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Effects of growth/differentiation factor 5 on the survival and morphology of embryonic rat midbrain dopaminergic neurones *in vitro*

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Abstract

Growth/differentiation factor 5 (GDF5) is a member of the transforming growth factor-β superfamily that is expressed in the developing CNS, including the ventral mesencephalon (VM). GDF5 has been shown to increase the survival of dopaminergic neurones in animal models of Parkinson's disease. This study was aimed at characterising the effects of GDF5 on dopaminergic neurones *in vitro*. Treatment with GDF5 induced a three-fold increase in the number of dopaminergic neurones in embryonic day 14 rat VM cultures after six days *in vitro*. A significant increase was also observed in the numbers of astrocytes in GDF5-treated cultures. GDF5 treatment also had significant effects on the morphology of dopaminergic neurones in these cultures; total neurite length, number of branch points and somal area were all significantly increased after six days *in vitro*. Analysis of neurite length and numbers of branch points at each level of the neuritic field revealed that the most pronounced effects of GDF5 were on the secondary and tertiary levels of the neuritic field. The specific type I receptor for GDF5, bone morphogenetic protein receptor (BMPR)-Ib, was found to be strongly expressed in freshly-dissected E14 VM tissue, but its expression was lost with increasing time in culture. Accordingly, treatment with GDF5 for 24 h from the time of plating induced increases in the numbers of dopaminergic neurones, while treatment with GDF5 for 24 h after six days *in vitro* did not. This study shows that GDF5 can promote both the survival and morphological differentiation of VM dopaminergic neurones *in vitro*, lending support to its potential as a candidate dopaminergic neurotrophin for use in the treatment of Parkinson's disease.

Introduction

The transforming growth factor-β (TGF-β) superfamily is a large family of structurally related growth factors. It can be divided into two main groups, based on phylogenetic relationships; the TGF-β/activin group and the bone morphogenic protein (BMP)/growth differentiation factor (GDF) group (for recent review, see de Caestecker, 2004). The BMPs represent one of the largest families of the TGF-β superfamily and have been shown to have effects on a variety of neuronal populations (for reviews, see Mehler *et al.*, 1997; Ebendal *et al.*, 1998). Various BMPs, as well as their receptors (BMPRs) and antagonists, have been shown to be expressed in the adult rat brain, including the substantia nigra (SN) and the striatum, suggesting that BMPs may modulate the function of these regions in adulthood (Tomizawa *et al.*, 1995; Soderstrom *et al.*, 1996; Mehler *et al.*, 1997; Zhang *et al.*, 1998; Soderstrom & Ebendal, 1999; Charytoniuk *et al.*, 2000; Strelau *et al.*, 2000; Chen *et al.*, 2003). BMP-2, -4 and -6 are expressed in the developing rat ventral mesencephalon (VM) at embryonic day (E) 13 and E15 (Jordan *et al.*, 1997). Recently, it has been reported that BMP-5, -6 and -7 increase the numbers of dopaminergic neurones in cultures of E14 rat VM (Brederlau *et al.*, 2002). These studies suggest that BMP family members may be involved in dopaminergic neurogenesis and/or provide neurotrophic support in the developing VM.

The GDFs belong to the same subfamily of the TGF-β superfamily as the BMPs and have been shown to be involved in a variety of developmental processes, ranging from chondrogenesis in the developing limb to the regulation of ovarian follicle development (for recent reviews, see Edwards & Francis-West, 2001; Balemans & Van Hul, 2002; Knight & Glister, 2003). The role of GDF5 in limb development has been

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well established (for reviews, see Luyten, 1997; Buxton *et al.*, 2001) and mutations in the GDF5 gene result in skeletal abnormalities in mice (Storm *et al.*, 1994) and humans (Thomas *et al.*, 1996). Recently it has emerged that a number of the GDFs are expressed in the nervous system (Lee, 1991; Bottner *et al.*, 1999; Nakashima *et al.*, 1999; Soderstrom & Ebendal, 1999; Zhao *et al.*, 1999; Vokes & Krieg, 2000; Schober *et al.*, 2001; Watakabe *et al.*, 2001). We (O'Keeffe *et al.*, 2004) and others (Krieglstein *et al.*, 1995a) have shown that GDF5 is expressed in the developing rat brain. Further, we have found that GDF5 is expressed in the developing rat VM during the period of dopaminergic neurogenesis, which peaks on E14 (O'Keeffe *et al.*, 2004). It has been shown that GDF5 can protect E14 rat VM dopaminergic neurones from neurotoxic agents (Krieglstein *et al.*, 1995b; Lingor *et al.*, 1999). Furthermore, GDF5 has neuroprotective effects on the adult nigrostriatal dopaminergic pathway in the 6-hydroxydopamine (6-OHDA) rat model of Parkinson's disease (PD) (Sullivan *et al.*, 1997, 1999; Hurley *et al.*, 2004) and improves the survival of grafts of E14 rat dopaminergic neurones in this rat model of PD (Sullivan *et al.*, 1998). Together, these studies suggest that GDF5 may act as a dopaminergic neurotrophin, both during the development of the VM and in the adult brain. Although GDF5 has been previously shown to increase the survival of dopaminergic neurones *in vitro* (Krieglstein *et al.*, 1995a), the effects of GDF5 on the morphological development of these neurones has not been reported. Here we show that GDF5 not only increases the numbers of dopaminergic neurones in cultures of E14 rat VM, but that it also has distinct effects on the morphological development of these neurones, by increasing their neuritic length and branching.

