Neurotrophic Effects of Growth / Differentiation Factor 5 in a Neuronal Cell Line.

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Abstract

The neurotrophin growth/differentiation factor 5 (GDF5) is studied as a potential therapeutic agent for Parkinson's disease as it is believed to play a role in the development and maintenance of the nigrostriatal system. Progress in understanding the effects of GDF5 on dopaminergic neurones has been hindered by the use of mixed cell populations derived from primary cultures or *in vivo* experiments, making it difficult to differentiate between direct and indirect effects of GDF5 treatment on neurones. In an attempt to establish a useful model to study the direct neuronal influence of GDF5, we have characterised the effects of GDF5 on a human neuronal cell line, SH-SY5Y. Our results show that GDF5 has the capability to promote neuronal but not dopaminergic differentiation. We also show that it promotes neuronal survival *in vitro* following a 6-hydroxydopamine insult. Our results show that application of GDF5 to SH-SY5Y cultures induces the SMAD pathway which could potentially be implicated in the intracellular transmission of GDF5's neurotrophic effects. Overall, our study shows that the SH-SY5Y neuroblastoma cell line provides an excellent neuronal model to study the neurotrophic effects of GDF5.

Keywords:

Growth/Differentiation Factor 5, neurotrophin, 6-hydroxydopamine, SH-SY5Y cells, Parkinson's disease

Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder characterised by the selective and progressive loss of the dopaminergic neurones of the *substantia nigra pars compacta* (SNpc) and the presence of intraneuronal proteinaceous inclusions known as Lewy bodies within the surviving neurones (Braak et al. 2003; Cookson 2005). Clinical symptoms usually appear when ~50% of dopaminergic neurones in the SNpc are lost, leading to a depletion of dopamine in the corpus striatum. Most of the available therapies aim to reduce the symptoms of the disease but cannot stop the progressive neurodegeneration or promote survival of the remaining neurones. Neuroprotective therapy could offer ways of preserving these neurones and, when administered with symptomatic treatments, could improve the long-term outcome for patients. Several compounds are being investigated as potential neuroprotectants (Toulouse and Sullivan 2008).

A group of dimeric proteins known for their neurotrophic properties has recently attracted much attention. Glial cell line-derived neurotrophic factor (GDNF) and neurturin (NRTN) have produced potent neurotrophic effects on dopaminergic neurones *in vivo* and *in vitro*. They have been shown to protect cultured dopaminergic neurones from a variety of insults (Akerud et al. 1999; Horger et al. 1998; Lin et al. 1993) and prevent 6-hydroxydopamine- (6-OHDA) and 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine- (MPTP) induced nigrostriatal damage in animal models (Gasmi et al. 2007a; Gasmi et al. 2007b; Herzog et al. 2007; Kordower et al. 2006). Intraputamenal injections of GDNF were initially successful in open-label trials but this was not replicated in a randomized double-blind trial (Gill et al. 2003; Patel et al. 2005; Slevin et al. 2005). Differences in the selection of patients, catheter design and drug dosage may explain the discrepancy. Intraputamenal injection of an adeno-associated virus type 2 (AAV2)-based NRTN expression vector initially proved to be very

efficient. Results from an open-label trial showed that 6 months after receiving the injection, patients showed a 25% reduction in their "off" medication UPDRS score, a 50% reduction in their "off" time and an increase in periods without dyskinesia (Marks et al. 2006). However, an 18 month assessment of a double-blind trial of intraputamenal AAV2-NTN showed only minor clinical improvements (Bartus 2009).

Growth/differentiation factor 5 (GDF5) is a member of the TGFB superfamily, that is related to GDNF and NRTN. In its active state, GDF5 forms a dimer that has binding affinity for various cell surface receptors; the bone morphogenetic proteins (BMP) receptors and the orphan receptor ROR2. Binding of GDF5 to BMPR1a or BMPR1b recruits BMPR2 to form a serine/threonine kinase receptor dimer that activates the SMAD family of nuclear transcription factors, SMAD 1/5/8 and the co-factor SMAD4 (ten Dijke et al. 2000). GDF5 has higher affinity for BMPR1b than BMPR1a (Nishitoh et al. 1996). Alternatively, the BMPR1b receptor can form a heterodimer with the ROR2 tyrosine kinase receptor in the presence or absence of GDF5 (Sammar et al. 2004). It has been shown that formation of this receptor complex inhibits SMAD signalling, most likely by sequestering BMPR1b and therefore providing a negative modulation loop (Sammar et al. 2009; Sammar et al. 2004).

GDF5 is expressed in many regions of the brain, including the midbrain. It is expressed in the ventral mesencephalon (VM) from embryonic day (E) 12, peaking at E14, the time of peak dopaminergic neurogenesis (Clayton and Sullivan 2007; O'Keeffe et al. 2004b). *In vitro* studies have shown that GDF5 treatment of VM cultures promotes the survival and the morphological differentiation of dopaminergic neurones and protects cultured dopaminergic neurones from MPP^+-induced cell death, suggesting that it may play a role in the development and maintenance of the nigrostriatal system (Krieglstein et al. 1995; O'Keeffe et

al. 2004a). *In vivo* studies using the 6-OHDA-lesioned rat model of PD have shown that intracerebral injection of GDF5 protects the nigrostriatal pathway (Hurley et al. 2004; Sullivan et al. 1997; Sullivan et al. 1999). Furthermore, GDF5 was found to be as effective as GDNF in promoting the survival and functional integration of embryonic VM grafts in 6-OHDA-lesioned rats (Sullivan et al. 1998).

It remains unclear whether GDF5's neurotrophic effects are mediated by direct actions on the dopaminergic neurones or are the results of secondary signalling from the surrounding glia. Most experiments conducted so far involved primary cultures or the use of animal models which do not allow separation of these effects. A paper by Wood et al. where primary cultures were treated with 5-FdU to prevent the growth of glial cell suggested that at least part of the dopaminergic neurotrophic effects of GDF5 may be through direct action on neurones (Wood et al. 2005); but otherwise, very little information is available. The experiments presented here aimed to assess the direct effects of GDF5 in a neuronal model, the SH-SY5Y cell line. Our results show that some of the neurotrophic effects of GDF5 can be reproduced in this model, mainly its capacity to promote neuronal survival and differentiation and that the SH-SY5Y is an ideal system for studying the effects of GDF5 on these parameters.

Materials and Methods

Cell culture

SH-SY5Y cells were maintained in Dulbecco's Modified Eagle's Medium:Ham's F12 mixture (1:1, DMEM:F12, Sigma-Aldrich) supplemented with 10% fetal calf serum (Sigma-

Aldrich), 100 mM L-glutamine (Sigma-Aldrich), 100 U/ml penicillin, and 10 μg/ml streptomycin (Sigma-Aldrich) in a 37°C humidified atmosphere supplemented with 5% CO_2. Where indicated, the cells were treated with 100 ng/ml recombinant human GDF5 (rhGDF5, Biopharm GmbH) or 10 μM retinoic acid (RA, Sigma-Aldrich).

