activating AKT and ERK pathways. Unexpectedly, VEGF-mediated survival signalling required the isoform-specific VEGF164 receptor NRP1, but not the pan-VEGF receptor KDR. Finally, we demonstrated that the combined loss of SEMA3A and VEGF164 led to a nearly complete failure in the development of the GnRH neuron system, suggesting that both neuropilin ligands cooperate to ensure that migrating neurons are neither misguided nor perish on their journey from the nasal placode to the hypothalamus.

MATERIALS AND METHODS

Mouse strains and ethics statement

All animal work was conducted according to local ethical and UK home office guidelines. We used *Nrp1*-null (Kitsukawa et al., 1997; Schwarz et al., 2004), *Vegfa*^{lacZ} (Miquerol et al., 1999), *Vegfa*$^{120/120}$ (Carmeliet et al., 1999), *Sema3a*-null (Taniguchi et al., 1997), floxed conditional *Nrp1*-null (Gu et al., 2003), floxed conditional *Kdr* mutants (a kind gift of Tom Sato, UT Southwestern, Texas, USA) as well as *Kdr*-null mutants (Shalaby et al., 1995) and mice lacking the NRP1 cytoplasmic domain (*Nrp1*$^{cyto\Delta/\Delta}$; Q.S., A. Fantin and C.R., unpublished). Conditional null mutants were mated to *Tie2-Cre* and/or *Nes-Cre* transgenic mice (Kisanuki et al., 2001; Petersen et al., 2002; Gu et al., 2003; Haigh et al., 2003).

Antibodies

Samples were immunostained with the following primary antibodies: rabbit anti-peripherin (Chemicon; 1:500), anti-GnRH (Immunostar; 1:400), anti-phosphohistone 3 (Upstate; 1:2000) or anti-activated caspase 3 (R&D Systems; 1:150); mouse anti-neuronal specific beta 3 tubulin (Covance; 1:5000) or goat anti-rat NRP1 or KDR (R&D Systems; 1:150). The NRP1 and KDR antibodies used for immunostaining and function-blocking experiments have been tested previously for specificity and functionality on knockout tissue and in endothelial growth assays (Fantin et al., 2010; Gerhardt et al., 2003; Erskine et al., 2011). Blood vessels were labelled with Alexa488-conjugated isolectin B4 (IB4; 1:400). Nuclei were counterstained with DAPI or Hoechst fluorochrome (Sigma). For immunoblotting, we used rabbit anti-pAKT (Ser 473; 1:100), mouse anti-AKT (1:150), mouse anti-pERK1/2 (Tyr 204; 1:150) and rabbit anti-ERK1/2 (1:1000), followed by horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Santa Cruz Biotechnology). Images were acquired with an MZ16 microscope (Leica) equipped with a ProgRes C14 digital camera (Jenoptiks, Jena, Germany) and OpenLab 3.5.1 software (Improvision), or with a Zeiss LSM510 confocal microscope and then processed with Adobe Photoshop CS4.

Beta-galactosidase staining

To visualise expression of the *Vegfa*^{lacZ} reporter, β-galactosidase activity was detected with the X-gal substrate (Sigma), as previously described (Miquerol et al., 1999).

RT-PCR

Total RNA from dissected nasal explants at different embryonic stages, GN11 cells and FACS-sorted GFP-GnRH neurons was isolated using RNeasy Micro Kit (Qiagen). Single-strand cDNA was synthesised with AMV reverse transcriptase and random hexamers (Promega). PCR was carried out using 0.5 μl cDNA, Taq DNA polymerase (Qiagen, UK) and the following oligonucleotide primers: *Vegfa*, 5′-CTGCTCTCTTG-GGTCCACTGG-3′ and 5′-CACCGCCTTGGCTTGTCACAT-3′; *Kdr*: 5′-AGCTTGGCTCACAGGCAACATCGG-3′ and 5′-CCTACGGACC-GTTAAGCGGGCCA-3′.

Proliferation and survival assays

GN11 cells were seeded in 24-well plates at a density of 5000 cells/well and grown for 24 hours in standard conditions (Dulbecco's MEM containing 1 mM sodium pyruvate, 100 mg/ml streptomycin, 100 U/ml penicillin and 10% FBS; Invitrogen). For proliferation assays, cells were then cultured overnight without serum and then for 24, 48 or 72 hours in the presence of 0.2% FBS with or without 10 ng/ml mouse recombinant VEGF164 (Peprotech). We then determined the number of mitotic nuclei (identified with rabbit anti-phosphohistone 3 antibody followed by a goat anti-rabbit Alexa488-conjugated secondary antibody), relative to the total number of DAPI-positive nuclei. Similar experiments were performed in the absence of FBS. For survival assays, cells were serum-starved for 72 hours and then grown for a further 12 hours in serum-free medium in the absence or presence of 10 ng/ml VEGF164. We determined the number of non-viable, propidium iodide (PI)-labelled cells relative to the total number of Hoechst fluorochrome-positive cells. In some experiments, 1 mg/ml control IgG, function-blocking goat anti-NRP1 or anti-KDR antibodies (R&D Systems) were added 1 hour prior to VEGF164 treatment. In other experiments, the PI3K inhibitor LY294 or the MAPK inhibitor U0126 (Sigma) were added at a concentration of 10 μM 1 hour prior to addition of VEGF164. All assays were repeated in three independent experiments.

Quantification and statistical analysis

To determine the total number of GnRH neurons in all mouse lines analysed, 25 μm sagittal sections through the whole head were cut and stained with GnRH antibody, as previously described (Cariboni et al., 2011). For each genotype, we analysed at least three embryos, and all GnRH-positive cells were counted in each section of each head under a 40× objective. To compare the relative number of apoptotic cells in the nasal area of *Vegfa*$^{120/120}$ mutants and wild-type littermates (three each), we determined the number of cells positive for activated caspase 3 in five adjacent 20 μm sagittal sections through the nose at the level of vomeronasal/olfactory axons. For proliferation and survival assays in GN11 cells, we determined the percentage of phosphohistone 3 (pH3)- or PI-positive cells out of all Hoechst-positive cells for each treatment group in 12 random pictures from three independent experiments. For immunoblotting, three independent experiments were performed for each condition, and optical density of the blots was measured with Image J. For all experiments, data are expressed as mean ± standard error of the mean. To determine statistical significance, we used a paired *t*-test with $P<0.05$ (one asterisk) considered to be statistically significant. Statistical analysis was performed using Prism4 software (GraphPad Software).

RESULTS

NRP1 is co-expressed on migrating GnRH neurons, nasal axons and blood vessels

GnRH neurons isolated by fluorescence-activated cell sorting (FACS) from embryonic GFP-GnRH transgenic mice express NRP1 (Cariboni et al., 2007a). To localise NRP1 expression in situ,