In contrast to our findings, a recent study has reported that BMP-5, -6 and -7, but not GDF5, induce increases in the numbers of dopaminergic neurones in E14 rat VM cultures (Brederlau *et al.*, 2002). One possible explanation for this discrepancy may be the timing of application of GDF5 to the cultures; that is, there may be changes in receptor expression during maturation of the cultures. We addressed this issue by examining the expression of the GDF5 receptor, BMPR-Ib, in freshly-dissected E14 VM tissue, as well as in E14 VM cultures. We found that the expression of BMPR-Ib was lost by six days *in vitro*, the time at which GDF5 was applied in the Brederlau study; this may explain the lack of an increase in dopaminergic neurones in response to GDF5 application in that study.

The results of our study suggest that, during the development of the VM, GDF5 may act as a dopaminergic neurotrophin and play a role in the morphological development of these neurones. Our data, taken together with those of the above-mentioned study (Brederlau *et al.*, 2002), suggest that the effects of GDF5 on these neurones are exerted at an earlier stage of development than those of the BMPs. These findings substantiate previous studies which showed that GDF5 can exert powerful protective effects on adult rat dopaminergic neurones and improve the survival of grafted embryonic dopaminergic neurones, suggesting that GDF5 may be a candidate dopaminergic neurotrophin for use in the treatment of PD.

Methods

E14 RAT VM CULTURES

E14 Sprague-Dawley rats were obtained by laparotomy under terminal anaesthesia induced by sodium barbiturate (150 mg/kg, i.p.). The embryos were decapitated and each VM dissected out and placed in ice-cold HBSS (Sigma). The tissue was incubated in 5 ml 0.1% trypsin-EDTA (Sigma) for 10 min at 37°C. 5 ml of DNase (25 μg/ml) was added and the tissue incubated at 37°C for a further 5 min. Soyabean trypsin inhibitor (0.5 mg/ml; Sigma) was then added and the tissue was triturated using fire-polished glass Pasteur pipettes. Following centrifugation at 1000 $\times$ g for 5 min, cells were resuspended in 1 ml of medium (Dulbecco's Modified Eagle's Medium (DMEM): Ham's F12 (1:1), with 2 mM L-glutamine, 1% PSF, 33 mM D-glucose, 1% foetal calf serum (all from Sigma), 2% B-27 (Gibco)). Cells were plated onto poly-D-lysine (0.1 mg/ml; Sigma) -coated four-chamber slides (Nunc), at a density of 1×10^5 cells per well, and incubated at 37°C, 5% CO_2. GDF5 (1 ng/ml or 10 ng/ml) was added to the culture medium at the time of plating (0 days *in vitro* (DIV)) and replaced every two days, then the cultures were processed for immunocytochemistry (as described below) after 6 DIV. In separate experiments, GDF5 (10 ng/ml) was added for 24 h at either the time of plating or after 6 DIV, before the cultures were processed for immunocytochemistry at 24 h or 7 DIV, respectively.

IMMUNOCYTOCHEMISTRY

Medium was removed from cultures, the cultures were washed and fixed in 4% paraformaldehyde (Sigma) for 20 min at room temperature, then washed three times in 10 mM phosphate-buffered saline (PBS) with 0.02% Triton-X (PBSTx). Cultures were incubated in blocking solution (5% normal horse serum, 0.2% Triton-X in 10 mM PBS) for 1 h at room temperature, then incubated in antiserum to either glial fibrillary acidic protein (GFAP, a glial cell marker) (1:200; Sigma), tyrosine hydroxylase (TH; a dopaminergic neuronal marker) (1:200; Chemicon), BMPR-Ib (1:10; Santa-Cruz), BMPR-II (1:10; Santa-Cruz) or BMPR-Ia (1:10; Santa-Cruz) diluted in blocking solution at 4°C overnight. Following removal of the primary antibody, cultures were then washed three times in PBSTx, before being incubated in secondary antibody (anti-rabbit IgG-FITC 1:50; Sigma) in blocking solution for 1 h at room temperature. Following three washes in PBSTx, cultures were counterstained with bisbenzimide (10 μg/ml; Sigma). For each experiment, separate cultures ("negative controls") were incubated in blocking solution alone, to control for non-specific binding of each of the primary antibodies. These cultures consistently displayed no

staining after being incubated in the FITC-linked secondary antibody.