Reverse-transcriptase polymerase chain reaction (RT-PCR)

For RT-PCR, total RNA was extracted from SH-SY5Y. RNA was extracted using the method described by Berk and Sharp (Berk and Sharp 1977). RNA samples were treated with RQ1 DNAse (Promega) for 20 min at room temperature before being neutralised. Complementary DNA (cDNA) synthesis was performed using 1 μg RNA following the ImProm-II™ kit protocol (Promega Inc.). Negative controls where the reverse transcriptase was left out of the reaction were also prepared (RT-). PCR was performed using the primers and conditions indicated in Table 1. Aliquots of the reactions were electrophoresed on 1.5% agarose gels and photographed.

Immunocytochemistry

10,000 SH-SY5Y cells were seeded in 24-well plates and grown for 7 days in the presence of GDF5 (100 ng/ml). Control cultures were left untreated. Cells were fixed in 4% paraformaldehyde or ice-cold methanol for 15 min followed by permeabilization in 0.2% Triton-X. Immunodetection was performed using the following antibodies: mouse monoclonal antibodies to BMPR1A (1:500, R&D Systems), BMPR1B (1:000, R&D Systems), BMPR2 (1:1000, R&D Systems), ROR2 (1:500, R&D Systems), TH (1:500, NovoCastra Laboratories), and rabbit polyclonal antibodies to DAT1 (1:500, Santa Cruz

biotechnology), SMAD 1/5/8 (1:1000, Santa Cruz biotechnology) and phospho-SMAD 1/5 (1:1000, Cell Signaling Technology). Alexa 488-conjugated donkey anti-rabbit and Alexa 594-conjugated donkey anti-mouse secondary antibodies (1:1500, Molecular Probes) were used. Cells were counterstained with DAPI. The cells were imaged on an Olympus IX70 inverted microscope. The fluorescence intensity of individual cells stained for phospho-SMAD 1/5/8 was measured using the Image J analysis software (Rasband, WJ, http://rsb.info.nih.gov/ij/). The relative fluorescence intensity was calculated as the intensity of individual cells after substraction of the background noise. Results were compared using a Student's *t*-test.

Immunoblotting

Total protein extracts were prepared by homogenizing cells in protein extraction buffer (70 mM TRIS-HCl pH 6.8, 10% glycerol, 3% SDS and 700 mM 2-mercaptoethanol) followed by centrifugation at 14000g. 20 μg were electrophoresed on 10% SDS-polyacrylamide gels and were transblotted to nitrocellulose membranes. Immunodetection was performed using rabbit polyclonal antibodies to SMAD 1/5/8 (1:2000, Santa Cruz biotechnology) and phospho-SMAD 1/5/8 (1:2000, Cell Signaling Technology) or rabbit polyclonal anti-actin (1:10000, Santa Cruz Biotechnology) antibodies. Results were visualized by chemiluminescence.

Cell growth assay

50,000 SH-SY5Y cells were seeded in 6-well plates. Cells were collected 2, 4 and 7 days after seeding, stained with trypan blue and counted using a haemacytometer. Growth curves

were plotted and differences were assessed by performing a one-way ANOVA followed by a Dunnett's *post-hoc* test.

Measurement of cellular morphology

10 to 15 microscopic fields were randomly selected from three independent experiments, photographed using an Olympus IX70 inverted microscope and all cells in each photograph were measured. Neurite branching was assessed by counting the numbers of "nodes" per cell. Primary nodes were considered branches from the cell body, and secondary nodes were considered branches of primary neurites. The length of the neuritic arborisation was estimated using standard stereological procedures (Mayhew 1992). A line grid was superimposed on the microscopic images and the number of times a neurite intersects the grid was recorded. The neurite length was calculated using the following formula;

$$NL = a \times T \times \pi/2$$

Where a = the number of times the neurite intersect the grid lines, T = the distance between the gridlines on the magnified image (taking into account the magnification factor). Results were compared using a Student's *t*-test.

Neuroprotection assays

100 000 cells per well were seeded in 24-well plates and grown in the presence or absence of rhGDF5 (100 ng/ml). After 24h, half of the wells were treated for 1h with 50 µM 6-OHDA. The cells were then rinsed three times in saline solution and fed either with culture medium or culture medium supplemented with 100 ng/ml rhGDF5 for an additional 24h. This treatment modality produced six experimental groups (Figure 4A). Following the second

incubation, an MTT assay was performed to assess cell viability. Thiazolyl Blue Tetrazolium Bromide (MTT) was added to cells at a concentration of 0.5 mg/ml in the culture medium and incubated for 3h. Cell culture medium was removed and the cells were lysed using a mixture of Isopropanol:HCl (24:1). Absorbance was measured at 540 nm with a reference wavelength of 690 nm. The mean of 9 independent samples were calculated for each group and results were compared using an ANOVA followed by Tukey's *post-hoc* analysis.

Results

GDF5 signalling machinery is expressed in SH-SY5Y cells

To assess their suitability as a neuronal model to further study the neurotrophic properties and downstream signalling pathways of GDF5, human neuroblastoma SH-SY5Y cells were tested for the expression of the various GDF5 receptors, BMPR1a, BMPR1b, BMPR2 and ROR2. RT-PCR experiments revealed that the mRNA for each of the four receptors is expressed in SH-SY5Y cells (Figure 1A). Immunocytochemistry confirmed that all four receptors are expressed on the surface of SH-SY5Y cells; although cell surface expression of BMPR1a was low compared to the other receptors (Figure 1B). Results also showed that the intracellular machinery for signal processing, SMAD proteins 1, 5, and 8, is present in both unphosphorylated (inactive) and phosphorylated (active) forms (Figure 2A). Controls performed for each secondary antibody confirmed the specificity of the staining (data not shown).

Given that the cells express the receptors and downstream transcription factors necessary for GDF-5 signalling, we next assessed whether GDF5 induced a physiological effect in SH-

SY5Y cells, by quantifying the relative intensity of phosphorylated SMAD 1, 5, 8 present in the nucleus. Results showed that treatment with 100 ng/ml GDF5 for 7 days resulted in a significant (35%) increase in phospho-SMAD 1/5 signal intensity compared to untreated cells (p<0.001, Figure 2A and Figure 2B). An immunoblot confirmed these findings. Protein extracts from GDF5-treated and untreated SH-SY5Y cells were probed with antibodies against SMAD 1/5/8 or their phosphorylated form. Comparison to an actin control revealed that an increase in phosphorylated SMAD proteins in the GDF5-treated sample (Figure 2C). These data suggest that GDF5 should be able to actively induce changes in gene expression as a result of nuclear accumulation of phospho-SMAD proteins, and this may induce phenotypic changes in the cells.