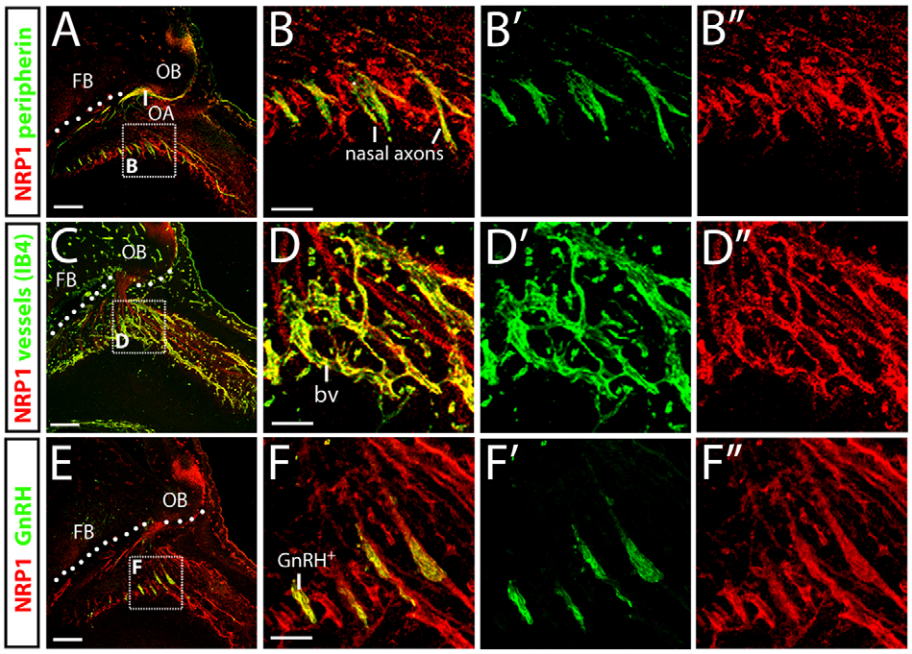

Fig. 1. NRP1 is expressed by olfactory and vomeronasal axons, blood vessels and GnRH neurons. (**A-F″**) Contiguous sagittal sections of E12.5 mouse heads were double labelled for NRP1 and the olfactory/vomeronasal axon marker peripherin (A,B), the blood vessel marker IB4 (C,D) and GnRH (E,F). Higher magnifications of the squared areas in A, C and E are shown in B, D and F, respectively. Single channels are shown in B′,B″,D′,D″,F′,F″. bv, blood vessels; FB, forebrain; OA, olfactory axons; OB, olfactory bulb. Scale bars: 150 μm in A,C,E; 50 μm in B,D,F.

we stained contiguous sagittal sections of E12.5 mouse heads by double label immunofluorescence with antibodies specific for NRP1 (Fantin et al., 2010; Ruiz de Almodovar et al., 2010) and GnRH (Cariboni et al., 2011; Cariboni et al., 2007a) (Fig. 1). Consistent with our previous finding that NRP1 cooperates with NRP2 to pattern vomeronasal and olfactory axons (Cariboni et al., 2011), we observed NRP1 on peripherin-positive axons in the nasal compartment (intermingled nasal and vomeronasal axons; Fig. 1A,B). As expected from previous studies on vascular patterning (Gerhardt et al., 2004; Kawasaki et al., 1999), we also detected NRP1 on blood vessels in the nasal compartment and forebrain (Fig. 1C,D). In addition, NRP1 was present on GnRH neurons migrating in the nasal compartment (Fig. 1E,F). These observations raised the possibility that NRP1 contributes to GnRH neuron development either by controlling the growth of neurotrophic blood vessels or by acting directly in these cells, perhaps to control their proliferation or survival.

Role for NRP1 in GnRH neuron development

To assess whether NRP1 plays a role in GnRH neuron development, we studied mice carrying the *Nrp1*-null mutation on a genetic background (JF1) that allows homozygous null mutants to survive the early phase of cardiovascular development (Schwarz et al., 2004). We analysed these mice at E14.5, when migrating GnRH neurons can be identified in the nose, at the nasal/forebrain junction and in the basal forebrain (Fig. 2A). This is also the latest stage at which *Nrp1*-null mutants are viable. Immunohistochemical analysis of sagittal sections through the entire head of mutant and wild-type littermates with GnRH antibody showed a significant reduction in the number of GnRH-positive cells in the heads of mutants compared with their wild-type littermates (Fig. 2B-D; $Nrp1^{+/+}$ 1197±69, n=4 vs $Nrp1^{-/-}$ 833±19, n=5; P<0.01; heterozygous $Nrp1^{+/-}$ mice contained a normal number of GnRH neurons). Importantly, a similar proportion of GnRH neurons were located in the forebrain of wild-type and mutant littermates at this stage ($Nrp1^{+/+}$ 504±37, corresponding to 41% of the total number vs $Nrp1^{-/-}$ 326±19, corresponding to 39% of the total number). The finding that the proportion of GnRH neurons was similar in both genotypes suggests that there is no significant delay in forebrain entry in these mutants. In agreement with the finding that GnRH neurons are able to enter the forebrain in a timely fashion, immunohistochemistry for peripherin showed that vomeronasal axons projected normally into the forebrain in three out of three of these mutants (see Fig. S1 in the supplementary material). NRP1 is, therefore, essential for the generation or survival of a significant subset of GnRH neurons, independently of its roles in axonal patterning.

Role for neuronal, not vascular NRP1, in GnRH neuron development

Following the observations that blood vessel patterning in the brain is perturbed by loss of NRP1 (Gerhardt et al, 2004; Kawasaki et al., 1999) and that GnRH neurons migrate in close association with blood vessels that express NRP1 (Fig. 1C,D), we asked next whether the reduced number of GnRH neurons in *Nrp1*-null mutants was indirectly caused by the loss of NRP1 from blood vessels that are important for GnRH neuron development or, instead, was due to the loss of NRP1 signalling in GnRH neurons themselves. Thus, we analysed E14.5 mouse mutants that lacked NRP1 specifically in blood vessels versus neurons. These mutants were created by targeting conditional *Nrp1*-null mutants ($Nrp1^{fl/fl}$) (Gu et al., 2003) with two well-characterised, tissue-specific transgenes, the *Tie2-Cre* line, which is active in endothelial, but not neural cells (Kisanuki et al., 2001), and the *Nes-Cre* line, which expresses CRE under the control of the promoter for nestin (Petersen et al., 2002). Nestin is an intermediate filament protein that is not only present in neural precursors of the central nervous system, but also in the precursors of GnRH-positive neurons (Kramer and Wray, 2000).

We observed that endothelial-specific *Nrp1* mutants contained fewer and larger blood vessels in the brain compared with control littermates (see Fig. S2 in the supplementary material), as previously reported (Gu et al., 2003). By contrast, the number of GnRH-positive cells was similar in endothelial-specific *Nrp1*-null mutants and control littermates (*Cre*-negative $Nrp1^{fl/+}$ controls 1234±75 vs *Tie2-Cre* $Nrp1^{fl/-}$ 1203±44, n=3 each; Fig. 2E; see Fig. S2 in the supplementary material). Conversely, dilated vessels were