WESTERN BLOTTING

Medium was removed from cultures, the cultures were washed and processed for Western blotting as described previously (O'Keeffe *et al.*, 2004). 30 μg aliquots of each sample were processed for immunoblotting, blots were incubated with primary antibodies at the following dilutions in 1% BSA in 10 mM PBS containing 0.1% tween-20: monoclonal antibody to β-III tubulin (Promega; 1:40,000), monoclonal antibody to GFAP (Sigma; 1:1000), rabbit anti-serum to TH (Chemicon; 1:1,500) and rabbit anti-serum to β-actin (Santa-Cruz; 1:10,000). The appropriate peroxidase-linked secondary antibody (Amersham) was used to detect each primary antibody on the blots and staining was visualised using ECL-plus (Amersham), as previously described (O'Keeffe *et al.*, 2004).

REVERSE TRANSCRIPTION (RT)-PCR

The VM of E14 Sprague-Dawley rat embryos were dissected as described above and stored at 4°C in "RNA Later" solution (Sigma) until RNA extraction was performed. Medium was removed from E14 rat VM cultures after 1, 2, 4 or 6 DIV, the cultures were washed in 10 mM PBS and stored in "RNAlater" solution as above. Total RNA was extracted from each sample using the "RNeasy" kit (Qiagen), according to the manufacturer's instructions. 1 μg of total RNA was reversed transcribed using the ImProm-II^{TM} reverse transcription system (Promega). PCR reactions were carried out in 25 μl volumes containing 50 mM KCl, 2 mM $MgCl_2$, 2 μl cDNA, 10 mM Tris pH 8.3, 200 μM dNTPs, 800 nM of each primer and 1U Taq. Primers for amplification of BMPR-Ib mRNA were designed using the Primer Select^{TM} program. The following primers were used to amplify mRNA for BMPR-II (Chen *et al.*, 2003) and BMPR-Ia (Panchision *et al.*, 2001).

Target mRNA	Sense Primer (5′ to 3′ direction)	Anti-sense primer (5′ to 3′ direction)
BMPR-II	GCTTCGCAGAA TCAAGAACG	GTGGACTGAGTGG TGTTGTG
BMPR-Ib	ACACGCCCATCCC TCATCAGA	ACGCCACTTTCCCA TCCACACT
BMPR-Ia	ACAGAAATTTAT GGCACGCAA	CATACGCAAAG AACAGCATCTC

Thirty-four cycles of PCR were performed with the following temperatures; denaturation at 94°C for 30 s, annealing at 57°C (BMPR-II and BMPR-Ib) or 55.1°C (BMPR-Ia) for 30 s and elongation at 72°C for 45 s. A final elongation step was performed at 72°C for 5 min. 20 μl of each PCR product was run on a 1% agarose-TBE gel.

ANALYSIS OF CELLULAR MORPHOLOGY

Fifty neurones per treatment group (control, 1 ng/ml and 10 ng/ml GDF5) from three independent experiments were analysed using established stereological methods (for review, see Mayhew, 1991). The formulas used to calculate neurite length and cell somal area were as follows:

$$\text{Neurite length} = n \times T \times \pi/2$$

$$\text{Somal area} = n \times B$$

Where n = the number of times the neurites intersect the grid lines, T = the distance between the gridlines (taking the magnification into account) and B = the area associated with each point (taking the magnification into account). An assessment of branching was done by counting the numbers of "nodes" per cell. Primary nodes were considered to be branches from the cell body, secondary nodes were considered branches from primary neurites and so on for tertiary and quarternary nodes. Data from these anaylses are presented as mean ± standard error of the mean (SEM) per dopaminergic neurone.

STATISTICAL ANALYSIS

Stastical comparison of cell counts and of morphological complexity of dopaminergic neurones was performed using ANOVA with post-hoc Tukey's test. Differences were considered significant at $P < 0.05$. Data are presented as mean ± SEM.

Results

GDF5 INCREASES THE NUMBERS OF DOPAMINERGIC NEURONES AND ASTROCYTES IN E14 RAT VM CULTURES

Treatment of E14 VM cultures for 6 DIV with GDF5 resulted in an increase in the numbers of TH-immunopositive neurones surviving for this period *in vitro* (Fig. 1A, B, E). Application of GDF5 at doses of 1 ng/ml and 10 ng/ml resulted in significant increases (3.1- and 2.6-fold, respectively) in the number of dopaminergic neurones, when compared to control values (Fig. 1E; $P < 0.001$). No significant difference was observed between the numbers of dopaminergic neurones in cultures treated with 1 ng/ml and 10 ng/ml GDF5.

GDF5 treatment for 6 DIV also induced a marked increase in the numbers of GFAP-immunopositive cells in the cultures (Fig. 1C, D, F). Treatment with GDF5 at doses of 1 ng/ml and 10 ng/ml resulted in significant increases (2.3- and 5.6-fold, respectively) in the number of astrocytes, when compared to control values (Fig. 1F; $P < 0.001$). In contrast to GDF5's effect on the numbers of dopaminergic neurones, its effect on astrocytes was dose-dependent, with a significant difference observed between the numbers of GFAP-immunopositive cells in cultures which had been treated with 1 ng/ml and 10 ng/ml GDF5 (Fig. 1F; $P < 0.001$).

There was no significant difference in either the total number of cells (as determined by bisbenzimide staining) or the number of β-tubulin- immunopositive cells between any of the treated or untreated cultures (data not shown).