GDF5 inhibits the growth of SH-SY5Y cells

To assess any potential phenotypic changes, we examined the growth and differentiation of the SH-SY5Y cells. Firstly we examined the growth rates of these cells in response to retinoic acid and GDF5. To assess the capacity of GDF5 to induce post-mitotic growth arrest of SH-SY5Y cells, growth rates were measured over a period of 7 days in the presence of 100 ng/ml GDF5. We used cells grown in 10 μM RA as a positive control, as RA has been shown to induce the post-mitotic neuronal differentiation of SH-SY5Y cells and inhibit their growth rates (Pahlman et al. 1995; Pahlman et al. 1984). As expected, RA strongly inhibited the growth of SH-SY5Y cells after 4 and 7 days of treatment (p<0.001, Figure 3). Interestingly, our results show that treatment with GDF5 also resulted in growth inhibition (Figure 3). While the difference is not as strong as the one elicited by RA, it is nonetheless significant compared to the control after 4 days and remains significant at 7 days (p< 0.05, Figure 3).

GDF5 stimulates neuronal differentiation of SH-SY5Y

While the results above may be an effect of GDF5 on cellular differentiation, they could also be a consequence of mitotic inhibition without differentiation. One of the morphological features of maturing neurones is the development of a neuritic arborisation and GDF5 has been shown to promote neurite outgrowth in primary VM cultures (O'Keeffe et al. 2004a). To assess the effect of GDF5 on neuronal differentiation, we analysed the number of primary and secondary neurites on GDF5-treated, RA-treated and untreated SH-SY5Y cells (Figure 4A). Our results show that after 7 days of treatment, 100 ng/ml GDF5 induced an increase in the number of neurites compared to untreated controls (Figure 4B). While the difference remained close to significance levels for the number of primary neurites (p=0.067), it reached statistical significance for second order neurites (p<0.05, Figure 4B). The control retinoic acid-treated cells showed a significant increase in the number of primary neurites (p<0.05) while there was a non-significant increase in the number of secondary neurites (figure 4B). Furthermore, GDF5-treated cells showed a 27% increase in total neurite length compared to control cells (p<0.05, Figure 4C) while the RA-treated cells showed a 42% increase in total neurite length (p<0.001). Altogether, these results suggest that GDF5 induces differentiation rather than simply inducing growth arrest.

Treatment with GDF5 does not affect tyrosine hydroxylase expression

Previous results from our lab showed that treatment of primary VM cultures with GDF5 leads to an increased survival/number of DA neurones (O'Keeffe et al. 2004a). SH-SY5Y cells have been reported by some groups to readily express tyrosine hydroxylase (TH, the rate-limiting enzyme in the synthesis of DA) (Gomez-Santos et al. 2002; McMillan et al. 2007),

while others failed to demonstrate expression (Mastroeni et al. 2008). To assess whether the neuroblastoma cell line model used here could recapitulate the results obtained using primary cultures, we analysed TH expression by RT-PCR, immuncytochemistry and immunoblotting in cultures maintained in the presence of 100 ng/ml GDF5 for 4 to 7 days. Using adult rat midbrain extracts or cryosections as positive controls, we were unable to demonstrate the presence of TH expression in SH-SY5Y cells in the presence or absence of GDF5 (data not shown).

Treatment with GDF5 protects SH-SY5Y cells from 6-OHDA induced toxicity

Having shown that GDF5 stimulates neuronal maturation we next investigated its neuroprotective properties. GDF5 has been previously shown to protect DA neurones from 6-OHDA-induced neurotoxicity both *in vitro* and *in vivo* (O'Keeffe et al. 2004a; Sullivan et al. 1997; Sullivan et al. 1999; Sullivan et al. 1998). To assess whether SH-SY5Y cells could represent a good cellular model to study the neuroprotective properties of GDF5, we demonstrated that SH-SY5Y cells express the dopamine transporter (DAT) involved in the uptake of 6-OHDA, on their surface (data not shown). Various treatment modalities with GDF5 and 6-OHDA were devised (Figure 5A) and neuronal survival was assessed using MTT assays.

Cell viability was decreased by 34% following a 6-OHDA treatment (50 µM, 1h) compared to untreated controls (group 2 *vs*. group 1, $p< 0.01$, Figure 5B). Continuous GDF5 treatment significantly protected cells from 6-OHDA-induced toxicity (group 3 *vs.* group 2, $p<0.05$, figure 5B). There was no observable difference between groups 1 and 3 (100% *vs.* 98% viability). GDF5 treatment applied only before or only after the 6-OHDA insult (groups 4 and

5 respectively) conferred significant neuroprotection (group 4 *vs.* group 2 $p<0.05$ and group 5 *vs.* group 2, $p<0.05$, Figure 4B). With the exception of group 2, there was no difference in viability between group 1 and any of the other groups (Figure 5).

Discussion

The neuroprotective properties of GDF5 have been well documented in primary neuronal cultures and in *in vivo* models of Parkinson's disease. However, due to the mixed cell populations of these models, it is not possible to determine whether the effects are direct or if they are mediated through other cell types, such as glial cells. In an attempt to establish a cell line model to further study the neuroprotective effects of GDF5, we have characterised the neuroblastoma cell line SH-SY5Y with regards to the expression of GDF5 surface receptors and its responsiveness to this neurotrophin.

This study confirmed that all four types of GDF5 receptor are expressed on SH-SY5Y cells. In addition, we showed that the principal signal transduction machinery for GDF5, SMAD proteins 1, 5 and 8, are present in SH-SY5Y cells and is activated in response to GDF5 treatment. This neuroblastoma cell line has previously been shown to respond to neuronal differentiating agents by exiting the mitotic cycle and acquiring a more complex dendritic arborisation (Pahlman et al. 1995; Pahlman et al. 1984). Our results showed that, while the effect of GDF5 is not as strong as that of RA, cell growth was nonetheless significantly inhibited when the cells were grown in the presence of GDF5. Analysis of the dendritic arborisation revealed that treatment with GDF5 for 7 days resulted in the development of a more extensive neurite network, particularly at secondary branching points.

Some authors have previously reported that TH is readily expressed by SH-SY5Y cells, while others reported that TH is not expressed in undifferentiated SH-SY5Y cells (Gomez-Santos et al. 2002; Mastroeni et al. 2008; McMillan et al. 2007). In addition, GDF5 has previously been reported to induce DA differentiation of rat primary VM cultures (O'Keeffe et al. 2004a). The absence of TH induction following GDF5 application found in the present paper could be due to a variety of factors, including clonal variations, defects in signaling pathways or in the TH gene promoter or to the dosage of GDF5 used. Gomez-Santos and collaborators (2002) previously showed that in SH-SY5Y cells, TH expression is induced through the activation of SMAD2/3 and not SMAD1/5/8. The latter group of SMADs are the targets of the BMPR pathway (ten Dijke et al. 2000) suggesting that the induction of the dopaminergic phenotype in primary VM cultures may have been an indirect effect of GDF5 treatment. This is further supported by results from Castelo-Branco et al. showing that secretion of Wnt5a by VM glia is an important event in the differentiation of VM dopaminergic neurones and that blockade of this signal results in reduced DA differentiation (Castelo-Branco et al. 2006). Interestingly, Wnt5a is a ligand for ROR2 and induces homodimerization of the receptor on the cell surface (Liu et al. 2008). While it is obvious that the SH-SY5Y cells do not recapitulate the events observed in GDF5-induced primary VM dopaminergic differentiation, we propose that the stimulation of TH expression observed in the O'Keeffe study was ultimately achieved via signals secreted from surrounding cells in primary cultures. For example, Wnt5a secreted by glial cells could have acted on the neurones but ultimately, GDF5 stimulation did not directly influence neuronal dopaminergic differentitation.