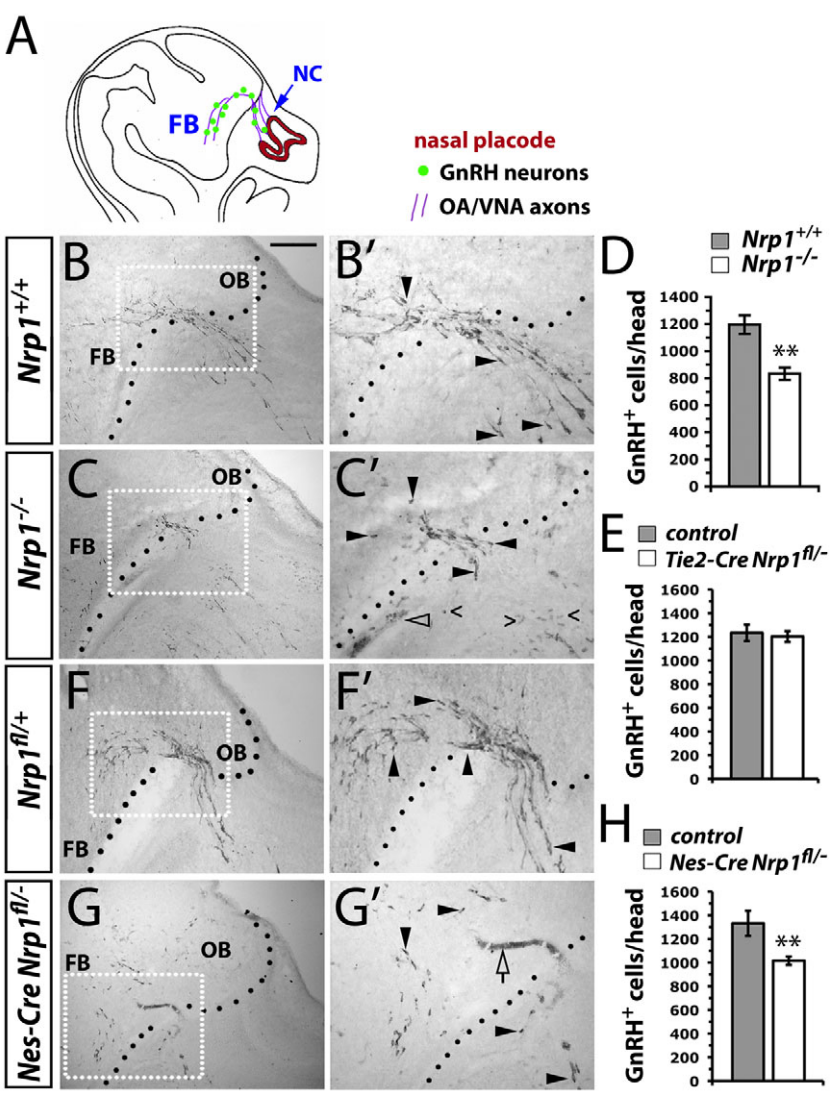

Fig. 2. Reduced number of GnRH neurons in mice lacking NRP1 in the neural lineage. (**A**) Schematic of an embryonic mouse head illustrating the migration of GnRH neurons (green dots); these neurons are born in the nasal placode that gives rise to the olfactory and vomeronasal epithelium (red) and migrate along olfactory and vomeronasal axons (OA/VNA; purple lines) through the nasal compartment (NC) to reach the forebrain (FB). (**B**,**B′**,**C**,**C′**,**F**,**F′**,**G**,**G′**) Sagittal sections of E14.5 mouse heads of the indicated genotypes were immunolabelled for GnRH to reveal neurons migrating in the nasal compartment and forebrain; the forebrain boundary is delineated with a dotted line. B′,C′,F′ and G′ show higher magnifications of the squared areas in the adjacent panels. Solid arrowheads indicate examples of migrating GnRH neurons; open arrowhead in C′ indicates abnormal blood vessels in the brain of *Nrp1*-null mutants; chevrons in C′ indicate peroxidase-positive blood cells, presumably present in the mutant tissues at high numbers due to blood vessel fragility; open arrow in G′ indicates a normal blood vessel in the *Nes-Cre Nrp1*$^{fl/-}$ mutant. Scale bar: 100 μm. (**D**,**E**,**H**) Quantitative analysis of the total GnRH neuron number in E14.5 heads of *Nrp1*-null mutants vs wild-type controls (D); *Tie2-Cre Nrp1*$^{fl/-}$ mutants vs controls (*Nrp1*$^{fl/+}$, no *Cre*) (E); and *Nes-Cre Nrp1*$^{fl/-}$ mutants vs heterozygous (*Nes-Cre Nrp1*$^{fl/+}$) and control (*Nrp1*$^{fl/+}$, no *Cre*) littermates (H). Data are expressed as the mean (± s.e.m.) of three or more independent experiments. $^{**}P<0.01$ mutants compared with controls. FB, forebrain; OB, olfactory bulb.

not present in neural-specific *Nrp1* mutants (Fig. 2G,G′, open arrow), but there was a significant reduction in the number of GnRH neurons (*Cre*-negative *Nrp1*$^{fl/+}$ and *Cre*-positive *Nrp1*$^{fl/+}$ controls 1340±67, n=3 each, vs *Nes-Cre Nrp1*$^{fl/-}$ 1016±48, n=4; $P<0.01$; Fig. 2F-H). Thus, the comparison of the two types of mutants demonstrated that NRP1 in neurons rather than blood vessels is essential for a normal GnRH neuron number.

VEGF164 enhances the survival of GnRH neurons by signalling through NRP1

Because NRP1 binds the VEGF isoform VEGF164 (Gitay-Goren et al., 1996; Soker et al., 1996; Soker et al., 1998), we next asked whether VEGF164 activates NRP1 to increase cell survival or proliferation of GnRH neurons in vitro. Because GnRH neurons are a small number of cells scattered over a large area in the developing head, it is not possible to isolate them in sufficient quantities for biochemical experiments. For this reason we used GN11 cells, which are immortalised GnRH neurons, for our biochemical studies (Radovick et al., 1991). These cells express NRP1, like primary GnRH neurons (Cariboni et al., 2007a), and have been used previously to identify cellular mechanisms and biochemical pathways regulating GnRH neuron proliferation and migration (Cariboni et al., 2011; Cariboni et al., 2007a; Cariboni et al., 2004; Cariboni et al., 2005; Giacobini et al., 2008; Giacobini et al., 2007; Maggi et al., 2000).

We found that addition of VEGF164 to the culture media did not affect the proliferation rate of GN11 cells either in the absence of serum (data not shown) or in the presence of a low serum concentration (0.2%; Fig. 3A-C). By contrast, VEGF164 clearly affected GN11 cell survival (Fig. 3D,E,H). For the survival experiments, we first determined how serum withdrawal affected GnRH neurons. We observed dying cells in the serum-starved cultures from 24 hours onwards, but cell death was greatest from 72 hours onwards, with 90% of cells dead after another 12 hours of serum withdrawal (Fig. 3D,H). When recombinant VEGF164 was added to the serum-free culture medium for the final 12 hours of serum starvation, only 25% of cells died (Fig. 3E,H; $P<0.001$). These findings suggest that VEGF164 is a potent pro-survival factor for GnRH neurons. We found further that inhibiting NRP1 with a previously validated function-blocking antibody (Erskine et al., 2011) abrogated the ability of VEGF to promote GN11 cell survival (compare Fig. 3E with 3F; Fig. 3H; $P<0.01$). Together with the in vivo analysis of GnRH neuron number in complete knockouts and neuronal