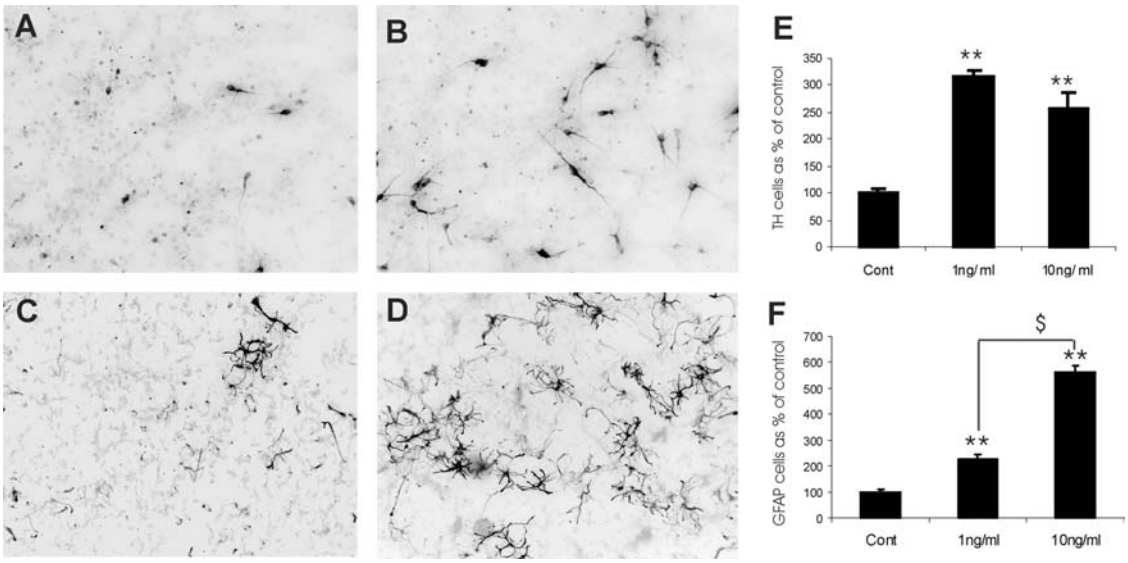

Fig. 1. Representative photomicrographs of (A, C) untreated and (B, D) GDF5 (10 ng/ml)-treated E14 rat VM cultures after 6 DIV, stained immuncytochemically for (A, B) TH or (C, D) GFAP. Treatment with GDF5 at doses of either 1 ng/ml or 10 ng/ml induced a significant increase in the percentage of total cells which were (E) TH-immunopositive and (F) GFAP-immunopositive. Data are expressed as mean ± SEM from three independent experiments. $^{**}P < 0.001$ vs. untreated cultures; \$ $P < 0.001$.

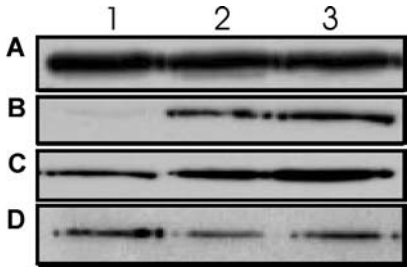

Fig. 2. Representative Western blots performed on protein extracts of E14 rat VM cultures after 6 DIV, immunostained for (A) β-III tubulin, (B) GFAP (C) TH and (D) β-actin. Lane 1 contained extracts from untreated cultures; lane 2, cultures treated with 1 ng/ml GDF5; lane 3, cultures treated with 10 ng/ml GDF5. Densitometric analysis (after normalisation to β-actin levels for the corresponding sample) showed that GDF5 treatment induced increases in the levels of expression of both GFAP and TH, but did not change β-tubulin expression levels.

The effects of GDF5 appeared to be predominantly exerted on dopaminergic neurones and astrocytes, since Western blotting experiments showed that GDF5 treatment increased the levels of expression of TH and GFAP protein, but did not alter that of the neuronal marker β-III tubulin (Fig. 2).

LOSS OF BMPR-IB EXPRESSION *in vitro* ABOLISHES THE ABILITY OF GDF5 TO INCREASE THE NUMBERS OF DOPAMINERGIC NEURONES

RT-PCR analysis demonstrated the expression of all three BMP receptors, BMPR-Ia, -Ib and -II, in extracts of E14 rat VM (Fig. 3A). However, when cells from the E14 VM were cultured, the expression of BMPR-Ib was dramatically down-regulated, even after 1 DIV (Fig. 3G). Low levels of BMPR-Ib mRNA were present at 1, 2 and 4 DIV (Fig. 3G), but expression was completely lost by 6 DIV (Fig. 3B and G). In contrast, the expression of BMPR-Ia and -II remained in the cultures after 6 DIV (Fig. 3B). Immunocytochemistry was used to confirm the RT-PCR results. Cells which were immunopositive for BMPR-II and BMPR-Ia were detected in the cultures after 6 DIV (Fig. 2C and D, respectively), but no positive immunostaining for BMPR-Ib was observed in any cultures at this time-point (Fig. 2E). This suggests a loss of expression of BMPR-Ib with maturation of these cultures. This may render them unresponsive to GDF5, since BMPR-Ib, but not BMPR-Ia, is a specific receptor for GDF5 (Nishitoh *et al.*, 1996). In agreement with this hypothesis, cultures treated with 10 ng/ml GDF5 for 24 h at the time of plating displayed a significant increase in the numbers of dopaminergic neurones when compared to untreated cultures (Fig. 3H; $P < 0.05$). However, when 10 ng/ml GDF5 was added at 6 DIV for 24 h, no increase in the numbers of dopaminergic neurones was observed (Fig. 3H), in agreement with a previous study (Brederlau *et al.*, 2002).