In our final series of experiments, we demonstrated that, while SH-SY5Y cells do not reproduce all the dopaminergic features of primary VM cultures, they nonetheless represent an excellent model to study the neuroprotective effects of GDF5. We confirmed that the SH-

SY5Y cells express the dopamine transporter protein DAT. Although some authors have shown that 6-OHDA neurotoxicity requires neuronal uptake by DAT *in vivo* (Glinka et al. 1997; Storch et al. 2004), results obtained from primary cell cultures and cell lines, including SH-SY5Y, suggest that 6-OHDA neurotoxicity *in vitro* may be mediated independently of DAT (Abad et al. 1995; Michel and Hefti 1990; Rosenberg 1988; Storch et al. 2000). Notwithstanding the mechanism, SH-SY5Y cells remain susceptible to the neurotoxic effects of 6-OHDA (Lopes et al. 2010; Storch et al. 2000). Our results showed that continuous treatment with GDF5 (group 3) could prevent neurotoxicity induced by 6-OHDA. Cells treated with GDF5 prior to a 6-OHDA pulse resisted its toxic effects (group 4, neuroprotection) but most importantly, application of GDF5 after the 6-OHDA pulse rescued most of the cells from the neurotoxic insult (group 5, neurorescue). Considering that neuroprotection following the onset of the disease is one of the major therapeutic avenues for the treatment of PD, our results suggest that GDF5 has enormous potential and the establishment of the model described here will greatly facilitate the elucidation of the pathways and intracellular machinery by which it confers neuroprotection.

GDF5 is one of the most potent neurotrophins characterised to date in animal models of PD but its molecular characterisation has been hindered by the lack of a good cellular model. While it remains an imperfect model for the characterisation of dopaminergic effects, our results show that SH-SY5Y cells are well suited to study some of the molecular events associated with GDF5 signalling and its role in neuronal differentiation and neuroprotection.

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Table 1: Gene-specific primers and PCR conditions.

Target gene	**Primer**	**Sequence (5'- 3')**	**$MgCl_2$ concentration (mM)**	**Annealing Temperature (°C)**	**Product Size (bp)**
BMPR1A	Forward	CGAAAAAGTGGCGGTGAAAGTATT	1.5	57	543
	Reverse	ATTAGGCCGAAGCTGTAGATGTCA			
BMPR1B	Forward	AAAGTGGCGTGGCGAAAAGGTAGC	1.5	57	406
	Reverse	TTTAACAGCCAGGCCCAGGTCAGC			
BMPR2	Forward	GCTTCGCAGAATCAAGAACG	1.5	57	349
	Reverse	GTGGACTGAGTGGTGTTGTG			
ROR2	Forward	ATCGCCCGCTCCAACCCtCTCATC	1.0	62	404
	Reverse	ATCCCCATCTTGCTGCcGTCTCG			
TH	Forward	GGCCGCCCTGCTCAGTGGTGTG	1.5	62	430
	Reverse	GGCCGCCCTGCTCAGTGGTGTG			

FIGURE LEGENDS

Figure 1: Expression of GDF5 receptors in SH-SY5Y cells. **A)** Representative gel electrophoresis of PCR products for BMPR1a, BMPR1b, BMPR2 and ROR2. MW: Molecular weight marker, +: RT-positive reaction, -: RT-negative control. **B)** Representative images showing immunocytochemical staining for the cell surface receptors for GDF5 (BMPR1a, BMPR1b, BMPR2, ROR2) as well as a negative control in which the primary antibody was omitted. The cells were counterstained with DAPI. Phase contrast images were also taken at 60X magnification (scale bar = 10 μm).

Figure 2: Expression of SMAD proteins in SH-SY5Y cells. **A)** Representative images showing immunocytochemical staining for SMAD 1/5/8 and phosphorylated-SMAD 1/5/8 proteins in untreated SH-SY5Y and GDF5-treated (100 ng/ml for 7 days) cells. Images were taken at 20X magnification (scale bar = 50 μm). **B)** The relative immunofluorescence intensity of untreated and GDF5-treated cells expressing phospho-SMAD 1/5/8. Data are presented as the mean relative fluorescence intensity ± S.E.M. (*** $p<0.001$ *vs.* untreated cells, Student's *t*-test, n=120 cells for each group). **C)** Western blot showing the effects of GDF5 treatment on SMAD proteins phosphorylation.

Figure 3: Growth rates of untreated (circles), GDF5-treated (100 ng/ml, squares) and RA-treated SH-SY5Y cells (10 μM, triangles). Data are presented as the mean ± S.E.M of 8 independent samples (* $p<0.05$ and *** $p<0.001$ compared to untreated cells; one-way ANOVA with Dunnett's post-hoc test).

Figure 4: GDF5 induces morphological changes in SH-SY5Y cells. **A)** Phase contrast microphotographs of untreated, GDF5-treated (100 ng/ml), and RA-treated (10 μM) SH-SY5Y cells. **B)** The numbers of primary and secondary neurites in untreated, GDF5-treated (100 ng/ml), and RA-treated (10 μM) cells after 7 days. Data are presented as the mean ± S.E.M. of 60 cells from 3 experiments (* $p<0.05$ compared to untreated cells, One way ANOVA with Dunnett's post-hoc analysis). C**)** The length of the neuritic arborisations in untreated, GDF5-treated (100 ng/ml), and RA-treated (10 μM) cells after 7 days. Data are presented as the mean ± S.E.M. length per cell of 60 cells from 3 experiments (* $p<0.05$ and *** $p<0.001$ compared to untreated cells, one-way ANOVA with Dunnett's post-hoc analysis).

Figure 5: Neuroprotective effects of GDF5 on SH-SY5Y cells. **A)** Experimental groups assessed. **B)** Cell viability in each treatment group after 48h, as measured by MTT assays. Mean absorbance values ± S.E.M. are presented, with untreated SH-SY5Y cells (group 1) considered as 100% viability (* $p<0.05$ compared to group 1, $ $p<0.05$ compared to group 2, ANOVA with *post-hoc* Tukey's test).