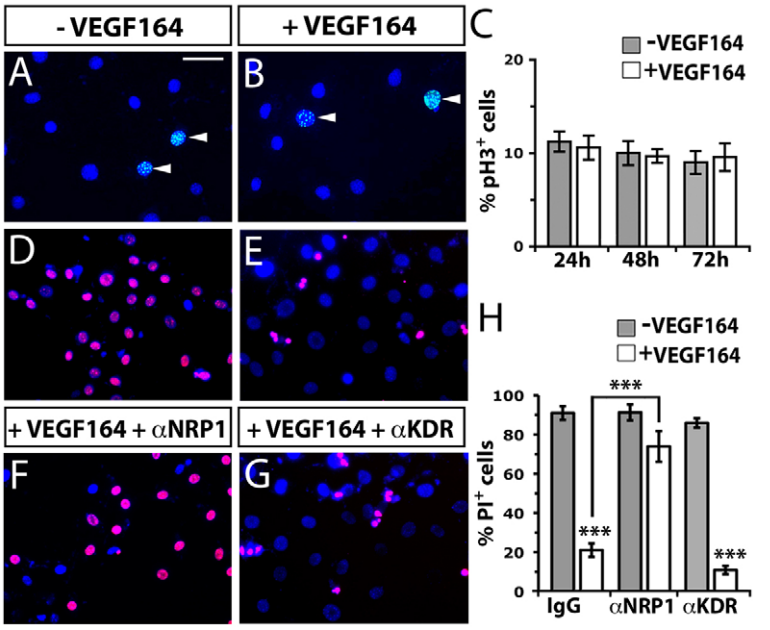

Fig. 3. VEGF164 promotes the survival of GnRH neurons. (**A-C**) The mitotic marker phosphohistone 3 (pH3) was used to identify proliferating GN11 cells (arrowheads) in cultures containing or lacking exogenous VEGF164; proliferation was quantified after 24 hours, 48 hours and 72 hours as the proportion of pH3-positive cells (green) in all cells (labelled with a nuclear counterstain in blue). (**D**,**E**) Cell death of serum-starved GN11 cells, grown in the absence or presence of exogenous VEGF164, was visualised by propidium iodide (PI) staining (red) of cultures labelled with a nuclear counterstain (blue), with dead cells being double-positive and therefore appearing pink. (**F**,**G**) An antibody that blocks NRP1 function (αNRP1) largely abolished the VEGF164-mediated rescue of GN11 cells (F), whereas an antibody that blocks KDR function (αKDR) did not affect rescue (G). (**H**) Quantification of cell death, expressed as the proportion of propidium-iodide permeable cells relative to Hoechst-positive cells in cultures containing control IgG, αNRP1 or αKDR. Quantitative data are expressed as the mean (± s.e.m.) from three or more independent experiments; ***P<0.001, compared with controls. Scale bar: 25 μm.

specific *Nrp1*-null mutants, these observations suggest that VEGF164 signals through NRP1 to promote GnRH neuron survival in a cell autonomous manner.

KDR does not convey VEGF survival signals in GnRH neurons

The classical receptor for VEGF-induced survival signalling in endothelial cells is the receptor tyrosine kinase KDR, with NRP1 usually considered to be a co-receptor that enhances KDR activity (e.g. Gerber et al., 1998; Soker et al., 2002; Whitaker et al., 2001). Even though in vivo evidence for a direct role of VEGF/KDR signalling in neurons is lacking, in vitro experiments had raised the possibility that KDR transduces VEGF survival signals in several different types of cultured neurons (for a review, see Ruiz de Almodovar et al., 2009). We therefore asked whether KDR was essential for GnRH neuron survival. However, immunofluorescence labelling and RT-PCR analysis suggested that GN11 cells did not express KDR at detectable levels (see Fig. S3 in the supplementary material). Consistent with their lack of KDR expression, treating GN11 cells with an antibody that blocks KDR signalling in endothelial cells (Gerhardt et al., 2003) did not affect the ability of VEGF164 to rescue neuronal cell death (compare

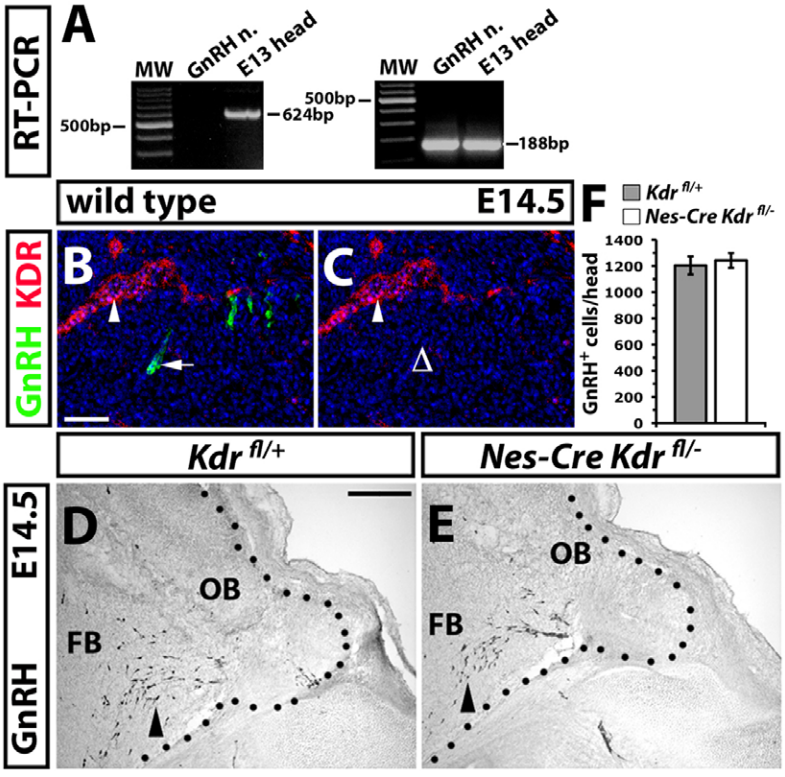

Fig. 4. KDR does not control GnRH neuron development. (**A**) RT-PCR analysis shows expression of KDR (624 bp) in E13.5 head tissue, but not in FACS-isolated primary GFP+ GnRH neurons. Positive control: GAPDH (188 bp). MW, molecular weight marker. (**B**,**C**) Sagittal cryosections of a E14.5 wild-type mouse embryo head, labelled by double immunofluorescence for KDR (red) and GnRH (green); KDR localises to blood vessels (white arrowheads, B,C), whereas GnRH-positive neurons (white arrow, B) do not appear to express KDR (Δ in C). (**D**,**E**) Sagittal sections of E14.5 mouse heads expressing KDR (*Kdr*$^{fl/+}$) or lacking KDR in the nestin-positive neuronal lineage (*Nes-Cre Kdr*$^{fl/-}$) were immunolabelled for GnRH to reveal migrating neurons (black arrowheads). Dotted line indicates the forebrain boundary. (**F**) Quantification of GnRH neuron number in heads of mice expressing or lacking KDR at E14.5. Quantitative data are expressed as the mean (± s.e.m.) from three independent experiments. Scale bars: 50 μm in B,C; 100 μm in D,E. FB, forebrain; OB, olfactory bulb.

