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RESEARCH ARTICLE

Growth differentiation factor 5 is a key physiological regulator of dendrite growth during development

Catarina Osório[1,‡], Pedro J. Chacón[1,2,‡], Lilian Kisiswa[1,*], Matthew White[1], Sean Wyatt[1], Alfredo Rodríguez-Tébar[2] and Alun M. Davies[1,§]

ABSTRACT

Dendrite size and morphology are key determinants of the functional properties of neurons. Here, we show that growth differentiation factor 5 (GDF5), a member of the bone morphogenetic protein (BMP) subclass of the transforming growth factor β superfamily with a well-characterised role in limb morphogenesis, is a key regulator of the growth and elaboration of pyramidal cell dendrites in the developing hippocampus. Pyramidal cells co-express GDF5 and its preferred receptors, BMP receptor 1B and BMP receptor 2, during development. In culture, GDF5 substantially increased dendrite, but not axon, elongation from these neurons by a mechanism that depends on activation of SMADs 1/5/8 and upregulation of the transcription factor HES5. *In vivo*, the apical and basal dendritic arbours of pyramidal cells throughout the hippocampus were markedly stunted in both homozygous and heterozygous *Gdf5* null mutants, indicating that dendrite size and complexity are exquisitely sensitive to the level of endogenous GDF5 synthesis.

KEY WORDS: Growth differentiation factor 5, Bone morphogenetic protein, Dendrite, Hippocampus, Mouse

INTRODUCTION

The size and shape of dendritic arbours and the distribution and nature of their synaptic connections play major roles in governing the functional properties of neurons and neural circuits (Gulledge et al., 2005; Häusser et al., 2000; Spruston, 2008), and many neurodevelopmental and acquired disorders of neural function are due primarily to structural abnormalities of dendrites and their connections (Kaufmann and Moser, 2000; Penzes et al., 2011). The characteristic morphologies of dendritic arbours result from the interplay of intrinsic genetic programmes and a wide variety of extrinsic signals that impinge upon dendrites as they grow and establish functional connections. In addition to the spatial and temporal patterns of neural activity, numerous cell surface and diffusible proteins, such as delta-notch, Eph-Ephins, cell adhesion molecules, agrin, neurotrophins, semaphorins and slits, orchestrate dendritic growth, branching and remodelling during the development and maturation of the nervous system (Parrish et al., 2007; Urbanska et al., 2008).

Here, we have focused on the role of GDF5 in the growth of pyramidal cell dendrites in the developing hippocampus. GDF5 is a member of the bone morphogenetic protein (BMP) subclass of the TGFβ superfamily that exerts its effects via a high-affinity receptor complex comprising BMPR1B and BMPR2 (Nishitoh et al., 1996). Originally identified by their osteogenic activity, BMPs are now recognised as having a great diversity of functions in many tissues (Bragdon et al., 2011). In the nervous system, various BMPs have been implicated in regulating patterning, neurogenesis, astrogliogenesis and neuronal specification (Bond et al., 2012). GDF5 is best characterised for its key roles in chondrogenesis, osteogenesis and joint morphogenesis (Mikic, 2004). GDF5 and its receptors have also been detected in the brain (Krieglstein et al., 1995; O'Keeffe et al., 2004; Miyagi et al., 2011), and although GDF5 enhances midbrain dopaminergic neuron survival *in vitro* and exogenous GDF5 is neuroprotective in animal models of Parkinson's disease (Sullivan and O'Keeffe, 2005), the role of endogenous GDF5 in the nervous system is not known.

Hippocampal pyramidal cells are large excitatory neurons that have distinctive, highly branched apical and basal dendritic trees that receive tens of thousands of excitatory and inhibitory synaptic inputs and project to neurons within and beyond the hippocampus (Piskorowski and Chevaleyre, 2012). They are among the most extensively studied cells of the mammalian nervous system and are one of the best-characterised models for studying the differentiation and growth of axons and dendrites during development (Dotti et al., 1988; Kaech and Banker, 2006; Spruston, 2008). In rodents, these neurons are generated during embryonic development and elaborate dendrites throughout late fetal and early postnatal stages (Danglot et al., 2006). We find that GDF5, BMPR1B and BMPR2 are expressed by these neurons during this stage of development, and show that GDF5 promotes hippocampal pyramidal dendrite growth *in vitro* by activating SMAD signalling and regulating the expression of the basic helix-loop-helix transcription factor HES5. In brachypod mice, which possess a spontaneous frame-shift null mutation in the *Gdf5* gene (Storm et al., 1994), the dendritic arbours of hippocampal pyramidal cells are substantially smaller and less complex than those of wild-type mice. These findings show for the first time a key role for endogenous GDF5 in a crucial aspect of neuronal development.

RESULTS

GDF5 and its receptors are expressed in the developing hippocampus

As a starting point to our investigation of the role of GDF5 in neural development, we used quantitative PCR (QPCR) and western blotting to detect and quantify the expression of GDF5 and its

[1]School of Biosciences, Cardiff University, Cardiff CF10 3AT, UK. [2]CABIMER, Americo Vespucio s/n, Isla de la Cartuja, 41092 Seville, Spain.
*Present address: Department Neuroscience, Karolinska Institute, 17177 Stockholm, Sweden.
‡These authors contributed equally to this work

§Author for correspondence (daviesalun@cf.ac.uk)

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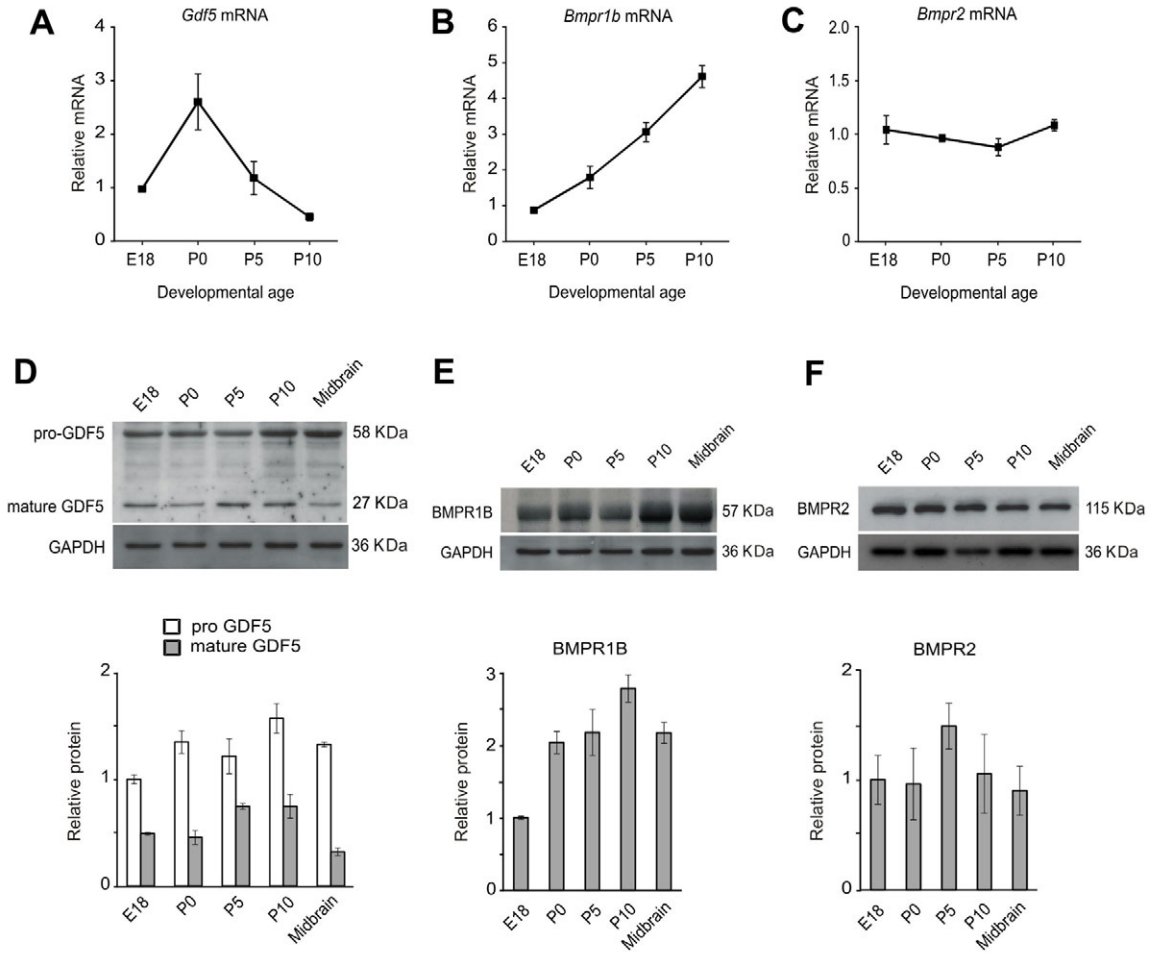

Fig. 1. Expression of GDF5, BMPR1B and BMPR2 in the developing mouse hippocampus. (A-C) Relative levels of *Gdf5* (A), *Bmpr1b* (B) and *Bmpr2* (C) mRNA in the hippocampus (mean ± s.e.m., *n*=4 per age). (D-F) Representative western blots probed for GAPDH and either GDF5 (D), BMPR1B (E) or BMPR2 (F) and graphs of pro-GDF5, mature GDF5, BMPR1B and BMPR2 relative to GAPDH in the hippocampus (mean ± s.e.m., *n*=3 per age). Lysates of E18 midbrain were used as positive control.

preferred receptors, BMPR1B and BMPR2, in the developing mouse hippocampus during the period of axon extension and dendrite elaboration from pyramidal cells. At stages from embryonic day (E) 18 to postnatal day (P) 10, GDF5 and its receptors and transcripts encoding these proteins were detectable (Fig. 1). GDF5, BMPR1B and BMPR2 were also detected in the midbrain at E18, a structure that has previously been reported to contain GDF5 protein (Fig. 1D-F). No band corresponding to mature GDF5 was detectable in extracts from *Gdf5*^{bp} mice (data not shown).