Figure 1A

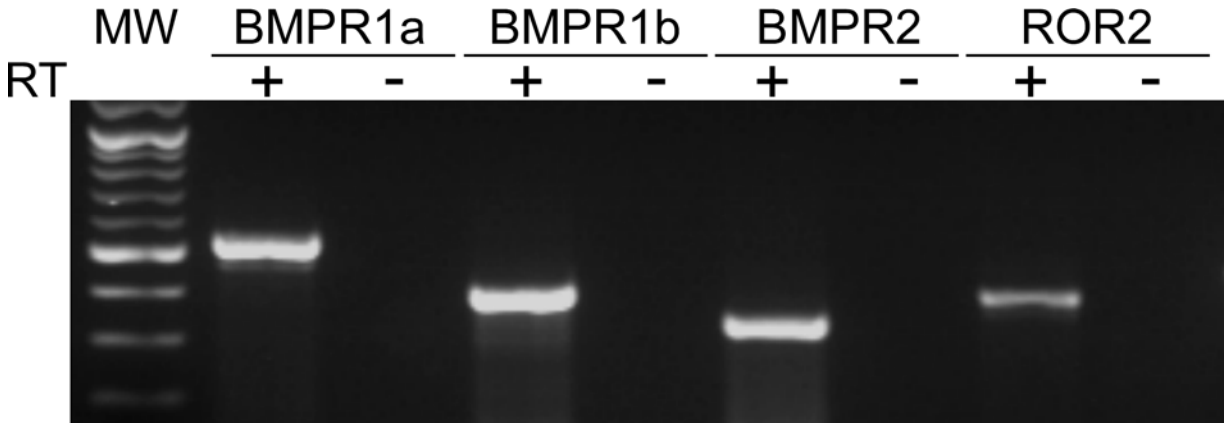


Figure 1B

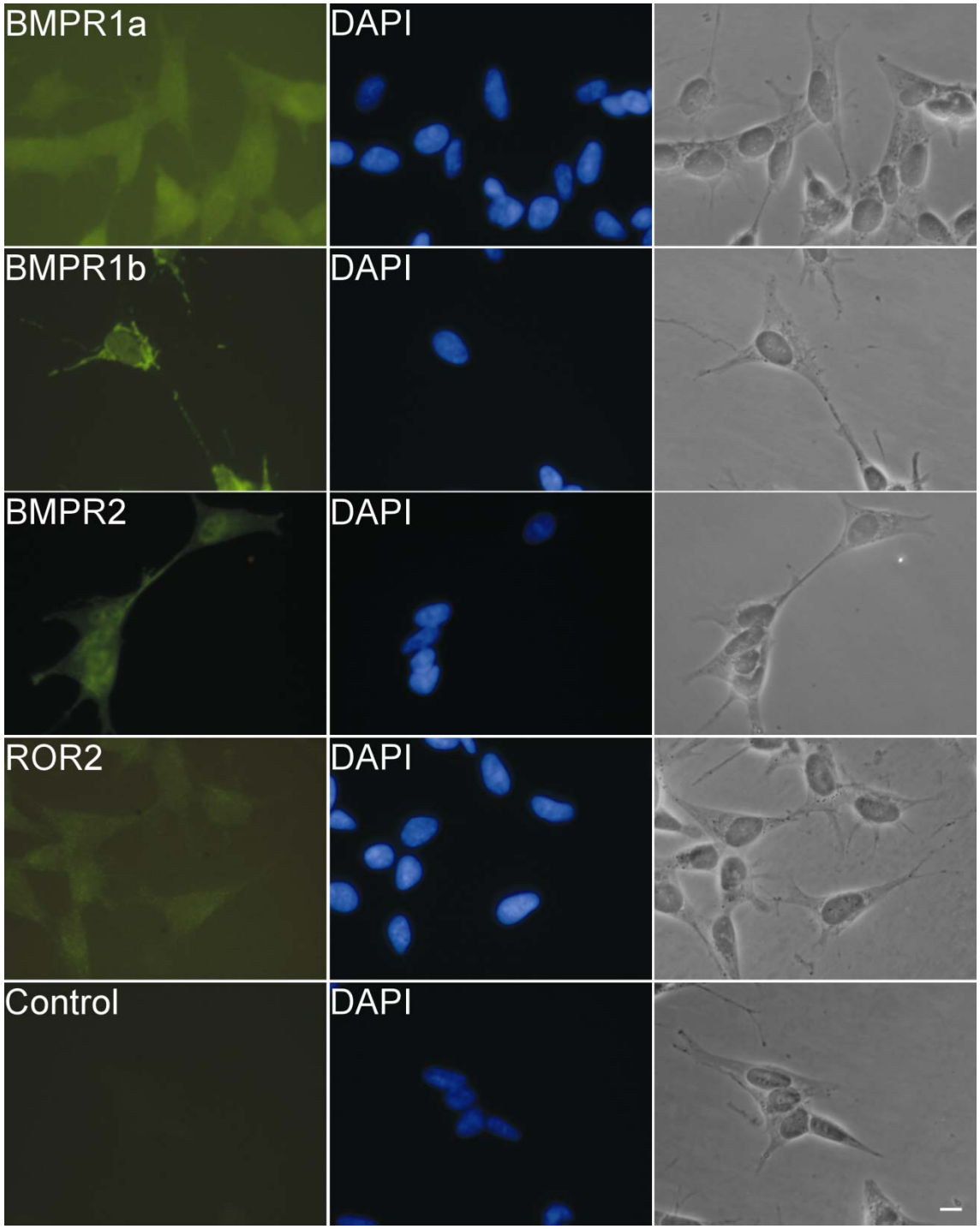


Figure 2A

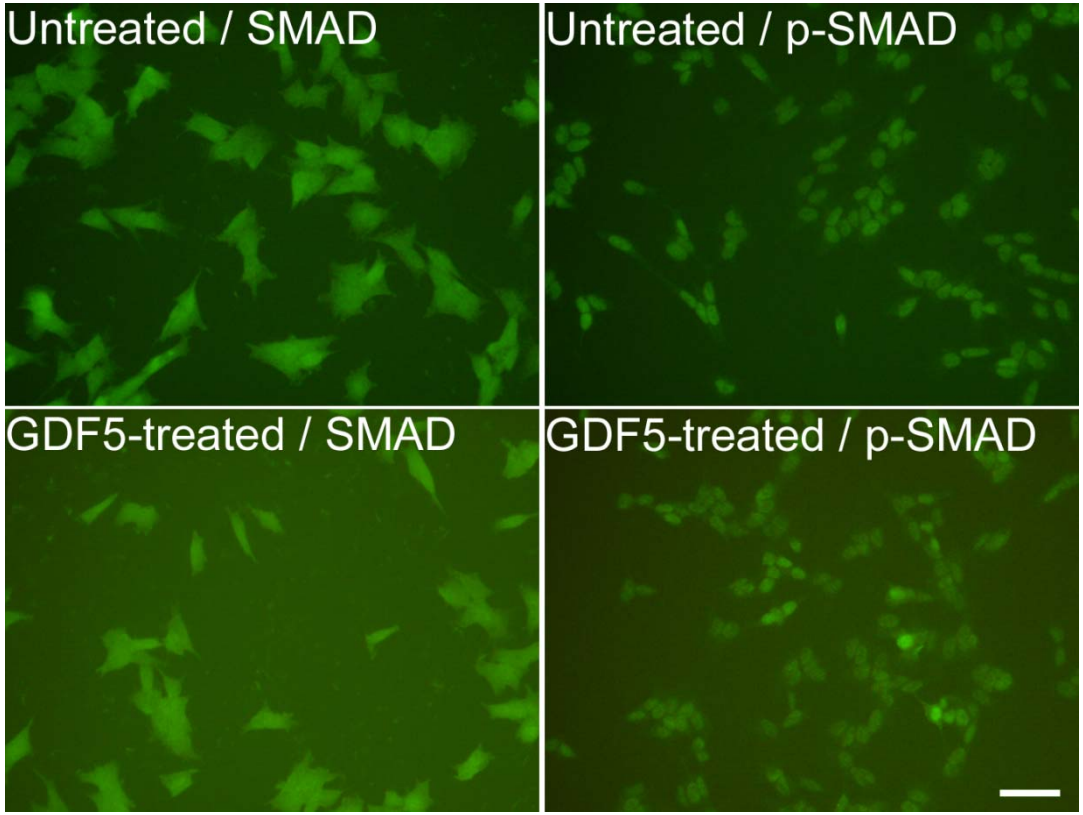

Untreated / SMAD
Untreated / p-SMAD
GDF5-treated / SMAD