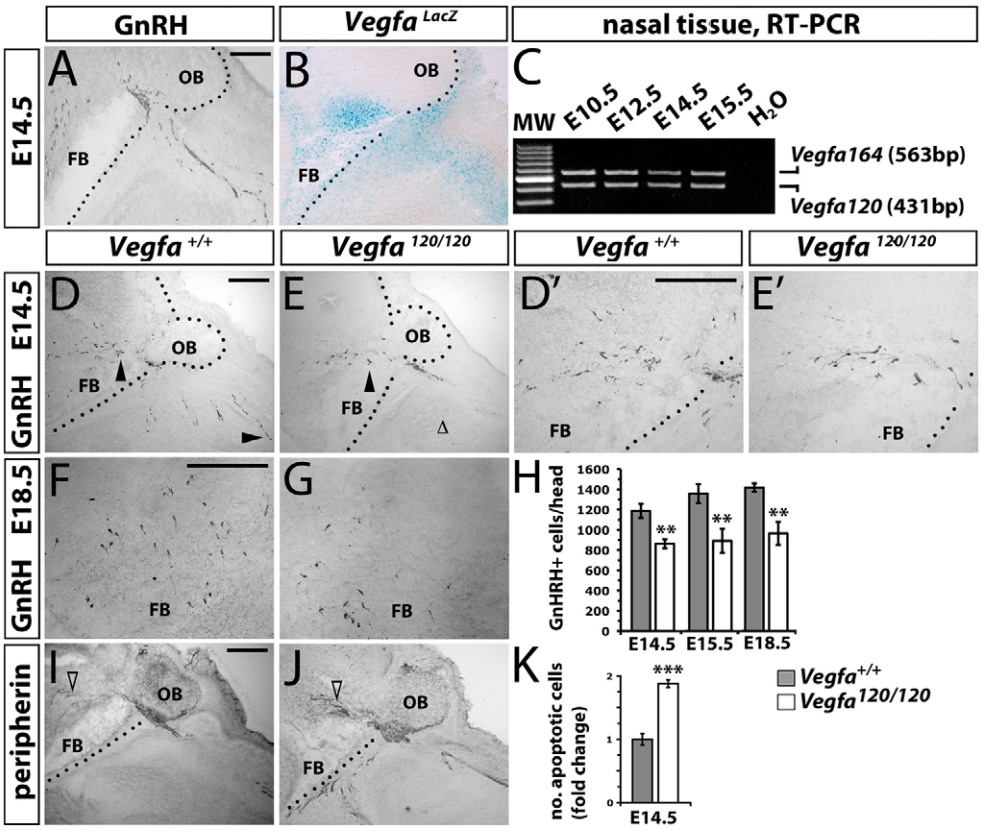

Fig. 5. VEGF164 is essential for GnRH neuron survival in vivo. (**A**,**B**) Contiguous sagittal sections of an E14.5 *Vegfa*^{lacZ} mouse head stained for GnRH protein (A) and β-galactosidase activity with X-Gal substrate (B). (**C**) RT-PCR analysis of embryonic nasal tissues showing the expression of mRNA for the VEGF164 and VEGF120 isoforms. (**D-G**) Sagittal sections of E14.5 (D-E′) and E18.5 (F,G) mouse heads expressing normal levels of all VEGF isoforms (*Vegfa*$^{+/+}$) or lacking the VEGF164 isoform (*Vegfa*$^{120/120}$) were labelled by immunohistochemistry for GnRH to reveal migrating neurons; examples of migrating neurons in the nasal compartment and forebrain are indicated with black arrowheads; the black dotted line indicates the forebrain boundary; D′ and E′ are higher magnifications of the forebrain entry site in the sections shown in D and E, respectively. (**H**) Quantification of GnRH neuron number in the head of mice expressing or lacking VEGF164 at E14.5, E15.5 and E18.5. (**I**,**J**) Sagittal sections of E14.5 *Vegfa*$^{+/+}$ and *Vegfa*$^{120/120}$ mouse heads, immunolabelled for the axon marker peripherin to reveal olfactory and vomeronasal axons in the nasal compartment and the caudal branch of the vomeronasal nerve in the forebrain (open arrowheads). (**K**) Quantification of apoptotic cells in the nasal area of mice expressing or lacking VEGF164 at E14.5. FB, forebrain; OB, olfactory bulb; MW, molecular weight marker. Scale bars: 100 μm in A,B,D,E,I,J); 150 μm in D′,E′,F,G. Error bars represent s.e.m. $^{**}P<0.01$, $^{***}P<0.001$, mutants compared with littermate controls.

Fig. 3E with 3G; Fig. 3H; $P<0.001$). *Kdr* expression was also not detected in FACS-sorted, primary GnRH neurons by RT-PCR (Fig. 4A), and immunofluorescence staining for KDR confirmed further that this VEGF receptor was not expressed by GnRH neurons at detectable levels in situ (Fig. 4B,C). Finally, the immunohistochemical analysis of sagittal sections from E14.5 mouse embryos carrying a conditional null *Kdr* allele in the neural lineage on a *Kdr*-null background (Haigh et al., 2003) showed that mutants had a similar number of GnRH neurons to control littermates (Fig. 4D-F; *Kdr*$^{fl/+}$ 1204±69 vs *Nes-Cre Kdr*$^{fl/-}$ 1243±56; n=3 each). The lack of a phenotype in neural-specific *Kdr* mutants was in striking contrast to the reduced number of GnRH neurons in mutants lacking NRP1 in the neural lineage (see above, Fig. 2G,H). We conclude that VEGF164 provides survival signals in GnRH neurons by signalling through NRP1, independently of KDR.

VEGF164 is essential for GnRH neuron development

To investigate whether VEGF164 is essential for GnRH neuron generation in vivo, we first studied its expression pattern during the embryonic period of GnRH neuron migration in the nasal compartment. For these experiments, we used a well-characterised β-galactosidase reporter for *Vegfa* gene expression (*Vegfa*^{lacZ}) (e.g. Miquerol et al., 1999; Ruhrberg et al., 2002; Schwarz et al., 2004). Immunohistochemistry of contiguous sagittal sections from these mice for GnRH and the β-galactosidase substrate X-Gal showed that VEGF was expressed along the migratory route of GnRH neurons, including the nose, nasal/forebrain junction and forebrain (Fig. 5A,B). RT-PCR analysis established further that both the VEGF164 and VEGF120 isoforms were expressed in the nasal compartment throughout the period of GnRH neuron formation and migration, i.e. between E10.5 and E15.5 (Fig. 5C).

We next analysed mouse embryos expressing VEGF120 as the only VEGF isoform (Carmeliet et al., 1999). In contrast to full *Vegfa* mutants, which die early in embryogenesis owing to lack of blood vessels (Carmeliet et al., 1996; Ferrara et al., 1996), *Vegfa*$^{120/120}$ mutants survive to birth because they assemble a basic blood vessel network (Carmeliet et al., 1999; Ruhrberg et al., 2002). They do, however, die after birth owing to cardiovascular complications. Immunohistochemical analysis of sagittal sections at E14.5 revealed a significantly lower number of GnRH-positive cells in *Vegfa*$^{120/120}$ mutants compared with wild-type littermates (Fig. 5D-E′,H;

Vegfa$^{+/+}$1187±69 vs *Vegfa*$^{120/120}$ 863±46; n=4 each; P<0.01). This decrease was similar at E15.5, confirming that the reduced number of GnRH neurons was not due to a developmental delay (Fig. 5H; *Vegfa*$^{+/+}$ 1358±93 vs *Vegfa*$^{120/120}$ 892±118; n=4 each; P<0.01). Moreover, a similar decrease in GnRH number was also seen at E18.5, when GnRH neuron migration is largely complete (Fig. 5F-H; *Vegfa*$^{+/+}$ 1437±19 vs *Vegfa*$^{120/120}$ 966±81; n=3 each; P<0.01). The comparable reduction in GnRH neuron number in mutants lacking VEGF164 and NRP1 confirmed our in vitro finding that VEGF164 signals through NRP1 to promote neuronal survival (compare Fig. 2D with Fig. 5H). By contrast, the patterning of olfactory and vomeronasal axons, which serve as substrates for migrating GnRH neurons, was normal in *Vegfa*$^{120/120}$ mutants (Fig. 5I,J).

The quantification of apoptotic cells in sections from E14.5 that had been immunostained for the apoptosis marker activated caspase 3 showed that *Vegfa*$^{120/120}$ mutants contained 1.8 times more apoptotic cells in the nose than wild-type littermates (Fig. 5K). Because double labelling for GnRH and activated caspase 3 was not possible owing to a lack of compatible antibodies, we stained contiguous sagittal sections from wild-type and *Vegfa*$^{120/120}$ mutant littermates for GnRH and activated caspase 3 (see Figs S4 and S5 in the supplementary material). As expected, few apoptotic cells were detected in the nose of wild-type embryos in areas where GnRH neurons migrated at E12.5 and E14.5 (see Fig. S4A,A′,C and Fig. S5A,D in the supplementary material). By contrast, many apoptotic cells were present in the mutant nose in the area containing migrating GnRH neurons at E12.5 (see Fig. S4B,B′,D in the supplementary material). Moreover, the forebrain entry site of nasal axons and GnRH neurons was enriched with apoptotic cells in mutants at E14.5 (see Fig. S5B,E in the supplementary material). A similar enrichment of apoptotic cells near nasal axons was observed in the nose of E14.5 *Nrp1*-null (see Fig. S5C,F in the supplementary material) and *Nes-Cre Nrp1*$^{fl/-}$ (see Fig. S6C,D in the supplementary material) mutants. Together with the in vitro and in vivo studies described above, these findings demonstrate that VEGF164 signals through NRP1 to promote the survival of a subset of GnRH neurons when they migrate through the nose.