GDF5 IMPROVES THE MORPHOLOGY OF DOPAMINERGIC NEURONES IN E14 RAT VM CULTURES

Morphological analysis of the cultured dopaminergic neurones revealed that treatment with GDF5 (at doses of either 1 ng/ml or 10 ng/ml) resulted in increased differentiation of these cells *in vitro*. A significant increase in total neurite length per dopaminergic neurone was

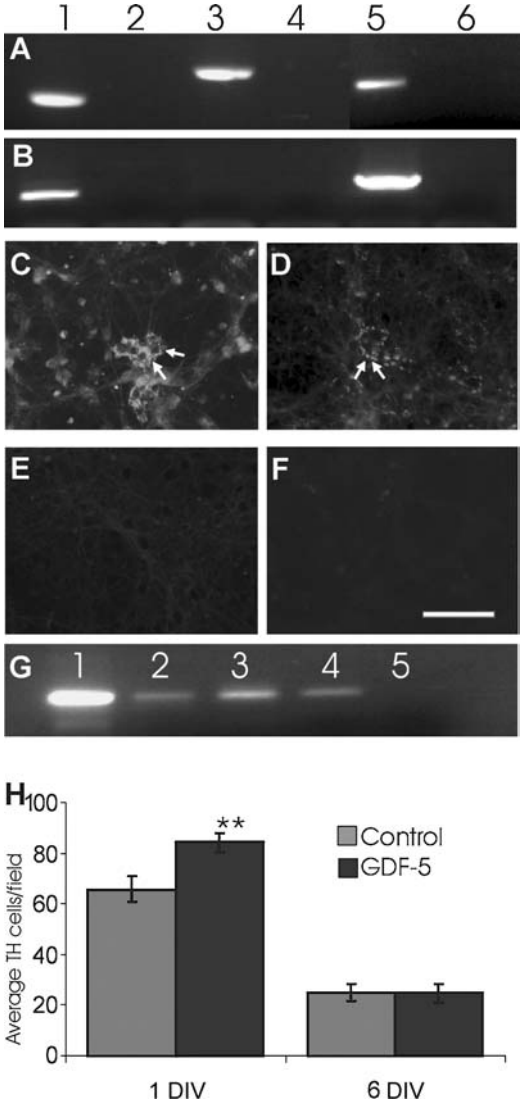

Fig. 3. (A) RT-PCR analysis of the expression of BMPR-II (lane 1, RT+; lane 2, RT-), BMPR-Ib (lane 3, RT+; lane 4, RT-) and BMPR-Ia (lane 5, RT+; lane 6, RT) in freshly-dissected E14 rat VM tissue. (B) RT-PCR analysis of the expression of BMPR-II (lane 1, RT+; lane 2, RT-), BMPR-Ib (lane 3, RT+; lane 4, RT-) and BMPR-Ia (lane 5, RT+; lane 6, RT) in E14 rat VM cultures after 6 DIV. Immunocytochemical analysis of (C) BMPR-II, (D) BMPR-Ia and (E) BMPR-Ib expression in E14 rat VM cultures after 6 DIV. (F) shows a "negative control" (no primary antibody) for the immuncytochemistry. Arrows in C and D indicate positively-stained cells. Scale bar = 50μm. (G) RT-PCR analysis of the time-course of BMPR-Ib expression in cultures of E14 rat VM (lane 1, 0 DIV; lane 2, 1 DIV; lane 3, 2 DIV; lane 4, 4 DIV; lane 5, 6 DIV). (H) Treatment of E14 VM cultures with 10 ng/ml GDF5 for 24 h at the time of plating induces a significant increase in the numbers of dopaminergic neurones per field, whereas treatment with 10 ng/ml GDF5 for 24 h at 6 DIV does not. $^{**}P < 0.05$ vs. untreated cultures.

observed in GDF5-treated cultures, when compared to control cultures (Fig. 4A; $P < 0.001$). Both doses of GDF5 significantly increased neurite length at all levels of the neuritic field ($P < 0.001$), with the greatest increases (at least four-fold) measured in the length of secondary and tertiary neurites and smaller increases observed in the length of primary and quaternary neurites (Fig. 5A). The average somal area per dopaminergic neurone was also significantly increased following GDF5 treatment (Fig. 4B; $P < 0.001$). The number of branch points per dopaminergic neurone (as measured by the mean number of nodes per dopaminergic neurone) was significantly increased by GDF5 treatment (Fig. 4C; $P < 0.001$). Analysis of the degree of branching at different levels of the neuritic field established that the greatest increases were at the level of secondary and tertiary branches, compared to those at primary and quaternary branches (Fig. 5B). There was no significant difference between the effects of the two doses of GDF5 on any of these morphological parameters.