To ascertain which cells express GDF5 and its receptors and to determine the distribution of these proteins within these cells, we localised these proteins in hippocampal sections and in hippocampal cultures. Sections were triple stained with the nuclear marker TOTO-3, the dendrite marker anti-MAP2 and either anti-GDF5, anti-BMPR1B or anti-BMPR2. At P10 (Fig. 2A), prominent GDF5 labelling was evident in the pyramidal cell layer of CA1, CA2 and CA3 and the granule cell layer of the dentate gyrus. GDF5 labelling was evident in both the nuclei and cytoplasm of these cells. Nuclear and cytoplasmic GDF5 staining has been reported in cell lines as a consequence of alternative splicing of *Gdf5* and the generated proteins that possess either a signal peptide or a nuclear localisation signal (Felin et al., 2010). Importantly, sections of brains obtained from *Gdf5*^{bp} mice were not labelled by anti-GDF5 (Fig. 2A). Anti-BMPR1B and anti-BMPR2 labelled the pyramidal cell layer of CA1, CA2 and CA3, although labelling was evident only in the cytoplasm. Labelling of the granule cell layer of the dentate gyrus by anti-BMPR1B and anti-BMPR2 was very weak in comparison, and was observed mostly in the suprapyramidal blade. Sections incubated with secondary antibody alone exhibited no background immunofluorescence.

In cultures of E18 hippocampi, essentially all pyramidal cells were labelled by anti-GDF5, anti-BMPR1B and anti-BMPR2 (Fig. 2B). GDF5 immunoreactivity was evident in the nuclei and cytoplasm. BMPR1B and anti-BMPR2 immunoreactivity was evident in cytoplasm. Dendrites, identified by double labelling with anti-MAP2, were particularly strongly labelled by anti-BMPR1B.

GDF5 promotes the growth of hippocampal pyramidal cell dendrites in culture

To investigate the potential role of GDF5 in hippocampal neuron development, we established dissociated cultures from mouse hippocampi at E18, a stage at which the predominant neuron type is the pyramidal cell. As described previously (Kaech and Banker, 2006), our cultures contained >90% pyramidal cells. We examined the effect of GDF5 treatment on axon growth and dendrite growth separately. After 3 days in culture, the single axon that emerges from these neurons is clearly distinguishable from the multiple, short

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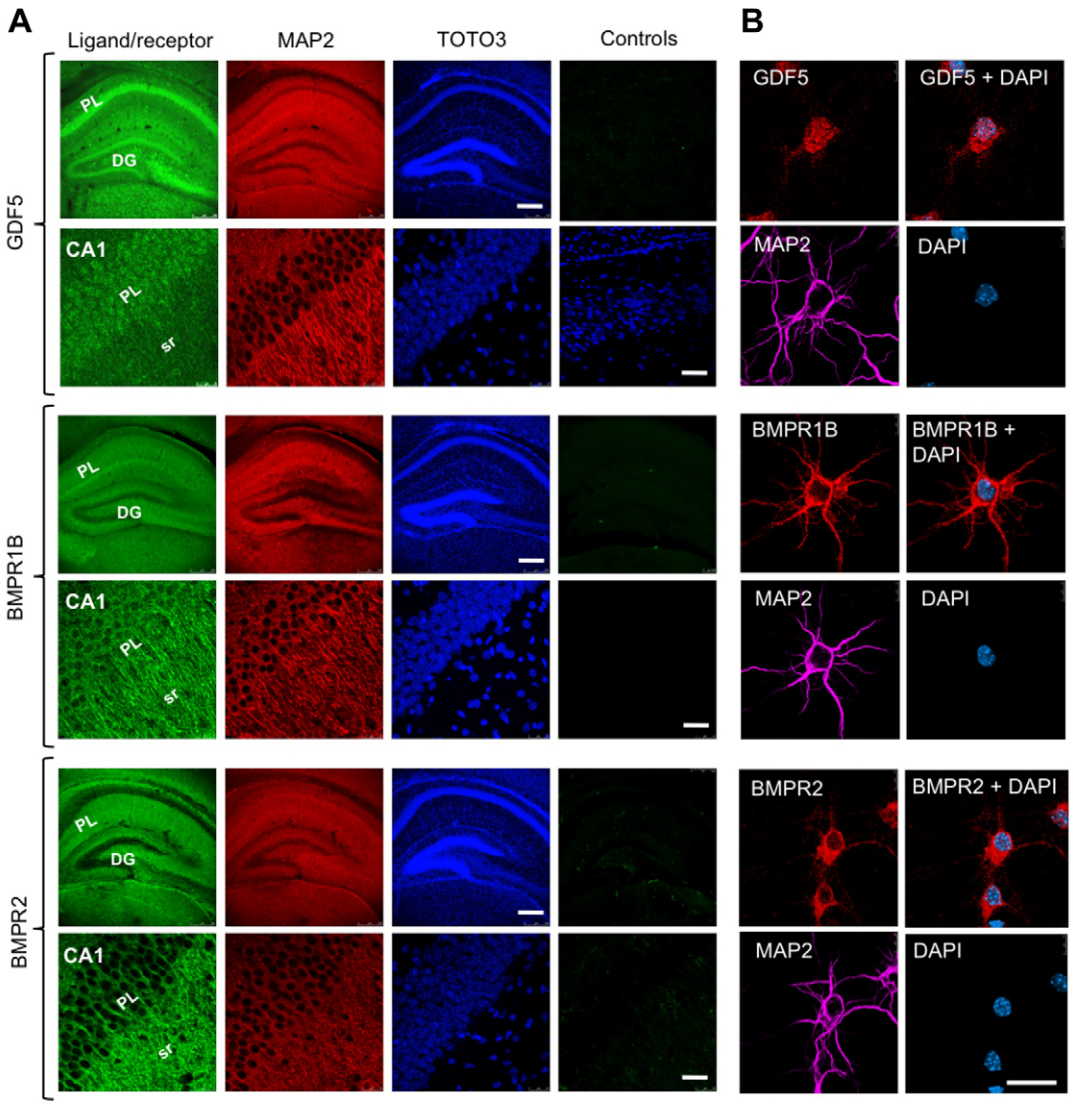

Fig. 2. Localisation of GDF5, BMPR1B and BMPR2 in the developing mouse hippocampus. (A) Sections of P10 hippocampus triple labelled with TOTO-3, anti-MAP2 and either anti-GDF5, anti-BMPR1B or anti-BMPR2. Upper panels show low-power images of CA regions and dentate gyrus and the lower panels show high-power images of CA1. *Gdf5*^{bp} hippocampus was used as a control for anti-GDF5. Controls for anti-BMPR1B and anti-BMPR2 received no primary antibodies. (B) E18 hippocampal neurons double stained with anti-MAP2 and either anti-GDF5, anti-BMPR1B or anti-BMPR2 after 7 days in culture. PL, pyramidal layer; DG, dentate gyrus; sr, stratum radiatum. Scale bars: 200 µm (A, upper panels); 25 µm (A, lower panels; B).

dendrites. To investigate whether GDF5 affects axon growth, the neurons were transfected with a GFP expression plasmid 2 days after plating and were treated with GDF5 for 18 hours prior to fixation and immunostaining for GFP. Examination of multiple neurons revealed no obvious effects of GDF5 treatment on axon length and morphology (Fig. 3A-C). Measurement of axon length revealed no significant differences between control cultures and cultures treated with GDF5 at concentrations ranging from 10 to 1000 ng/ml (Fig. 3B).

After 7 days in culture, MAP2-positive dendrites are well developed. To investigate whether GDF5 treatment affects dendrite growth, neurons were transfected with a GFP expression plasmid 6 days after plating, treated with GDF5 and immunostained for GFP 18 hours later. The dendrite arbours of neurons treated with GDF5 were clearly larger than in control cultures (Fig. 3D-F). To compare the effect of different concentrations of GDF5 on dendrite growth, the percentage of dendrites longer than 50 µm was quantified. This revealed that GDF5 at concentrations ranging from 10 to 1000 ng/ml caused highly significant increases in dendrite elongation, with 100 ng/ml being maximally effective (Fig. 3E). These results show that GDF5 treatment selectively enhances dendrite growth from developing hippocampal pyramidal cells.

To ascertain whether secreted GDF5 affects dendrite elongation, we quantified dendrite elongation after adding function-blocking anti-GDF5. In these experiments, hippocampal cells were cultured for 6 days before adding anti-GDF5 and quantifying the percentage of dendrites longer than 50 µm 24 hours later. Anti-GDF5 caused a highly significant reduction in dendrite elongation compared with IgG-treated control cultures (5.9±0.6 versus 9.39±0.84% dendrites >50 µm, mean ± s.e.m.; P=0.0005, n=150 neurons per condition, three independent cultures, t-test). Anti-GDF5 also eliminated the effect of exogenous GDF5 on dendrite elongation (6.22±0.79% in cultures treated with GDF5 plus anti-GDF5 versus 34.59±1.53% in cultures treated with GDF5 alone). These results suggest that cells secrete sufficient GDF5 to influence dendrite elongation.