VEGF164 promotes GnRH neuron survival by activating both PI3K and MAPK signalling

VEGF activates the intracellular PI3 kinase (PI3K) and MAP kinase (MAPK) signalling pathways to support cell survival of cultured hippocampal neurons after glutamate-induced cell stress or serum starvation (Jin et al., 2000; Matsuzaki et al., 2001). However, KDR rather than NRP1 was implicated in these studies as the VEGF receptor mediating survival signalling. We therefore asked whether either of these intracellular signalling pathways was also activated by VEGF164 in the absence of KDR. For these experiments, we compared phosphorylation of the PI3K substrate AKT1 and the MAP kinases ERK1 (MAPK3 – Mouse Genome Informatics) and ERK2 (MAPK1 – Mouse Genome Informatics) in GN11 cells that were serum-starved overnight or for 72 hours and then treated with recombinant VEGF164 (Fig. 6). VEGF164 treatment increased the phosphorylation of AKT1 and ERK1/2, with maximal activation for each pathway 5 minutes after VEGF addition and with comparable activation kinetics, either after serum starvation overnight (Fig. 6A,B) or for 72 hours (Fig. 6C,D). A similar increase in phosphorylation was seen in both conditions (compare Fig. 6B with 6D).

We next used established inhibitors for AKT (LY294) and MAP kinases (U0126) to investigate whether both pathways were activated in a co-dependent fashion or independently of each other.

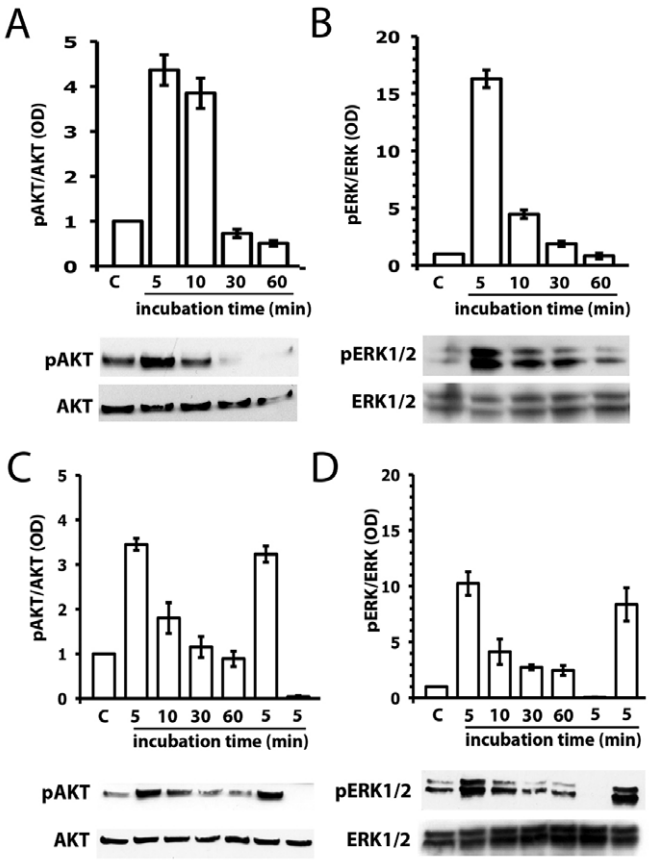

Fig. 6. VEGF164 induces AKT and ERK phosphorylation in serum-starved GnRH neurons. Immunoblotting showed that 10 ng/ml VEGF164 increased the phosphorylation of AKT and ERK1/2 in GN11 cells that had been serum-starved overnight (**A**,**B**) or for 72 hours (**C**,**D**). Treatment with the PI3K inhibitor LY294 abolished AKT phosphorylation (C) and treatment with the MAPK inhibitor U0126 abolished ERK1/2 phosphorylation (D), but not vice versa. The bar graphs show the level of phosphorylation at each time point in three independent experiments relative to the level of phosphorylation just prior to VEGF164 treatment (0 min), expressed as optical density (OD) ratios of pAKT/AKT or pERK/ERK. Error bars represent s.e.m.

We found that LY294 abrogated AKT phosphorylation after VEGF164 treatment of serum-starved GN11 cells, but did not affect ERK1/2 phosphorylation (Fig. 6C,D). Conversely, U0126 inhibited ERK1/2 phosphorylation after VEGF164 treatment, but did not affect the phosphorylation of AKT (Fig. 6C,D). VEGF164, therefore, activates the AKT and ERK signalling in GnRH neurons simultaneously and independently of each other.

We then investigated whether AKT and MAP kinase activation are essential mediators of the pro-survival effect of VEG164 in serum-starved GN11 cells (Fig. 7). We found that VEGF treatment rescued GN11 cells from death by serum starvation (Fig. 7A,B), but that addition of either LY294 (Fig. 7C) or U0126 (Fig. 7D) prior to VEGF treatment prevented rescue (Fig. 7E). Both pathways are, therefore, essential for VEGF survival signalling. We conclude that VEGF164/NRP1 signalling co-activates the PI3K/AKT and MAPK pathways to promote GnRH neuron survival, independently of KDR (Fig. 7H). This mechanism differs from the pathway models previously proposed for endothelial cells and other types of neurons, which were derived exclusively from