Discussion

EFFECTS OF GDF5 ON THE CELLULAR COMPOSITION OF E14 RAT VM CULTURES

This study examined the effect of GDF5 on cultures of E14 rat VM. When applied at the time of plating, GDF5 increased the numbers of dopaminergic neurones present in the cultures after 6 DIV, in agreement with previous studies (Krieglstein *et al.*, 1995a). We chose to add GDF5 to the culture medium at this early stage because we previously observed that during the development of the rat VM, GDF5 protein levels peak on E14 (O'Keeffe *et al.*, 2004). It is known that dopaminergic neurones in the rat VM undergo their final mitosis at E14 or E15 (Altman & Bayer, 1981; Bayer *et al.*, 1995), thus factor(s) involved in the induction of a dopaminergic fate or in regulating the survival of these neurones would be expected to peak in the VM at about E14.

Previous reports have shown that addition of the neurotrophic factors, glial cell line-derived neurotrophic factor (GDNF), TGF-β1/2/3 or activin A, to E14 rat VM cultures at 24 h after plating, induced 1.5-, $\sim$2.3- and 1.6-fold increases, respectively, in the numbers of dopaminergic neurones after 8 DIV when compared to controls (Krieglstein *et al.*, 1995b). In the present study, we have found that GDF5 treatment from the time of plating resulted in a $\sim$3-fold increase in dopaminergic neuronal survival after 6 DIV. Thus, GDF5 has powerful neurotrophic effects on mesencephalic dopaminergic neurones in culture.

It has recently been reported that GDF5 treatment does not increase the numbers of dopaminergic neurones in cultures of E14 rat VM (Brederlau *et al.*, 2002). This study proposed that GDF5 affects mainly non-neuronal cells in such cultures. The apparent discrepancy between this study and the present one may be explained by the fact that, in our study, GDF5 was added from the time of plating of the cultures (*i.e.* 0 DIV), whereas Brederlau and co-workers did not apply GDF5

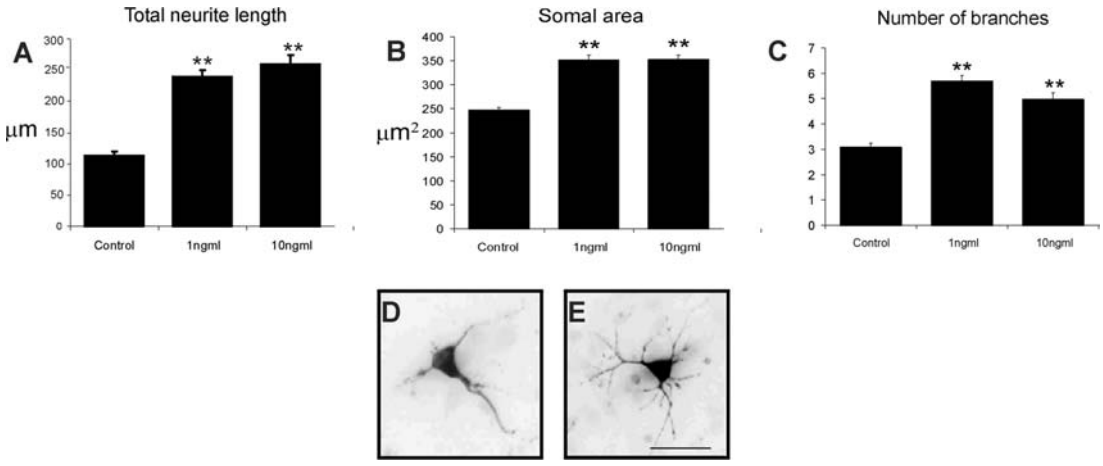

Fig. 4. Treatment of E14 VM cultures with GDF5 had significant effects on (A) total neurite length, (B) somal area and (C) number of branches in dopaminergic neurones in these cultures. Data are expressed as mean ± SEM of measurements of fifty neurones in three independent experiments. $^{**}P < 0.001$ vs. untreated cultures. Representative photomicrographs of dopaminergic neurones in (E) untreated and (F) 10 ng/ml GDF5-treated cultures after 6 DIV. Scale bar = 20 μm.