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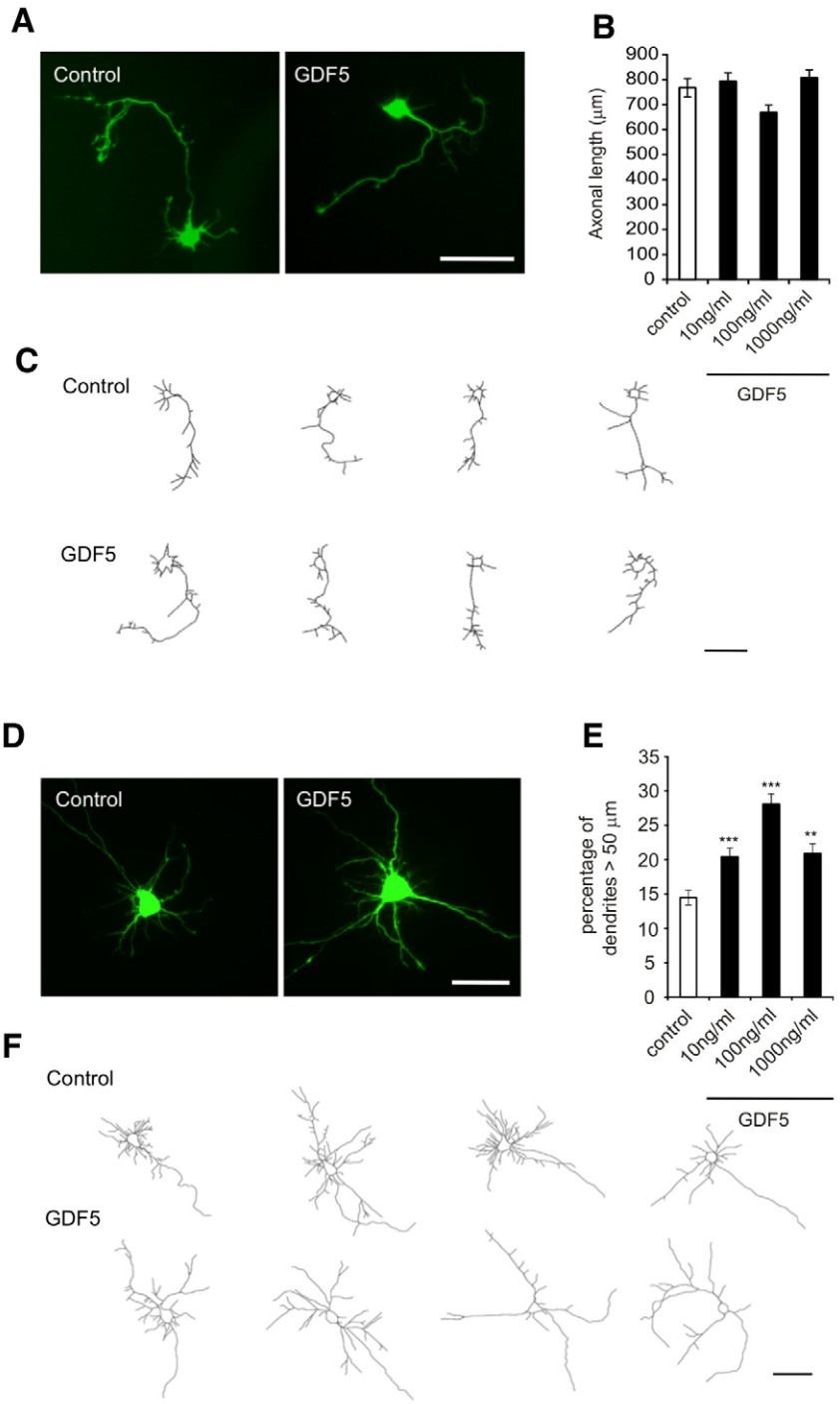

Fig. 3. GDF5 selectively enhances dendrite growth from cultured mouse hippocampal pyramidal cells. (A-C) Axonal growth from E18 neurons after 3 days. (A) Representative neurons incubated with or without 100 ng/ml GDF5. (B) Axon length in control cultures and cultures treated with GDF5. (C) Camera lucida drawings of representative control neurons and neurons treated with 100 ng/ml GDF5. (D-F) Dendrite growth from E18 neurons after 7 days. (D) Representative neurons incubated with and without 100 ng/ml GDF5. (E) Percentage of dendrites longer than 50 μm in control and GDF5-treated cultures. (F) Camera lucida drawings of representative control and GDF5-treated neurons. Mean ± s.e.m. of data from >150 neurons per condition from at least three separate experiments are shown. $^{**}P<0.001$, $^{***}P<0.0001$, statistical comparison with control. Scale bars: 100 μm (A,C); 50 μm (D,F).

BMPR1B and BMPR2 are required for GDF5-promoted dendrite growth

Like other BMPs, GDF5 binds a receptor complex composed of type I and type II BMP receptors (Nishitoh et al., 1996). Although GDF5 binds BMPR1A, BMPR1B and BMPR2, its affinity for BMPR1B is an order of magnitude higher than its affinity for BMPR1A (Nickel et al., 2005).

To determine if the BMPR1B-BMPR2 receptor complex is essential for the effects of GDF5 on dendrite growth, we manipulated the function of these receptors in hippocampal neurons by transfecting them with a plasmid vector that expresses both GFP and a mutated BMP receptor protein. The neurons were transfected after 6 days *in vitro* and were grown for a further 18 hours with or without GDF5 before being immunostained for GFP. GDF5 treatment markedly enhanced dendrite growth from neurons transfected with a vector expressing GFP alone (Fig. 4). Enhanced dendrite growth of a comparable level was observed from neurons grown without GDF5 that had been transfected with a vector that expresses constitutively active BMPR1B, although the extent of dendrite growth was not further enhanced in GDF5-treated neurons expressing constitutively active BMPR1B (Fig. 4A,B). Likewise, overexpression of wild-type BMPR2 significantly enhanced dendrite growth in the absence of GDF5 (Fig. 4C,D). The extent of dendrite growth from neurons expressing dominant-negative versions of either BMPR1B or BMPR2 was not significantly different from control-transfected neurons in the absence of GDF5. However,

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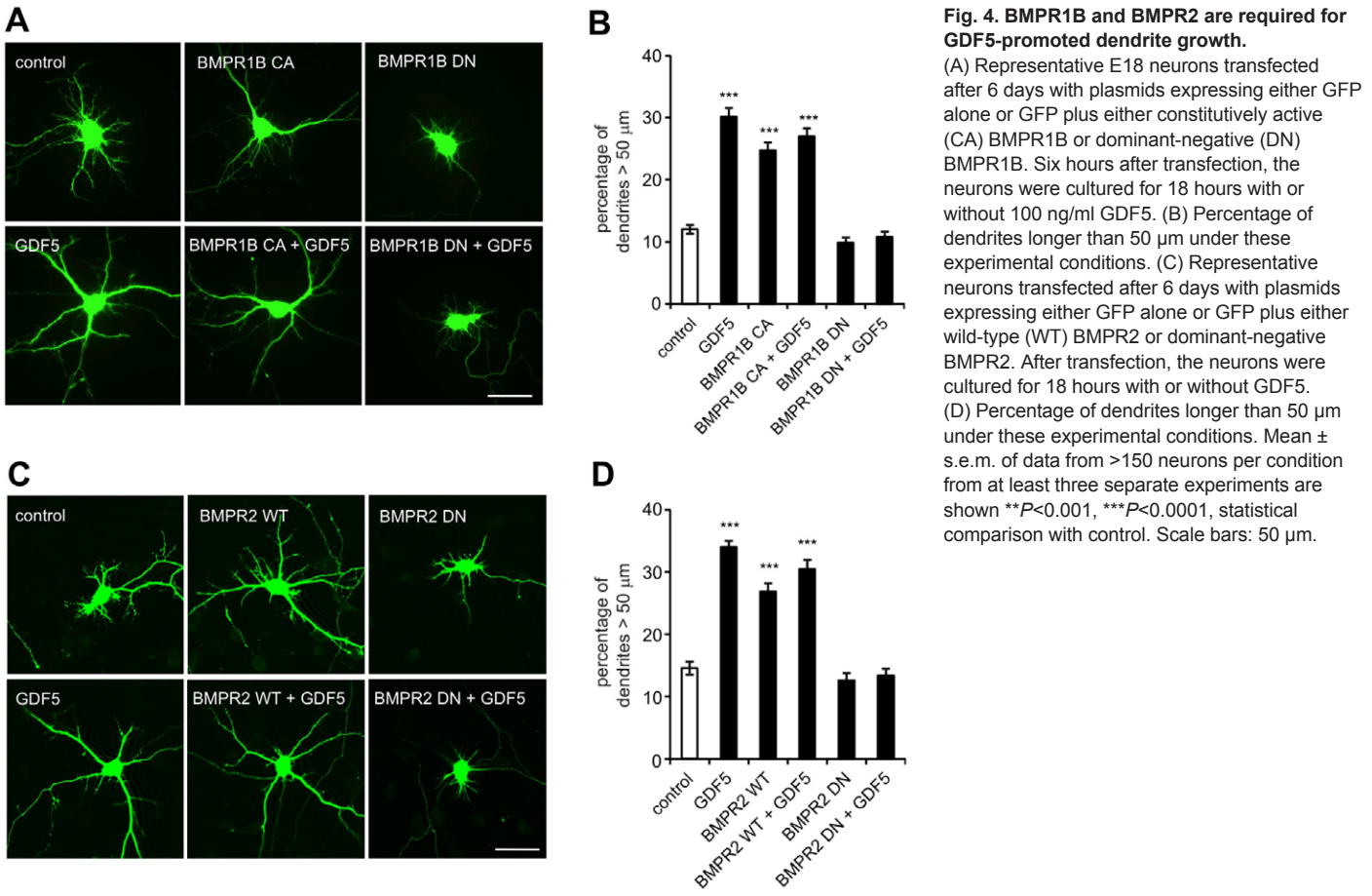