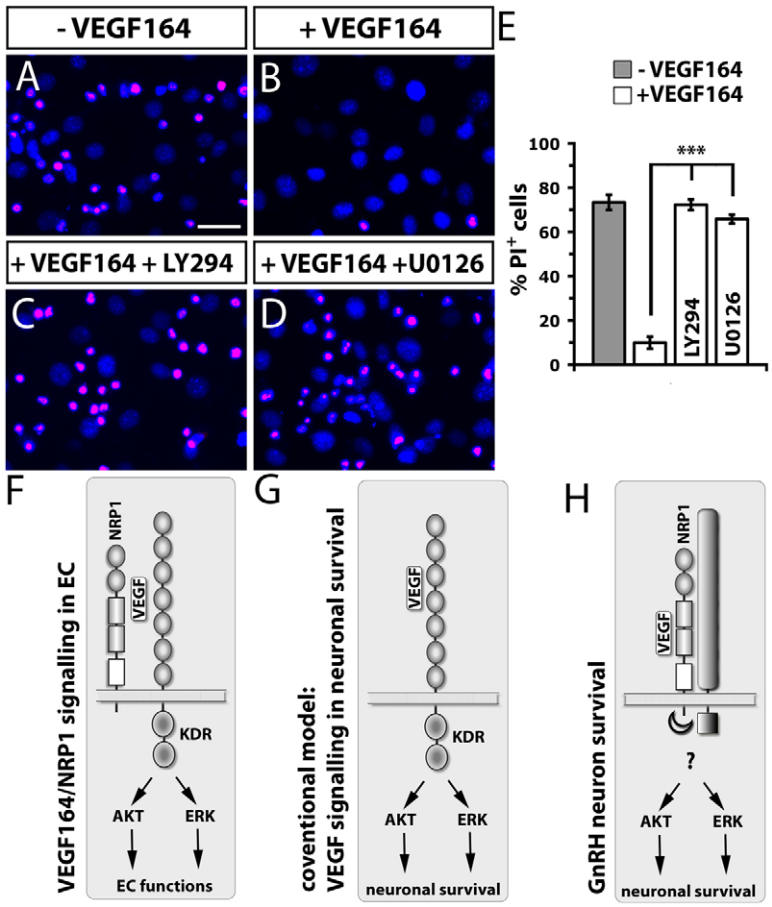

Fig. 7. AKT and ERK pathways are essential for VEGF-induced GnRH neuron survival. (**A-D**) The death of GN11 cells that had been serum-starved for 72 hours (A) is rescued by the addition of 10 ng/ml VEGF164 (B), but not in the presence of LY294 (C) or U0126 (D). Cultures are stained with propidium iodide (red) and with a nuclear counterstain (blue); dead cells are double-positive and therefore appear pink. (**E**) Quantification of the extent of cell death in the presence of VEGF164 plus or minus LY294 or U0126, expressed as the proportion of propidium iodide-permeable cells relative to Hoechst-positive cells in untreated control cultures. Quantitative data are expressed as the mean (± s.e.m.) from three independent experiments; ***P<0.001, compared with control. Scale bar: 25 μm. (**F-H**) Schematic of conventional and novel aspects of neuropilin signalling. In endothelial cells, VEGF164 interacts with KDR and NRP1 to activate AKT and ERK signalling (F). In several different neuronal culture models, VEGF164 promotes survival via KDR (G). In GnRH neurons, KDR is not involved in survival signalling; instead, survival signalling in vitro and in vivo depends on NRP1 (H). It is not known how NRP1 activates AKT and ERK, but it might involve unidentified co-receptors because loss of the NRP1 cytoplasmic domain did not perturb GnRH neuron survival.

in vitro experiments (Fig. 7F,G; see Discussion). Importantly, the genetic ablation of the NRP1 cytoplasmic domain did not decrease GnRH neuron number at E17.5 (see Fig. S7A,B in the supplementary material; *Nrp1*$^{+/+}$ 1064±58 vs *Nrp1*$^{cyto\Delta/\Delta}$ 1150±25), and GnRH neurons were distributed normally in the adult brain (see Fig. S7C,D in the supplementary material). This observation is consistent with a model in which NRP1 does not convey VEGF164 signals through intracellular adaptors, but recruits an unidentified co-receptor to activate AKT and ERK signalling (Fig. 7H).

Synergistic action of VEGF164 and SEMA3A in development of the GnRH neuron system

Because VEGF164 signalling is required to maintain a normal number of GnRH neurons (Fig. 5), and SEMA3A is essential to organise the axonal substrates that guide GnRH neurons (Cariboni et al., 2011), we hypothesised that mutants lacking both ligands would fail to assemble the GnRH neuron system. Consistent with this idea, the analysis of sagittal sections from compound *Vegfa*$^{120/120}$ *Sema3a*$^{-/-}$ mutants at E14.5 by immunohistochemistry showed a striking reduction in the total number of GnRH neurons in double homozygous mutants (Fig. 8A-C; *Vegfa*$^{120/120}$ *Sema3a*$^{-/-}$ 683±120 vs wild-type littermates 1202±5; n=2 each). The surviving GnRH neurons in *Vegfa*$^{120/120}$ *Sema3a*$^{-/-}$ mutants adopted an aberrant pattern of migration that led to their accumulation beneath the meningeal tissue (Fig. 8A,B). This distribution was similar to that reported previously for single *Sema3a*-null and compound *Nrp2*$^{-/-}$ *Nrp1*$^{Sema-/-}$ mutants (Cariboni et al., 2011). As a result of the combined failure to retain a normal number of GnRH neurons and to direct the migration of the remaining ones appropriately, very few GnRH neurons had entered the forebrain of double *Vegfa*$^{120/120}$ *Sema3a*$^{-/-}$ mutants at E14.5 (Fig. 8C; *Vegfa*$^{120/120}$ *Sema3a*$^{-/-}$ 32±2 vs wild-type littermates 573±18; n=2 each). We conclude that VEGF164 and SEMA3A play distinct, but synergistic roles in the development of the GnRH neuron system (Fig. 8D-G).

DISCUSSION

Gonadotropin releasing hormone (GnRH) neurons are neuroendocrine cells of the hypothalamus, whose axons release the decapeptide into the portal circulation to initiate puberty and regulate fertility in all mammalian species. Recent studies have identified a number of cues that control GnRH neuron migration during embryonic development to ensure that they reach the hypothalamus (for reviews, see Cariboni et al., 2011; Cariboni et al., 2007b). By contrast, little is known about the factors that control the survival of these cells. We have shown here that VEGF164, acting through NRP1, is one significant survival factor for developing GnRH neurons during their migration from the nasal placode to the hypothalamus.

Besides its key roles during vasculogenesis and angiogenesis, VEGF has been proposed to be a neuroprotective factor in both the developing and adult brain (for a review, see Ruiz de Almodovar et al., 2009). However, owing to the early embryonic lethality of complete VEGF and VEGF receptor null mutants, it was not previously established whether VEGF promotes the survival of neurons in vivo directly by activating neuronal VEGF receptors, or acts solely by promoting the growth of neurotrophic

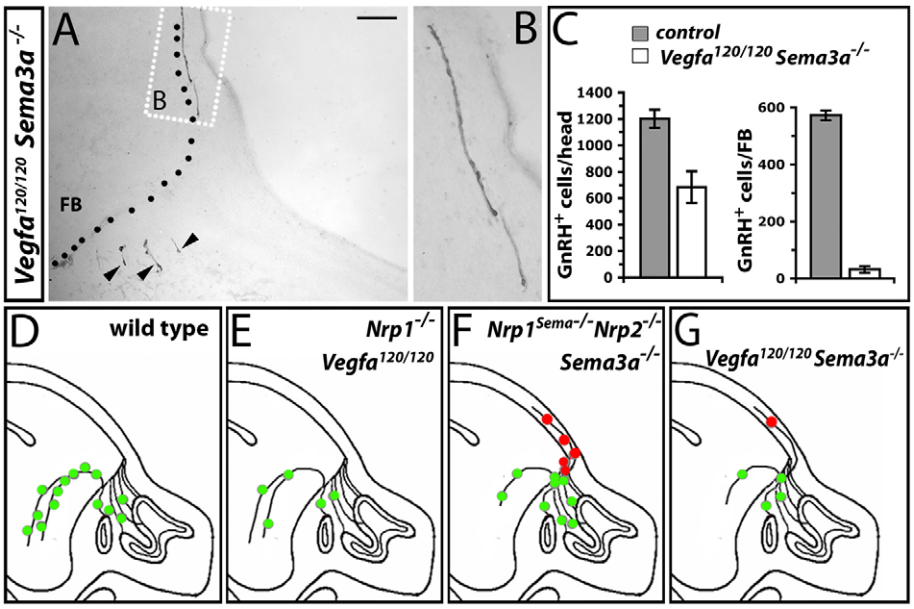

Fig. 8. Reduced number and mis-positioning of GnRH neurons in mice lacking both VEGF164 and SEMA3A. (**A**,**B**) Sagittal sections of E14.5 *VEGF*$^{120/120}$ *Sema3a*$^{-/-}$ mouse heads were immunolabelled for GnRH to reveal migrating neurons in the nasal compartment and forebrain (FB). A few surviving neurons are located in the nasal compartment; examples are indicated with arrowheads in A. The area outlined with a white square in A contains ectopic neurons and is shown at higher magnification in B. The forebrain boundary (FB) is indicated with a black dotted line. Scale bar: 100 μm. (**C**) Quantification of the GnRH neuron number in the entire head or forebrain of E14.5 *Vegfa*$^{120/120}$ *Sema3a*$^{-/-}$ mutants and their wild-type littermates. (**D-G**) Schematics of GnRH neuron migration and olfactory/vomeronasal axon projection at E14.5 in wild-type mice (D), mutants examined in this study (E,G) and mutants described in Cariboni et al. (Cariboni et al., 2011) (F). Green dots indicate neurons that migrate normally, red dots indicate ectopic neurons.

blood vessels. Thus, *Vegfa*-, *Kdr*- and *Flt1*-null mutants die owing to severe vascular defects before overt neuronal differentiation, and *Nrp1*-null mutants die between E10.5 and E12.5 in the genetic backgrounds available in other laboratories (e.g. Kawasaki et al., 1999; Jones et al., 2008). Accordingly, our studies on GnRH neuron survival in full *Nrp1*-null mutants on the JF1 background, combined with analysis of neural- versus vascular-specific NRP1 mutants and *Vegfa*$^{120/120}$ mice, provide the first proof-of-principle that VEGF plays a direct role in neuronal survival in vivo.