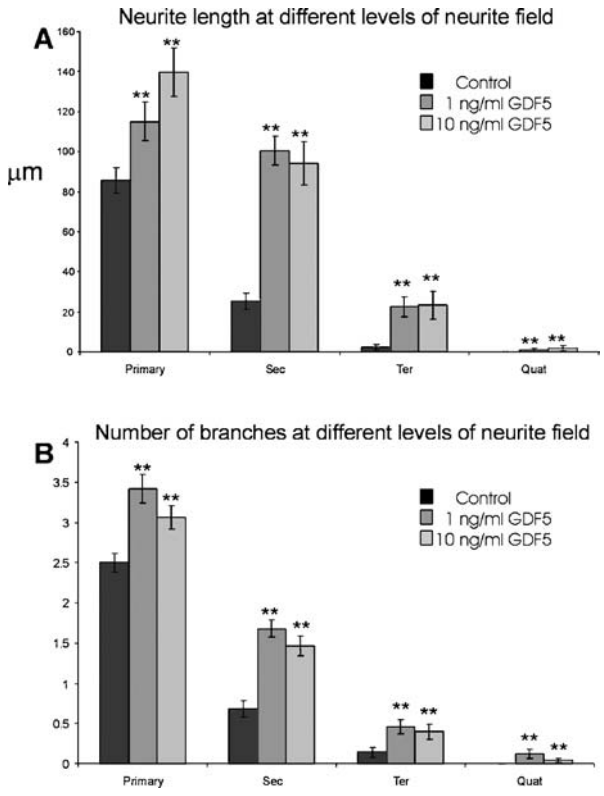

Fig. 5. Treatment of E14 VM cultures with GDF5 had significant effects on (A) neurite length and (B) number of branches at each of the levels (primary, secondary, tertiary and quarternary) of the neuritic field of dopaminergic neurones in these cultures. Data are expressed as mean ± SEM of measurements of fifty neurones in three independent experiments. $^{**}P < 0.001$ vs. untreated cultures.

to their cultures until after 7 DIV. Given that GDF5 protein levels in the developing rat VM peak on E14 and are subsequently down-regulated, reaching lowest levels around birth (O'Keeffe *et al.*, 2004), it is possible that dopaminergic neurones may become less responsive to GDF5 as they mature. This may be due to changing receptor expression patterns with maturation *in vitro*, as discussed below.

In agreement with previous reports (Krielgstein *et al.*, 1995a; Brederlau *et al.*, 2002), we found that GDF5 treatment resulted in an increase in the numbers of GFAP-positive cells in these cultures. This raises the possibility that GDF5 may mediate its effect on neuronal survival in the cultures indirectly, by increasing the numbers of astrocytes and thus increasing the levels of astrocyte-derived neurotrophic factors. It has been shown that the presence or absence of astrocytes affects the morphological development of E14 rat VM dopaminergic neurones *in vitro* (Wood *et al.*, 2003). Thus, GDF5 may indirectly increase the numbers of dopaminergic neurones and improve their morphological development by increasing the numbers of astrocytes present in the culture. The effect of GDF5 to increase the number of astrocytes in these cultures was dose-dependent, unlike its effect on the number of dopaminergic neurones. Thus, the increase in the number of astrocytes with increasing dose of GDF5 was not paralleled by an increase in the numbers of dopaminergic neurones. This suggests that the effect of GDF5 on these neurones is not solely due to an astrocyte-mediated action. Indeed, another study in our laboratory has found that the effects of GDF5 on the survival of dopaminergic neurones *in vitro* are not significantly reduced by depletion of astrocytes in E14 rat VM cultures (Wood *et al.*, 2005). A direct action on dopaminergic neurones is substantiated by the fact that GDF5 application to E14 rat VM cultures at 1 DIV results in nuclear accumulation of phosphorylated smad proteins in these cells (Sullivan & O'Keeffe, 2005). The possibility of a direct action of GDF5 on dopaminergic neurones is also supported by the fact that mRNA for both BMPR-Ib and

BMPRII, the receptors which are necessary for GDF5 signalling (Nishitoh *et al.*, 1996), have been found in the adult rat SN and striatum (Chen *et al.*, 2003). Furthermore, the expression of BMPR-II mRNA has been localised to dopaminergic neurones in the adult rat SN (Charytoniuk *et al.*, 2000).

The actions of GDF5 on astrocytes are an important consideration, if this protein is to be proposed as a candidate neurotrophic factor for the treatment of PD. Previous *in vivo* studies have detected no evidence of astrogliosis after intracerebral administration of recombinant human GDF5 to adult rats (Sullivan *et al.*, 1997, 1998, 1999; Hurley *et al.*, 2004). Furthermore, we have recently carried out a comprehensive examination of the immunological reaction in the adult rat brain after administration of both recombinant human GDF5 and a GDF5-producing cell line; no evidence of astrogliosis was detected in this study (Costello *et al.*, in preparation).