Fig. 4. BMPR1B and BMPR2 are required for GDF5-promoted dendrite growth.
(A) Representative E18 neurons transfected after 6 days with plasmids expressing either GFP alone or GFP plus either constitutively active (CA) BMPR1B or dominant-negative (DN) BMPR1B. Six hours after transfection, the neurons were cultured for 18 hours with or without 100 ng/ml GDF5. (B) Percentage of dendrites longer than 50 µm under these experimental conditions. (C) Representative neurons transfected after 6 days with plasmids expressing either GFP alone or GFP plus either wild-type (WT) BMPR2 or dominant-negative BMPR2. After transfection, the neurons were cultured for 18 hours with or without GDF5. (D) Percentage of dendrites longer than 50 µm under these experimental conditions. Mean ± s.e.m. of data from >150 neurons per condition from at least three separate experiments are shown $^{**}P<0.001$, $^{***}P<0.0001$, statistical comparison with control. Scale bars: 50 µm.

expression of either dominant-negative protein completely inhibited the effect of GDF5 on dendrite growth (Fig. 4). These results suggest that the effect of GDF5 on dendrite growth is mediated by the BMPR2-BMPR1B receptor complex.

GDF5 promotes dendrite growth by activating SMAD signalling

Binding of GDF5 to the BMPR2-BMPR1B receptor complex results in the phosphorylation and activation of the transcription factors SMAD1, SMAD5 and SMAD8 (also known as SMAD9) (Nishitoh et al., 1996). These activated SMADs form a complex with a common mediator SMAD4, which translocates to the nucleus (Ross and Hill, 2008). Alternatively, binding of GDF5 to an independent heterodimeric receptor complex comprising BMPR1B and the receptor tyrosine kinase ROR2 leads to inhibition of SMAD1/5 signalling (Sammar et al., 2004). To ascertain whether GDF5 activates SMAD signalling in hippocampal neurons, we carried out western blot analysis to determine whether SMADs 1, 5 and 8 become phosphorylated following GDF5 treatment. The neurons were cultured for 7 days before being treated with GDF5 for different times prior to protein extraction and western blot analysis. This revealed a marked increase in the levels of phospho-SMADs 1/5/8 within 15 minutes of GDF5 treatment (Fig. 5A,B). GDF5 treatment also resulted in a marked increase in the reporter signal from neurons transfected with a reporter construct in which luciferase is under the control of SMAD-binding elements (Fig. 5C). These findings demonstrate that GDF5 rapidly activates SMAD signalling in hippocampal neurons, leading to SMAD-dependent gene transcription.

To determine the importance of SMAD signalling in GDF5-promoted dendrite growth, we co-transfected hippocampal neurons with a GFP plasmid and either one that expresses an inhibitor of SMAD signalling or an empty control plasmid. To inhibit SMAD signalling, we either overexpressed the inhibitory SMADs SMAD6 and SMAD7 (Hata et al., 1998; Hayashi et al., 1997) or expressed a SMAD4 protein with a C-terminal truncation (Zhang et al., 1996). Dendrite growth from neurons transfected with plasmids expressing either SMAD6 or SMAD7 was not significantly different from control-transfected neurons grown in the absence of GDF5. However, overexpression of either inhibitory SMAD eliminated GDF5-promoted dendrite growth (Fig. 5D,E). Dendrite growth from neurons transfected with a plasmid expressing dominant-negative SMAD4 was significantly less than that from control-transfected neurons grown in the absence of GDF5, and expression of dominant-negative SMAD4 eliminated GDF5-promoted dendrite growth (Fig. 5D,E). These findings suggest that SMAD signalling mediates the effect of GDF5-promoted dendrite growth.

GDF5-promoted dendrite growth depends on HES5 upregulation

Dendrite elongation from cultured hippocampal neurons is promoted by increasing the expression of hairy and enhancer of split 1 and 5 (HES1 and HES5) basic helix-loop-helix transcription factors, which are nuclear targets of Notch signalling (Salama-Cohen et al., 2005). This, together with the demonstration of cross-talk between TGFβ signalling and Notch signalling (Blokzijl et al., 2003; Larrivée et al., 2012; Zavadil et al., 2004), raised the possibility that GDF5 might exert its effects on dendrite growth by upregulating the expression

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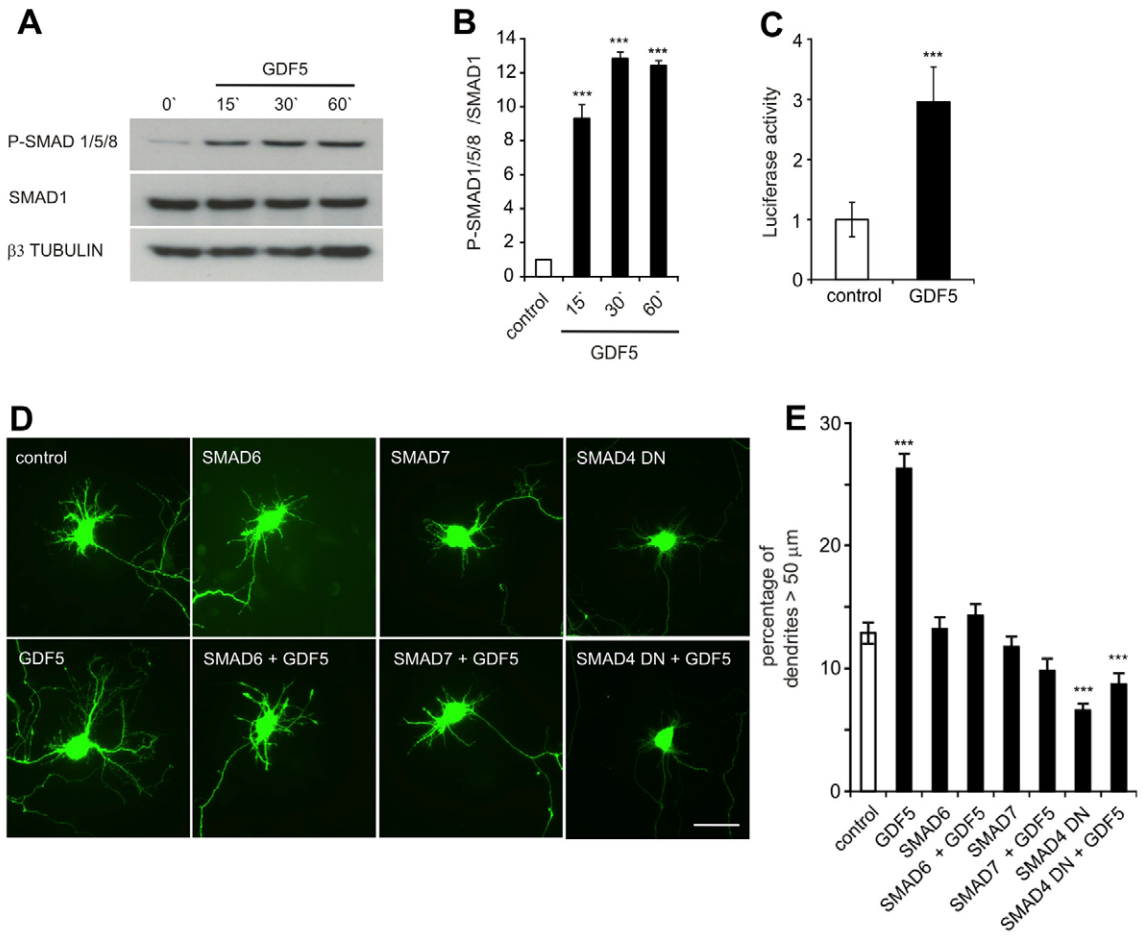

Fig. 5. GDF5 promotes dendrite growth by activating SMAD signalling. (A) Representative western blot probed for phospho-SMADs 1/5/8, total SMAD1 and β3 tubulin in lysates of E18 hippocampal neurons of treated with 100 ng/ml GDF5 after culturing the neurons for 7 days. (B) Levels of phospho-SMAD1/5/8 relative to total SMAD1 from three separate western blot experiments (mean ± s.e.m.). (C) Reporter signal from E18 hippocampal neurons transfected with the SMAD1/5/8-responsive BRE-luciferase reporter after 6 days *in vitro* and cultured for a further 24 hours with or without 100 ng/ml GDF5 (mean ± s.e.m. from three independent experiments, signal normalised to 1 for controls). (D) Representative neurons transfected after 6 days *in vitro* with plasmids expressing either GFP alone or GFP and either SMAD6, SMAD7 or dominant-negative (DN) SMAD4. After transfection, the neurons were cultured for a further 18 hours with or without 100 ng/ml GDF5. (E) Percentage of dendrites longer than 50 µm under these experimental conditions. Mean ± s.e.m. of data from >150 neurons per condition from at least three separate experiments are shown. ***P<0.0001, statistical comparison with control. Scale bar: 50 µm.

of these transcription factors. To test this possibility, we first quantified the levels of *Hes1* and *Hes5* mRNAs in cultured hippocampal neurons following GDF5 treatment. E18 neurons that had been in culture for 6 days were treated with GDF5, and RNA was extracted for QPCR at intervals following treatment. Fig. 6A shows that GDF5 promoted a statistically significant, greater than twofold increase in *Hes5* mRNA. Given this sustained elevation of *Hes5* mRNA, we assayed *Hes5* promoter activity in response to GDF5 by transfecting hippocampal neurons with the *pHes5-pGL3* reporter in which a fragment of the *Hes5* promoter drives expression of luciferase (Nakashima et al., 2001). GDF5 treatment significantly increased the luciferase reporter signal in these neurons, but not in neurons transfected with the *pHes5-SM-pGL3* reporter in which the SMAD-binding consensus sequence in the *Hes5* promoter is mutated and incapable of binding and responding to the activated SMAD complex (Takizawa et al., 2003) (Fig. 6B). These findings suggest that GDF5 increases *Hes5* expression by a SMAD-dependent mechanism in hippocampal neurons.