GDF5-treated / p-SMAD

Figure 2B

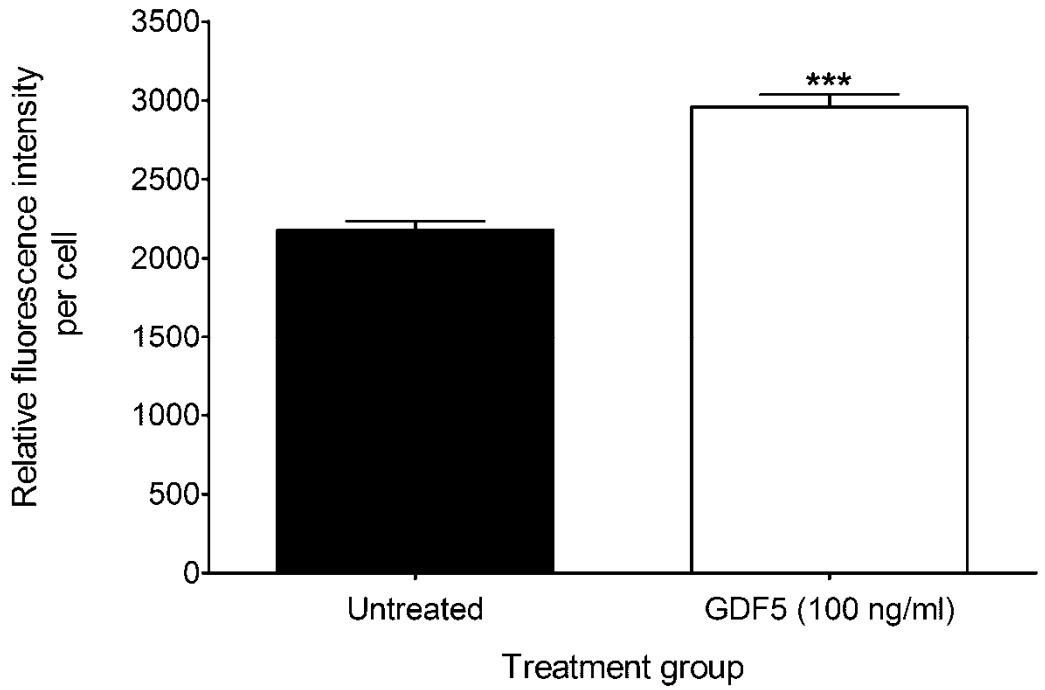

3500
3000
2500
2000
1500
1000
500
0
Relative fluorescence intensity per cell
***
Untreated
GDF5 (100 ng/ml)
Treatment group