EXPRESSION OF BMP RECEPTORS IN THE RAT VM

In agreement with previous studies on chick embryos (Ming *et al.*, 2002), we have found that BMPR-II, -Ia and BMPR-Ib are expressed in the rat VM at E14. The expression of various BMPs (Jordan *et al.*, 1997) and GDF5 (O'Keeffe *et al.*, 2004) in the VM at this stage suggests that cells in this tissue at this stage of development are responsive to BMP and GDF5 signalling. It has been reported that the expression of BMPs generally peaks around the perinatal period (for review, see Mehler *et al.*, 1997). However, we have found that GDF5 expression peaks much earlier during rat brain development (at E14 in both whole brain and VM), reaching its lowest level around the perinatal period before being up-regulated in the post-natal period to reach high levels in the adult brain, including the SN and striatum (O'Keeffe *et al.*, 2004). The expression of BMPR-II, -Ia and BMPR-Ib in the adult rat striatum and SN (Chen *et al.*, 2003), suggests that BMPs/GDF5 play roles in these regions in the adult rat brain. It has been suggested that the maturation state of dopaminergic neurones in E14 VM cultures at 7 DIV are representative of these cells at around the time of birth (Brederlau *et al.*, 2002). Brederlau and co-workers have shown that neurones at this stage of development are affected by BMP signalling, as evidenced by an increase in TH-immunopositive neurones and nuclear translocation of smad proteins, but that they are largely unresponsive to GDF5 (Brederlau *et al.*, 2002). Our findings complement these results, since we show that the expression of BMPR-Ib is down-regulated in these cultures after 6 DIV, suggesting that they become unresponsive to GDF5 at this stage in culture. In agreement, we found that addition of GDF5 for 24 h at 0 DIV (when BMPR-Ib is present) induced a significant increase in the numbers of dopaminergic neurones in these cultures, whereas addition of GDF5 for 24 h after 6 DIV (when BMPR-Ib is no longer expressed) did not. This suggests that the actions of GDF5 and various other BMPs on the development and survival of rat VM dopaminergic neurones may be exerted at different stages during embryonic development, with GDF5 acting at an earlier stage than the BMPs. Given its earlier expression, GDF5 may be involved in the induction of a dopaminergic phenotype in progenitor cells in this area of the brain, an action which has been bestowed on other members of the TGF-β superfamily (Farkas *et al.*, 2003).

EFFECTS OF GDF5 ON THE MORPHOLOGY OF DOPAMINERGIC NEURONES *in vitro*

Having shown that GDF5 increases the numbers of dopaminergic neurones in E14 VM cultures, we assessed whether this factor might also affect the morphological development of these neurones. Previous studies have shown that BMPs can affect the morphological development of striatal neurones (Gratacòs *et al.*, 2001). Another member of the TGF-β superfamily, GDNF, has been shown to be a potent neurotrophic factor for dopaminergic neurones in VM cultures (Lin *et al.*, 1993; Krieglstein *et al.*, 1995b; Hou *et al.*, 1996). It has been reported that treatment with 10 ng/ml GDNF for 7 DIV resulted in an increase in neurite length and branching of dopaminergic neurones in E14 rat VM cultures (Widmer *et al.*, 2000). In that study, GDNF resulted in a 1.5-fold increase in the total neurite length per dopaminergic neurone (Widmer *et al.*, 2000). Our findings are similar, showing that GDF5 treatment for 6 DIV resulted in a 2.5-fold increase in the total neurite length per dopaminergic neurone. Cell somal area was reported to be increased by 1.3-fold by GDNF treatment in the Widmer study, while we found a 1.4-fold increase in this parameter due to GDF5 treatment. Widmer and co-workers also found that treatment with GDNF resulted in a 1.4-fold increase in the number of primary nodes per dopaminergic neurone (Widmer *et al.*, 2000). In agreement, we have found that GDF5 treatment resulted in a 1.4-fold increase in the numbers of primary nodes per dopaminergic neurone. Our detailed morphological analysis showed that GDF5 treatment resulted in an increase in neurite branching and neurite length at all levels of the neuritic field. The greatest effects were seen on the secondary and tertiary neurites, where GDF5 induced a $\sim$5-fold increase in length (in comparison to $\sim$1.6-fold increase in length of primary neurites).

These results show that GDF5, like GDNF, has potent effects on the morphological development of VM dopaminergic neurones *in vitro*. The observed increases in dopaminergic neuronal survival and in the size of their neuritic field have important implications for research on intrastriatal neuronal transplantation for PD, where efforts are concentrated on promoting the

survival of dopaminergic neurones and their functional integration into the host striatum after transplantation. It has been reported that GDNF treatment can increase fiber outgrowth from embryonic rat dopaminergic VM tissue after transplantation into the striatum of 6-OHDA lesioned rats (Rosenblad *et al*., 1996; Wang *et al*., 1996; Granholm *et al*., 1997). It has also been shown that GDF5 is at least as effective as GDNF in improving the survival of grafts of E14 VM in a rat model of PD (Sullivan *et al*., 1998). Further, our previous studies have found that during the development of the rat VM, GDF5 expression peaks at E14 (O'Keeffe *et al*., 2004), while that of GDNF peaks at a later date (O'Keeffe & Sullivan, in preparation). These findings suggest that GDF5 may be a candidate neurotrophic factor for use in attempts to improve the survival, neurite outgrowth and functional integration of transplanted embryonic dopaminergic neurones for the treatment of PD.

Abbreviations

6-OHDA: 6-hydroxydopamine; BMP: bone morphogenetic protein; BMPR: bone morphogenetic protein receptor; DIV: days *in vitro*; E: embryonic day; GFAP: glial fibrillary acidic protein; GDF: growth/differentiation factor; GDNF: glial cell line-derived neurotrophic factor; PD: Parkinson's disease; PBS: phosphate-buffered saline; SEM: standard error of the mean; SN: substantia nigra; TGF-β: transforming growth factor-β; TH: tyrosine hydroxylase; VM: ventral mesencephalon

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