To determine whether GDF5-promoted HES5 upregulation mediates the effect of GDF5 on dendrite growth, we transfected neurons with a plasmid that expresses *Hes5* shRNA to interfere with HES5 expression (Matsuda et al., 2012). We first confirmed efficiency of HES5 knockdown by this plasmid in HEK 293T cells. Western blot analysis 24 hours after transfection revealed that cells transfected with this plasmid had a much lower level of HES5 protein than did cells transfected with a plasmid that expresses the scrambled RNA sequence (Fig. 5C). In hippocampal neurons, the *Hes5* shRNA plasmid, but not the scrambled sequence plasmid, completely prevented GDF5-promoted dendrite growth (Fig. 6D), suggesting that GDF5-promoted HES5 upregulation is required for the effect of GDF5 on dendrite growth. To confirm the requirement for HES5 in GDF5-promoted dendrite growth, we transfected hippocampal neurons with a plasmid that expresses a truncated HES5 protein that dimerises with endogenous HES5 normally but lacks the DNA-binding domain, thereby interfering with the transcriptional activity of endogenous HES5 (Liu et al., 2006). Dendrite growth from neurons expressing truncated HES5 was not significantly different from that of control-transfected neurons grown in the absence of GDF5. However, truncated HES5 eliminated GDF5-promoted dendrite growth (Fig. 6E).

To ascertain whether upregulation of HES5 is sufficient to enhance dendrite growth in the absence of GDF5, we transfected

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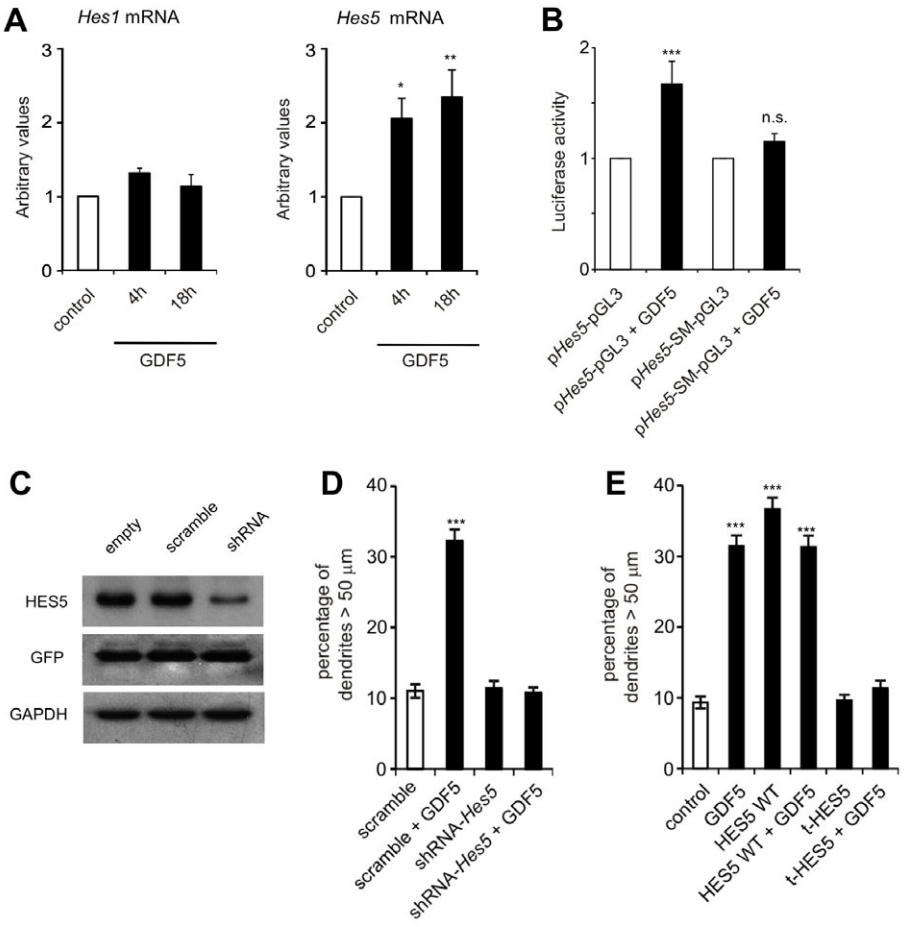

Fig. 6. GDF5 promotes hippocampal dendrite growth by inducing HES5 expression. (A) Levels of *Hes1* and *Hes5* mRNAs relative to reference mRNAs in hippocampal neurons treated with 100 ng/ml of GDF5 for 4 or 18 hours after 6 days in culture. *Hes* mRNA levels are normalised to untreated cultures (controls). (B) Luciferase reporter signal from E18 hippocampal neurons transfected with either the *pHes5-pGL3* or *pHes5-SM-pGL3* reporter after 6 days *in vitro* and cultured for a further 24 hours with or without 100 ng/ml GDF5. The reporter signal in GDF5-treated cultures is normalised to the signal in untreated cultures (controls). (C) Representative western blot probed for HES5, GFP and GAPDH in lysates of HEK 293T cells transfected with either an empty expression plasmid or plasmids expressing either *Hes5* shRNA or a scrambled RNA sequence. (D) Percentage of dendrites >50 µm growing from E18 neurons that were transfected after 6 days *in vitro* with a GFP plasmid together with a plasmid that either expresses *Hes5* shRNA or a *Hes5* shRNA scrambled sequence. After transfection, the neurons were cultured for a further 18 hours with or without GDF5. (E) Percentage of dendrites longer than 50 µm from E18 neurons that were transfected after 6 days *in vitro* with either pGFP alone or pGFP and plasmids that express either wild-type HES5 or truncated HES5. Six hours after transfection, the neurons were cultured for 18 hours with or without 100 ng/ml GDF5. The mean ± s.e.m. of three experiments is shown for each dataset. (A) **P*<0.05, ***P*<0.01, compared with control, REST test. (B,D,E) ****P*<0.0001, compared with control, Mann–Whitney U-test. n.s, not significant.

hippocampal neurons with a plasmid that expresses wild-type HES5. The extent of dendrite growth from neurons overexpressing HES5 was comparable to that of neurons treated with GDF5. Furthermore, dendrite growth from neurons overexpressing HES5 was not further enhanced by GDF5 treatment (Fig. 6E). Together with the findings described above, these results suggest that GDF5-promoted HES5 upregulation is both necessary and sufficient for the effect of GDF5 on dendrite growth.

Because BMP9 increases NGF expression in cultured forebrain neurons (Schnitzler et al., 2010; Lopez-Coviella et al., 2011) and NGF increases the expression of HES1 and HES5 in cultured hippocampal neurons, resulting in enhanced dendrite elongation (Salama-Cohen et al., 2005), we tested whether GDF5 induces NGF expression. In these experiments, E18 neurons were cultured for 6 days prior to treatment with GDF5 for 4 hours or 18 hours. Quantification of *Ngf* mRNA by QPCR revealed no significant changes in *Ngf* mRNA relative to reference mRNAs in cultures treated with GDF5 for 4 hours (0.92±0.076, mean ± s.e.m.) or 18 hours (0.916±0.083, mean ± s.e.m.) compared with untreated control cultures (1.04±0.098, mean ± s.e.m., n=7 experiments). These results suggest that GDF5 does not indirectly influence HES5 expression by regulating NGF expression.