Defective neuronal survival occurred in our *Nrp1*-null mutants on the JF1 background in the absence of obvious vomeronasal axon defects (see Fig. S1 in the supplementary material). By contrast, we reported previously that mutants lacking semaphorin signalling through NRP1 on a CD1 background displayed a mild vomeronasal axon defect, but no decrease in the number of GnRH neurons (*Nrp1*$^{Sema-/-}$ mutants) (Cariboni et al., 2011). Our preliminary analysis of axon patterning in *Nrp1*-null mutants on the same CD1 background at E12.5, the latest time point at which they are still viable, suggested vomeronasal axon defects similar to those of *Nrp1*$^{Sema-/-}$ mutants and excess cell death in the area into which ectopic axons were projecting (A.C., K.D. and C.R., unpublished observations). Vomeronasal axon defects in *Nrp1*-null mutants are, therefore, ameliorated in some background strains by unknown mechanisms, but when the defects are present, the dependence of GnRH neurons on VEGF164/NRP1 survival signalling is increased.

An explanation for the importance of VEGF164 as a survival factor for GnRH neurons may be its ability to suppress the activation of caspase 3 in vitro (Jin et al., 2001) and in vivo (Fig. 5; see Figs S4, S5 in the supplementary material). However, because there are different GnRH neuron subpopulations with diverse molecular characteristics (e.g. Tobet et al., 1993), VEGF probably cooperates with other signalling pathways to ensure that all GnRH neurons survive the changing environment they encounter during their migration. In support of this idea, a small subset of GnRH neurons depends on AXL/TYRO3 signalling (Pierce et al., 2008), and it therefore seems likely that further survival signals remain to be identified.

Biochemical experiments in endothelial cells had raised the possibility that VEGF isoform signalling could use both NRP1 and the related NRP2 (Gluzman-Poltorak et al., 2000). However, NRP2 is unlikely to contribute to VEGF-mediated GnRH neuron survival for two reasons. First, loss of NRP2, unlike NRP1, does not reduce the overall number of GnRH neurons that are generated, but instead affects GnRH neuron migration along their axonal substrates and, accordingly, their entry into the forebrain (Cariboni et al., 2011; Cariboni et al., 2007a). Second, the number of GnRH neurons that are lost in *Vegfa*$^{120/120}$ mice is similar to that seen in mice lacking NRP1, and both mutants therefore phenocopy each other with respect to GnRH neuron survival (compare Figs 2 and 5; see Fig. S5 in the supplementary material).

Our studies on the VEGF signalling pathway that mediates GnRH neuron survival challenge two widely held models derived from in vitro studies, which suggest that (1) the lack of a catalytic intracellular domain forces NRP1 to use KDR as a co-receptor to transduce VEGF signals [discussed by Fantin and colleagues (Fantin et al., 2009)], and that (2) KDR is the main VEGF receptor promoting neuronal survival (e.g. Ogunshola et al., 2002; Oosthuyse et al., 2001; Sondell et al., 1999). In contrast to these models, we found that VEGF164 signalling in GnRH neurons does not require KDR (Figs 3 and 4; see Fig. S3 in the supplementary

material). This finding does, however, agree with the observation that mice lacking KDR in the neural lineage are viable, with no gross anatomical defects (Haigh et al., 2003).

A previous study (Pan et al., 2007) showed that VEGF120 binds NRP1 in endothelial cells to promote VEGF signalling through KDR. However, for several reasons it is unlikely that a similar pathway operates in GnRH neurons. First, GnRH neurons do not express KDR, so the signalling receptor complex must be different to that in endothelial cells. Second, *Vegfa*$^{120/120}$ mutants retain VEGF120, but VEGF120 did not rescue the GnRH neuron survival defect caused by loss of VEGF164, as the severity of the GnRH neuron survival defect was similar in *Vegfa*$^{120/120}$ mutants and NRP1 knockouts. Third, the observation that VEGF120 cannot compensate for VEGF164 in NRP1-dependent neurons has precedence in our previous studies of facial branchiomotor neuron migration and retinal ganglion axon guidance (Schwarz et al., 2004; Erskine et al., 2011). Both types of neurons, like GnRH neurons, lack obvious expression of KDR. Therefore, the ability of VEGF120 to bind NRP1 does not appear to be relevant in the context of KDR-deficient neurons.

Interestingly, we found that VEGF164/NRP1 signalling promotes the survival of immortalised GnRH neurons via activation of AKT and ERK (Fig. 7), as recently described for endothelial cells (Wang et al., 2007). As NRP1 does not itself have a kinase domain and loss of the NRP1 cytoplasmic domain does not impair GnRH neuron survival (see Fig. S7 in the supplementary material), an unidentified co-receptor might be required to activate AKT and ERK signalling in these neurons (Fig. 7H). Our unpublished data suggest that GnRH neurons express a large number of signal transducing transmembrane proteins that have been shown to interact with neuropilin directly or indirectly, for example FLT1, MET and several plexins (A.C. and J.G.P., unpublished observations). Future work should therefore aim to identify the unknown NRP1 co-receptor that transmits VEGF164/NRP1 survival signals in GnRH neurons.

Finally, we have extended our recently published work showing that the other NRP1 ligand, SEMA3A, patterns the olfactory and vomeronasal axons that guide migrating GnRH neurons from the nose into the brain (Cariboni et al., 2011; Cariboni et al., 2007a). Thus, the analysis of compound *Sema3a*$^{-/-}$ *Vegfa*$^{120/120}$ mutants demonstrated that VEGF164 cooperates with SEMA3A to ensure that migrating GnRH neurons do not perish on their way to the hypothalamus. Strikingly, the reduction in GnRH neuron number was greater in compound than in single *Nrp1*-null or *Vegfa*$^{120/120}$ mutants (compare total number of GnRH-positive cells in Fig. 8C with Fig. 2D and Fig. 5H). This observation might be explained by more neurons being misplaced in mutants lacking SEMA3A than in mutants lacking NRP1 signalling, because NRP2 partially compensates for loss of NRP1 (Cariboni et al., 2011), and by ectopic neurons undergoing cell death when VEGF164 signalling is lacking, adding to the total pool of dying GnRH neurons in the nose. Consistent with this idea, a preliminary experiment showed ectopic cell death outside the forebrain of *Nrp1*-null mutants on the CD1 background, in the region where ectopic axons reside (A.C., K.D. and C.R., unpublished observations). Accordingly, compound *Sema3a*$^{-/-}$ *Vegfa*$^{120/120}$ mutants show the most severe developmental defects of the GnRH neuron system described for any animal model, and the severity of the phenotype also exceeds that described for the single case of a human foetus with X-linked KS studied so far, in which GnRH neurons accumulated on the dorsal surface of the cribriform plate, outside the forebrain (Schwanzel-Fukuda et al., 1989).

In summary, we have shown that VEGF164 is a cell-autonomous survival factor for developing GnRH neurons that signals through NRP1 and cooperates with SEMA3A-mediated axon guidance to ensure the normal development of the neuroendocrine system that regulates sexual reproduction. These observations will advance our understanding of the molecular mechanisms that underlie the development of the GnRH neuron system and could shed light on the aetiology of KS and related disorders.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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