Figure 2C

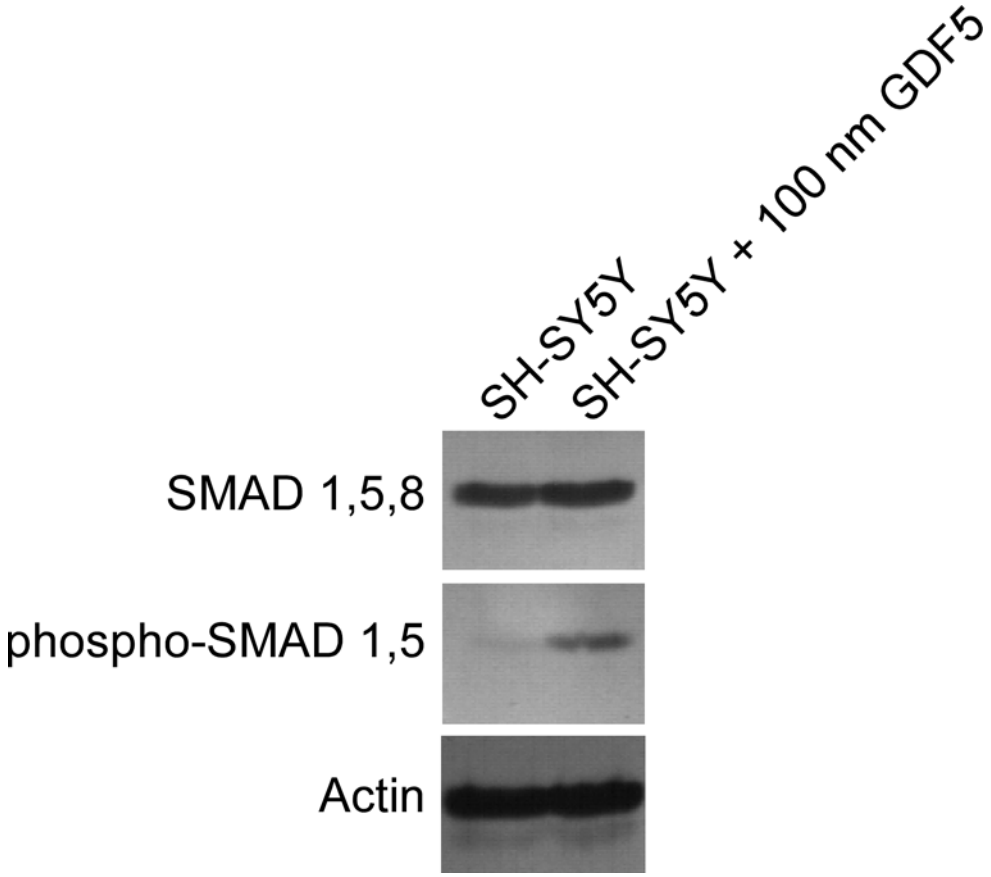


Figure 3

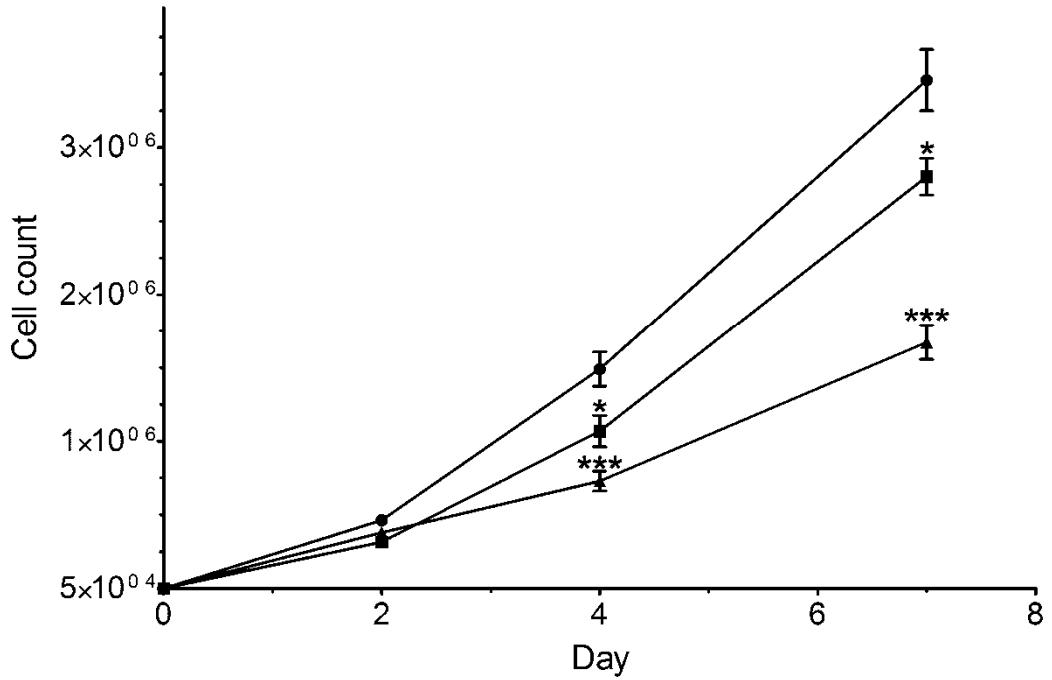


Figure 4A

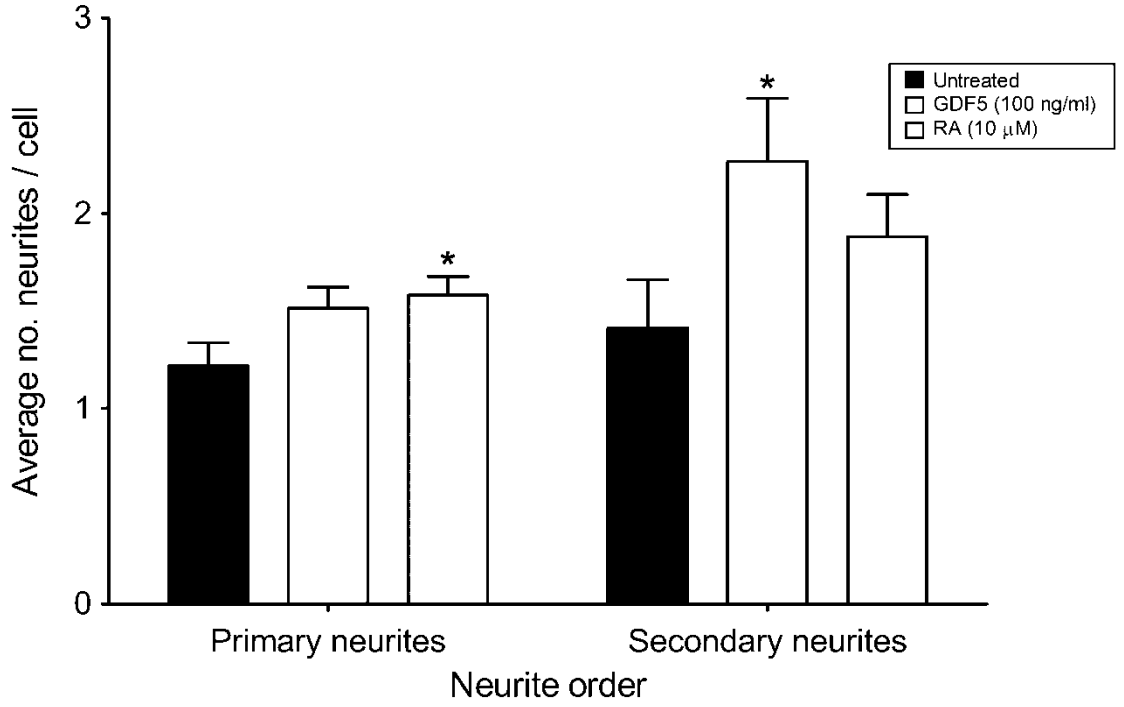

3
2
1
0
Average no. neurites / cell
*
*
Untreated
GDF5 (100 ng/ml)
RA (10 μM)
Primary neurites
Secondary neurites
Neurite order