Hippocampal pyramidal dendrites are greatly reduced in size in brachypod mice

To assess the physiological and developmental significance of the effects of GDF5 on dendrite growth, we used Golgi staining to visualise the dendritic arbours of pyramidal cells in the CA1 and CA3 fields of the hippocampus of P10 wild-type and *Gdf5*^{bp} mice. These preparations revealed that pyramidal dendrite arbours of mice that are either heterozygous or homozygous for the *Gdf5*^{bp} mutation were dramatically stunted compared with those of wild-type mice in both CA1 (Fig. 7A) and CA3 (Fig. 7B). Because the dendrite arbours of pyramidal cells in CA1 are normally less exuberant than those of CA3, they were especially amenable to analysis, which was carried out separately on the apical and basal parts of the dendritic arbours of large numbers of these neurons in all three genotypes. This analysis revealed highly significant reductions in total dendrite length and branch point number in both components of the dendrite arbours of

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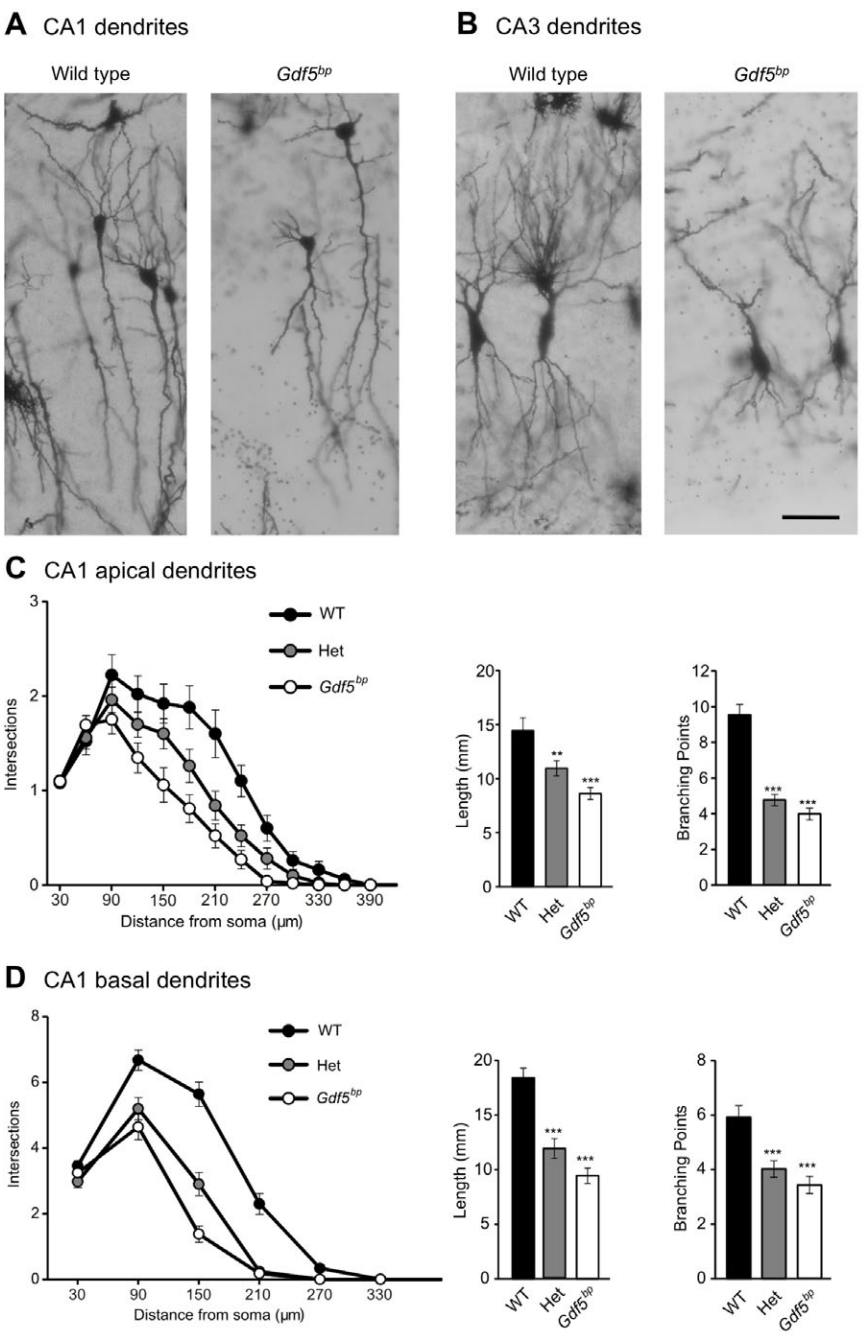

Fig. 7. Reduced size and complexity of pyramidal dendrites in *Gdf5*^{bp} mice. (A,B) Representative images of Golgi-stained preparations of the hippocampi of P10 wild-type and *Gdf5*^{bp} mice showing pyramidal cells in CA1 (A) and CA3 (B). Sholl plots, total length and number of branch points in the apical (C) and basal (D) dendrites of the CA1 field of P10 hippocampi of P10 wild-type, *Gdf5*^{bp} and heterozygous mice. The dendrites of at least 50 neurons from at least six separate mice of each genotype were analysed (mean ± s.e.m., $^{**}P<0.001$, $^{***}P<0.0001$, statistical comparison with wild type). Scale bar: 50 μm.

both heterozygous and homozygous mice compared with wild-type mice (Fig. 7C,D). Accordingly, the reductions in the size and complexity of both dendritic compartments were evident in Sholl plots (Fig. 7C,D). The length and branching data and the Sholl profiles for heterozygous mice were intermediate between those of wild type and homozygous, suggesting a gene dosage effect. These findings indicate that GDF5 plays a major role in regulating the growth and elaboration of pyramidal dendrites in the developing hippocampus *in vivo*.

DISCUSSION

We have demonstrated a crucial role for BMP signalling in controlling dendrite growth and elaboration in the developing nervous system *in vivo*. The dendrites of hippocampal pyramidal cells in postnatal mice lacking GDF5 were markedly stunted compared with those of wild-type mice. The intermediate, though highly significant, reductions in the size and complexity of hippocampal pyramidal dendrites in mice that were heterozygous for the *Gdf5* null mutation demonstrates a gene dosage effect, implying that dendrite size and complexity are exquisitely sensitive to the level of endogenous GDF5 synthesis. Several BMPs, notably BMP7, have been reported to enhance the growth of dendrites from cultured sympathetic (Lein et al., 1995), cortical (Esquenazi et al., 2002; Le Roux et al., 1999), hippocampal (Withers et al., 2000) and striatal projection neurons (Gratacòs et al., 2001). BMP2 and GDF11 also stimulate dendrite initiation from cultured *Xenopus* retinal ganglion cells and blocking BMP/GDF signalling with a secreted antagonist or inhibitory receptors reduces the number of primary dendrites extended *in vivo* (Hocking et al., 2008).

In accordance with the very stunted dendritic arbours of hippocampal pyramidal cells in postnatal *Gdf5*^{bp} mice, we found that

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recombinant GDF5 had a pronounced effect on the dendrite growth *in vitro*. By contrast, GDF5 did not affect axon elongation, suggesting that GDF5 has a selective effect on dendrite growth. Similarly, BMP7 selectively promotes the growth of dendrites from cultured hippocampal pyramidal cells without affecting axon growth (Withers et al., 2000). Both GDF5 and BMP7 bind and signal via the same preferred receptor complex composed of BMPR2 and BMPR1B (de Caestecker, 2004), both of which are expressed by postnatal hippocampal pyramidal cells. There is considerable ligand/receptor promiscuity within the BMP/GDF family of the TGFβ superfamily (de Caestecker, 2004). In addition to GDF5 and BMP7, several other members of this family utilise the BMPR2-BMPR1B receptor complex, including BMP2, BMP4, BMP6, GDF6 (also known as BMP13) and GDF9b (BMP15) (de Caestecker, 2004). However, the pronounced dendritic phenotype of mice lacking GDF5 indicates that this member of the BMP family is a major, physiologically relevant regulator of dendritic growth *in vivo*.

The dendritic arbours of pyramidal cells throughout the hippocampus were markedly stunted in P10 *Gdf5* mutant mice. Quantitative analysis in CA1 indicated that both the apical and basal components of the dendritic arbours were considerably smaller and much less complex than those of age-matched wild-type mice. Although both components of the dendritic arbours of these neurons were markedly stunted in *Gdf5* mutant mice, they were nonetheless morphologically discernible. This suggests that the early differentiation of the dendritic arbour of pyramidal cells into apical and basal components is unaffected in *Gdf5* mutant mice.

We have shown that pyramidal cells express both GDF5 and its preferred receptors, BMPR2 and BMPR1B, during hippocampal development from at least E18 onwards. This raises the possibility that GDF5 acts directly on pyramidal cells by an autocrine mode of action *in vivo*. Autocrine signalling is well documented in the TGFβ superfamily, and has been demonstrated for several BMPs (Cho et al., 2012; Sporn and Todaro, 1980; Thériault and Nachtigal, 2011). In future work it will be important to ascertain how the synthesis of GDF5 is controlled as this has a bearing on understanding how GDF5 participates in the regulation of dendrite growth and elaboration.

Although GDF5 is a secreted protein, we also observed specific GDF5 immunoreactivity in pyramidal cell nuclei. Nuclear GDF5 has been reported in cell lines, and GDF5, BMP2 and BMP4 possess a bipartite nuclear localisation signal that overlaps the cleavage site of the respective proproteins (Felin et al., 2010). *Gdf5*, *Bmp2* and *Bmp4* mRNAs are initiated from alternative start codons to generate proteins that either possess an N-terminal signal peptide and are secreted or lack a signal peptide, retain an intact nuclear localisation signal and are translocated to the nucleus (Felin et al., 2010). Our demonstration that function-blocking anti-GDF5 antibody reduced dendrite growth in high-density cultures of pyramidal cells suggests that GDF5 is secreted in part by these neurons. This, together with our demonstration that exogenous GDF5 increased dendrite growth, implicates secreted GDF5 in dendrite growth regulation. Whether nuclear GDF5 plays any role in dendrite growth regulation or whether it performs some other function remains to be ascertained.

Although the preferred receptor complex for GDF5 consists of BMPR2 and BMPR1B, GDF5 can also signal via the BMPR1A-BMPR2 receptor complex (Nickel et al., 2005) and can also utilise an independent receptor complex consisting of BMPR1B and ROR2 (Sammar et al., 2004). Our demonstration that ectopic expression of dominant-negative versions of either BMPR2 or BMPR1B completely inhibited the effect of GDF5 on dendrite growth suggests that the BMPR1B-BMPR2 receptor complex mediates the effects of GDF5 on pyramidal cells. Furthermore, our finding that expression of either constitutively active BMPR1B or overexpression of wild-type BMPR2 enhanced dendrite growth in the absence of GDF5 suggests that activation of the BMPR1B-BMPR2 receptor complex is sufficient to enhance dendrite growth from cultured hippocampal pyramidal cells. The demonstration that knockdown of ROR2 in cultured hippocampal neurons reduces neurite extension (Paganoni and Ferreira, 2005) raises the possibility that ROR2 might also contribute to the observed GDF5-mediated effects.

Given the importance of the BMPR1B-BMPR2 receptor complex in mediating the effects of GDF5 on dendrite growth *in vitro* together with the grossly abnormal dendritic arbours of hippocampal pyramidal cells in brachypod mice, detailed analysis of the hippocampus of *Bmpr1b* knockout mice will be particularly informative. To date, only abnormalities in chrondrogensis (Yi et al., 2000) and defects in axon guidance and cell survival in the developing retina (Liu et al., 2003) have been described in these mice. An absent or mild pyramidal dendrite phenotype in *Bmpr1b* knockout mice would suggest functional redundancy between BMPR1B and BMPR1A in the regulation of dendrite growth, whereas a more severe dendrite phenotype than that observed in brachypod mice might suggest the involvement of additional GDF or BMP proteins in the control of dendrite growth *in vivo*. Although studies of single *Bmpr1a* and *Bmpr1b* knockout mice have revealed that these receptors perform a variety of distinctive functions *in vivo* (Ahn et al., 2001; Andl et al., 2004; Jamin et al., 2002; Liu et al., 2003; Mishina et al., 1995; Yi et al., 2000), evidence for functional redundancy has come from the finding that in certain instances one receptor can compensate for the loss of the other (Hébert et al., 2003; Wine-Lee et al., 2004). Although an abnormal hippocampal phenotype has not been reported in *Bmpr1b* knockouts, in mice that are constitutively deficient in *Bmpr1b* and conditionally deficient in *Bmpr1a* in the dorsal telencephalon, the dentate gyrus is much smaller than in control mice as a result of decreased production of granule neurons at the peak period of neurogenesis (Caronia et al., 2010).

Depending on the receptor complex utilised, GDF5 can either activate or inhibit SMAD signalling. In accordance with the ability of the BMPR2-BMPR1B complex to activate SMAD signalling, we showed that treating pyramidal cells with GDF5 led to the rapid phosphorylation of the receptor-regulated SMAD proteins SMAD1, SMAD5 and SMAD8, and activation of SMAD-dependent gene transcription. Furthermore, we found that expression of inhibitory SMAD6, inhibitory SMAD7 or truncated SMAD4 completely eliminated GDF5-promoted dendrite growth, suggesting that SMAD signalling is required for GDF5-promoted dendrite growth. Although a link between SMAD activation and dendrite growth has been reported *in vitro*, this is not an invariable link. In cultured sympathetic neurons, BMP7 treatment leads to phosphorylation and nuclear translocation of SMAD1, and expression of dominant-negative SMAD1 inhibits BMP7-induced dendritic growth (Guo et al., 2001). However, in cultured cortical neurons, BMP7-promoted dendrite growth occurs by a SMAD-independent mechanism that involves BMPR2-dependent activation of LIMK1 and JNK (also known as MAPK8) (Lee-Hoeflich et al., 2004; Podkowa et al., 2010). Furthermore, activation of SMAD signalling is not invariably associated with enhanced growth of neural processes. For example, although BMP2 activates SMADs 1, 5 and 8 in cultured cerebellar neurons, BMP2 inhibits neurite growth from these neurons (Matsuura et al., 2007). Thus, although we have demonstrated that SMAD signalling plays a crucial role in mediating the striking effects of GDF5 on pyramidal dendrite growth in the developing hippocampus, SMAD signalling does not provide a consistent link between BMPs and neural process growth. Our finding that dendrite

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growth in cultures not treated with GDF5 is only significantly reduced by dominant-negative SMAD4 but not by dominant-negative BMPR1B, dominant-negative BMPR2, shRNA-*Hes5* or truncated HES5 raises the possibility that other BMP/TGFβ ligands influence hippocampal dendritic growth.

Phosphorylated receptor-regulated SMADs form complexes with SMAD4 that translocate to the nucleus where they regulate the transcription of a wide variety of target genes by binding to the respective promoter elements of these genes (Ross and Hill, 2008). The *Hes5* transcription factor gene is among the many genes that possess a SMAD-binding motif in its promoter (Nakashima et al., 2001), and, together with its homologue *Hes1*, it has been linked to the regulation of dendrite growth by Notch/delta signalling and NGF/$p75^{NTR}$/NF-κB signalling in cultured hippocampal neurons (Salama-Cohen et al., 2006), although induction of Notch target genes by TGFβ signalling mostly involves SMAD2/3 rather than SMAD1/5/8 signalling (Blokzijl et al., 2003; Zavadil et al., 2004). Our demonstration that GDF5 enhances the activity of an intact *Hes5* promoter, but not one that has a disrupted SMAD-binding motif, and that it promotes sustained upregulation of *Hes5* mRNA in hippocampal pyramidal cells suggests that *Hes5* is a transcriptional target of GDF5-promoted SMAD signalling. *Hes5* upregulation plays a key role in mediating the effects GDF5 on dendrite growth because shRNA knockdown of *Hes5* and expression of truncated HES5 completely eliminated the ability of GDF5 to enhance dendrite growth. The extent of dendrite growth from neurons overexpressing HES5 was similar to that promoted by GDF5 treatment, and dendrite growth was not further enhanced by treating HES5-overexpressing pyramidal cells with GDF5. These observations suggest that *Hes5* upregulation is necessary and sufficient for the effect of GDF5 on dendrite elongation.

In summary, we have demonstrated that GDF5 plays a crucial role in regulating the elongation and elaboration of pyramidal cell dendrites in the developing hippocampus *in vivo* and that it does this by SMAD-dependent upregulation of the transcription factor HES5. These findings reveal an unsuspected major function for this cytokine in the establishment of a key feature of neuronal morphology that has a major bearing on central nervous system function.

MATERIALS AND METHODS

Mice

C57BL6/J and brachypod (*Gdf5*^{bp}) mice were obtained from the Jackson Laboratory. Although *Gdf5*^{bp} mice are recognised by their short limb phenotype, wild-type and heterozygous mice are phenotypically indistinguishable and cannot be genotyped. Wild-type mice were obtained by crossing C57BL6/J mice. To generate litters consisting of mice that are homozygous and heterozygous for the *Gdf5*^{bp} mutation, a female *Gdf5*^{bp} mouse was crossed with a male mouse that is heterozygous for the *Gdf5*^{bp} mutation. Heterozygous male mice were identified as phenotypically normal mice that produced litters comprising phenotypically normal mice and *Gdf5*^{bp} mice when crossed with *Gdf5*^{bp} females. All other studies were carried out on tissues obtained from CD-1 mice.

Neuron culture

Hippocampal neuron cultures were prepared as described previously (Dotti et al., 1988; Kaech and Banker, 2006). The neurons grown on polylysine-coated dishes at a density of 50,000 cells/cm^2 in Neurobasal medium (Invitrogen) supplemented with 2% B27 (Invitrogen) and 0.5 mM GlutaMAX I (Invitrogen).

Neuronal transfection

After 2 or 6 days *in vitro*, 1.2 μg of DNA mixed with 4 μl of Lipofectamine (Invitrogen) was added to the cultures, which were washed with culture medium after 3 hours.

Expression vectors and reporter constructs

The vector expressing GFP (pCDH-CMV-MCS-EF1-copGFP) was obtained from Cambridge Bioscience; vectors expressing constitutively active and dominant-negative forms of BMPR1B (pcDNA4-BMPR1B-Q203D and pcDNA4-BMPR1B-Q203/D265A) (Ho and Bernard, 2009) were provided by Daniel Bernard; and vectors expressing wild-type and dominant-negative forms of BMPR2 (pEGFPN1-BMPR2-WT and pEGFPN1 BMPR2-D485G) (Jiang et al., 2011) were provided by Nicholas Morrell. To generate pCDH-CMV-BMPR1B-Q203D-EF1-copGFP, pCDH-CMV-BMPR1B-Q203D/D265A-EF1-copGFP, pCDH-CMV-BMPR2-WT-EF1-copGFP and pCDH-CMV-BMPR2-D485G-EF1-copGFP, the corresponding cDNA sequences were inserted in pCDH-CMV-MCS-EF1-copGFP. Vectors expressing SMAD6 (pCS2-SMAD6-FLAG) (Hata et al., 1998), SMAD7 (pCMV5-SMAD7-HA) (Hayashi et al., 1997) and truncated SMAD4 (pRK-DPC4-deltaC-Flag) (Zhang et al., 1996) were obtained from Addgene (plasmids 14960, 11733 and 12628, respectively). The vector expressing HES5 (pCLIG-HES5) (Hojo et al., 2000) was provided by Ryoichiro Kageyama. To obtain pCDH-CMV-HES5-EF1-copGFP, the corresponding cDNA encoding sequence was inserted in pCDH-CMV-MCS-EF1-copGFP. To obtain pCDH-CMV-HES5 DN-EF1-copGFP, the sequence lacking the DNA-binding domain was inserted in pCDH-CMV-MCS-EF1-copGFP. For pGFP-VRS shRNA HES5, the targeting sequence 5′-GACACGCA-GATGAAGCTGCTTTACCACTT-3′ was inserted in the pGFP-VRS vector (Origene Technologies, Rockville, MD, USA). For the scramble construct, 5′-GCACTACCAGAGCTAACTCAGATAGTACT-3′ was used.

Reporter gene studies were carried out using the Dual-Luciferase Reporter Assay Kit (Promega). Two hundred thousand neurons/well were transfected using Lipofectamine and 1.25 μg of the corresponding reporter plasmid carrying firefly luciferase (*pGL3-BRE-Luc*) (Liu et al., 2009) provided by Xiao-Fan Wang or *pHes5-pGL3-Luc* or *pHes5-SM-pGL3-Luc* (Takizawa et al., 2003) provided by Tetsuya Taga and 125 ng of a plasmid carrying *Renilla* luciferase (phRL-TKluc, Promega). After 18-24 hours, luciferase and *Renilla* activities were quantified using a GloMax 20/20 Luminometer (Promega).

Analysis of axon and dendrite growth

Eighteen hours after transfection, neurons were fixed for 30 minutes in 4% paraformaldehyde, permeabilised for 15 minutes with 0.5% Triton X-100 in PBS and blocked for 1 hour with 10% goat serum in PBS. The neurons were incubated overnight at 4°C with primary antibodies (anti-Cop/turbo GFP, 1:250, TA150041, Origene Technologies; mouse anti-FLAG, 1:500, F3165, Sigma-Aldrich; or mouse anti-HA, 1:250, ab18181, Abcam) in PBS containing 10% goat serum and 0.1% Triton X-100. After washing, the cultures were incubated with secondary antibody (Alexa Fluor488 goat anti-mouse IgG, Alexa Fluor488 goat anti-rabbit IgG or Alexa Fluor546 goat anti-rabbit IgG, 1:500) (Invitrogen) in PBS containing 10% goat serum and 0.1% Triton X-100. Labelled neurons were visualised using an Axioplan confocal microscope (Zeiss). For the analysis of axonal length, neurons were transfected with GFP-plasmids after 2 days *in vitro* and were fixed in 4% paraformaldehyde after a further 24-hour incubation. Axonal length was measured using ImageJ software. The analysis of dendrite morphology was performed as described previously (Salama-Cohen et al., 2005).

shRNA efficiency experiments

To check the efficiency of HES5 knockdown, HEK 293T cells (ATCC) were seeded in 12-well plates (100,000 cells/well) in DMEM containing 10% fetal bovine serum and 2 mM GlutaMAX. The following day, the cells were co-transfected with pCDH-CMV-HES5-EF1-GFP with either pGFP-VRS-shRNA Hes5, pGFP-VRS-scramble or empty pGFP-VRS using the calcium phosphate method (Sambrook and Russell, 2001). After 24 hours, the cells were lysed and the proteins were separated on 12% SDS-PAGE gels and transferred to PVDF membranes that were probed with either rabbit anti-HES5 (1:500, ab25374, Abcam), mouse anti-Cop/turbo GFP (1:1000, TA150041, Origene Technologies) or rabbit anti-GAPDH (1:500, 247 002, Synaptic Systems).

Golgi preparations

Modified Golgi-Cox impregnation of neurons was performed using the FD Rapid GolgiStain kit (FD NeuroTechnologies) on 80 μm sections of P10

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mouse brains. Sholl analysis (Sholl, 1953) was carried out separately on the apical and basal dendritic arbours of pyramidal cells in the CA1 field.

Immunoblotting

Neurons (200,000 cells/well) were lysed in RIPA buffer containing proteinase and phosphatase inhibitor cocktail mix (Sigma-Aldrich) and insoluble debris was removed by centrifugation. Equal quantities of protein were separated on 10% SDS-PAGE gels and were transferred to PVDF membranes using the Bio-Rad Trans-Blot. The blots were probed with either rabbit anti-phospho-SMAD1/SMAD5/SMAD8, anti-total SMAD1 (1:1000, #9511 and #9743, respectively, Cell Signaling) or anti-β3 tubulin (1:10,000, MAB1195, R&D), anti-GDF5, anti-BMPR1B, anti-BMPR2 (1:200, ab38546, ab78417 and ab96826, respectively, Abcam) or anti-GAPDH (1:500, 247 002, Synaptic Systems). Binding of primary antibodies was visualised with HRP-conjugated secondary antibodies (1:5000, W4011or W4021, Promega) and Chemiluminescence Luminol Reagent (Santa Cruz Biotechnology). Densitometry was carried out using Gel-Pro Analyzer (Media Cybernetics).

Immunohistochemistry and immunocytochemistry

For immunohistochemistry, brains were fixed in 4% paraformaldehyde for 24 hours, cryoprotected in 30% sucrose, embedded in OCT and snap frozen in isopentene cooled with dry ice and then serially sectioned at 15 µm. Sections were blocked for 1 hour in PBS containing 5% goat serum, 2% bovine serum albumin and 0.1% Triton X-100, then incubated for 18 hours at 4°C with primary antibodies in blocking buffer diluted fivefold. Primary antibodies were: anti-GDF5, anti-BMPR1B, anti-BMPR2 (1:100, ab93855, ab78417 and ab124463, respectively, Abcam) and anti-MAP2 (1:250, ab5392, Abcam). After incubation, sections were washed then incubated for 1 hour with biotin-conjugated rabbit IgG (1:500, Vector Laboratories). After a 30-minute incubation with DyLight 488-conjugated streptavidin (1:500, Vector Laboratories), the sections were washed and mounted. Nuclei were counterstained with TOTO-3 (1:10,000 Invitrogen). For immunocytochemistry, cultured neurons (50,000 cells/cm^2) were fixed for 30 minutes in 4% paraformaldehyde, permeabilised for 15 minutes with 0.5% Triton X-100 in PBS and blocked for 1 hour with 10% goat serum in PBS containing 0.1% Triton X-100. Neurons were then incubated with the same primary antibodies at 4°C in PBS containing 10% goat serum and 0.1% Triton X-100. After washing, the cells were incubated with secondary antibody (either Alexa Fluor546 goat-anti rabbit IgG, Alexa Fluor546 goat anti-mouse IgG or Alexa Fluor647 goat anti-chicken IgG, 1:500, Invitrogen) in PBS containing 10% goat serum and 0.1% Triton X-100.

QPCR

Gdf5, *Bmpr1b*, *Bmpr2* and *Ngf* mRNAs were quantified relative to a geometric mean of mRNAs for glyceraldehyde phosphate dehydrogenase (*Gapdh*) and succinate dehydrogenase (*Sdha*). Total RNA was extracted using the RNeasy Mini Lipid extraction kit (Qiagen) and 5 µl was reverse transcribed for 1 hour at 45°C using the AffinityScript kit (Agilent) in a 25 µl reaction. cDNA (2 µl) was amplified in a 20 µl reaction volume using Brilliant III Ultrafast QPCR Master Mix reagents (Agilent). QPCR products were detected using dual-labelled (FAM/BHQ1) hybridisation probes (MWG/Eurofins). The PCR primers were: *Gdf5* forward, 5′-TAATGAACTCTATGGACC-3′ and reverse, 5′-GATGAAGAGGAT-GCTAAT-3′; *Bmpr1b* forward, 5′-AGTGTAATAAAGACCTCCA-3′ and reverse, 5′-AACTACAGACAGTCACAG-3′; *Bmpr2* forward, 5′-ACTAGAGGACTGGCTTAT-3′ and reverse, 5′-CCAAAGTCACTG-ATAACAC-3′; *Ngf* forward. 5′-AAACGGAGACTCCACTCACC-3′ and reverse, 5′-GTCCTGTTGAAAGGGATTGTACC-3′; *Gapdh* forward, 5′-GAGAAACCTGCCAAGTATG-3′ and reverse, 5′-GGAGTTGCTGTT-GAAGTC-3′; *Sdha* forward, 5′-GGAACACTCCAAAAACAG-3′ and reverse, 5′-CCACAGCATCAAATTCAT-3′. Dual-labelled probes were: *Gdf5*, FAM-TGAATCCACACCACCCACTTG-BHQ1; *Bmpr1b*, FAM-CCACTCTGCCTCCTCTCAAG-BHQ1; *Bmpr2*, FAM-CACAGAAT-TACCACGAGGAGA-BHQ1; *Ngf*, FAM-TGTTCAGCACCCAGCC-TCCACCCA-BHQ1; *Gapdh*, FAM-AGACAACCTGGTCCTCAGTGT-BHQ1; *Sdha*, FAM-CCTGCGGCTTTCACTTCTCT-BHQ1. Forward and reverse primers were used at 150 nM and dual-labelled probes were used at 300 nM. PCR was performed using the Mx3000P platform (Agilent) using the following conditions: 45 cycles of 95°C for 12 seconds and 60°C for 35 seconds. Standard curves were generated in every 96-well plate, for each cDNA in each PCR run, by using serial threefold dilutions of reverse-transcribed adult mouse brain total RNA (Ambion). TaqMan probes and primers for *Hes1* and *Hes5* and the housekeeping gene ribosomal (r)*RNA 18S* (*Rn18s*) were selected as the Assay-on-Demand gene expression products (Applied Biosystems-Invitrogen Life Technologies).

Statistical analysis

Pair-wise comparisons were made using Student's *t*-test and Mann–Whitney U-test. The relative expression software tool (REST) method was used for statistical analysis of real-time PCR data (Pfaffl et al., 2002).

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Competing interests

The authors declare no competing financial interests.

Author contributions

C.O. and P.J.C. conducted the majority of the cell culture experiments; L.K. and C.O. analysed the Gdf-5bp mice; P.J.C. carried out the immunocytochemical studies; P.J.C. and M.W. performed the immunohistochemical studies; S.W. carried out QPCR; C.O. and A.M.D. wrote the manuscript; A.R.-T. and A.M.D. supervised the project.

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