Figure 4B

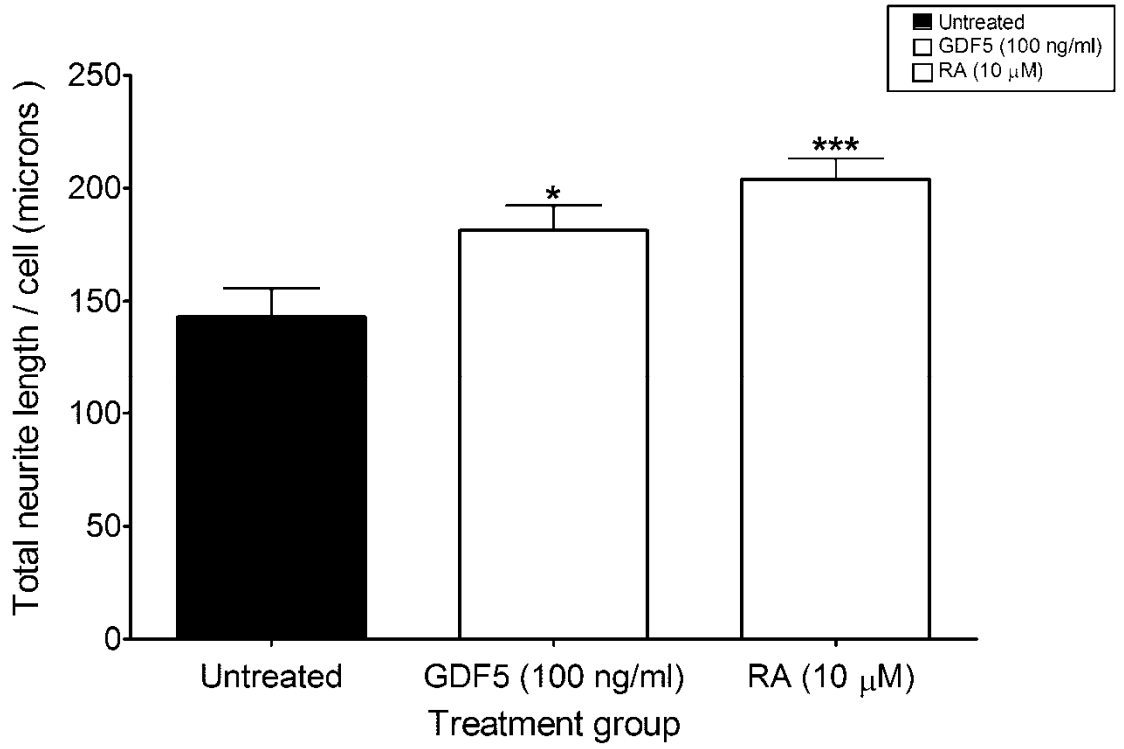

Untreated
GDF5 (100 ng/ml)
RA (10 μM)
Total neurite length / cell (microns)
250
200
150
100
50
0
*
***
Untreated
GDF5 (100 ng/ml)
RA (10 μM)
Treatment group

Figure 4C

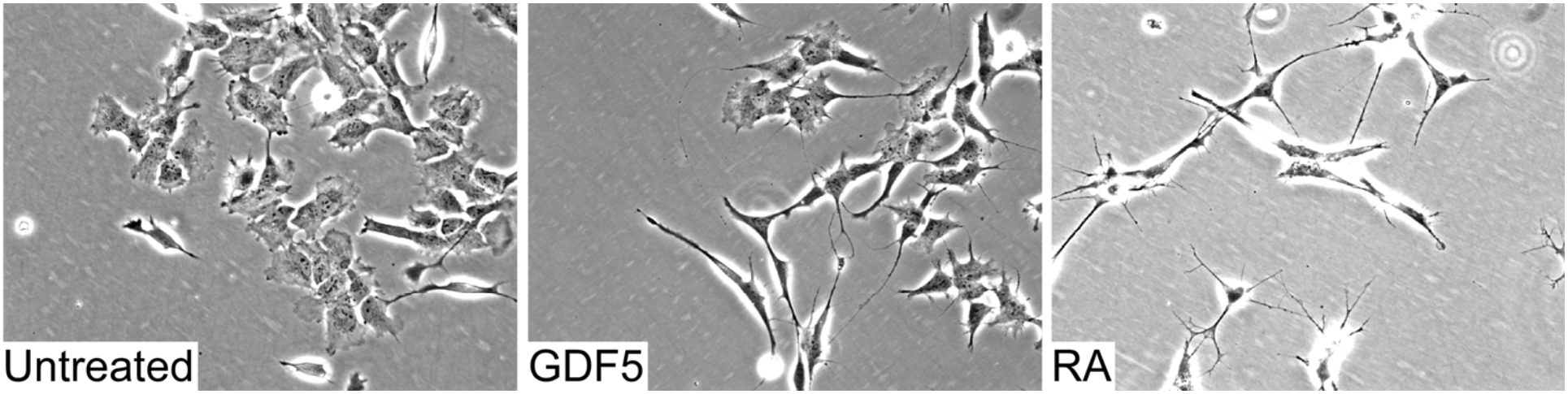
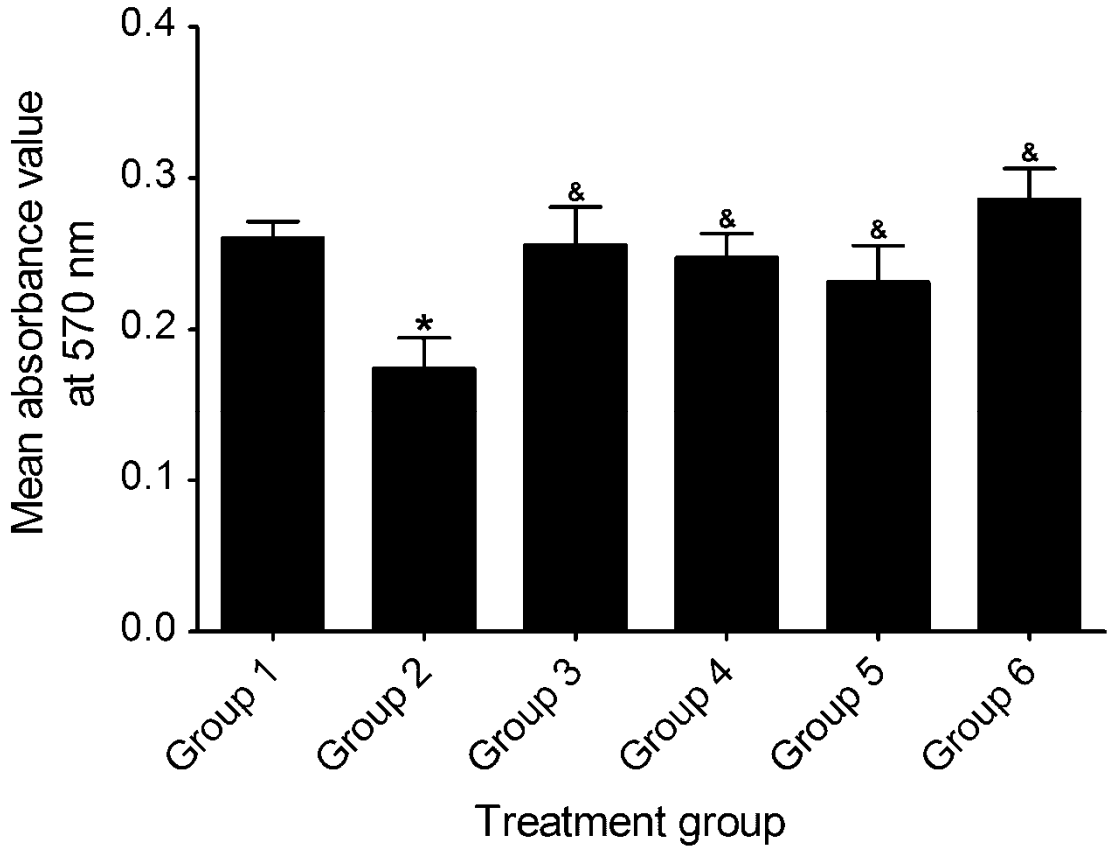


Figure 5A

Experimental Group	24h incubation	50 µM 6-OHDA (1h)	24h incubation
1	Medium	No	Medium
2	Medium	Yes	Medium
3	GDF5 (100 ng /ml)	Yes	GDF5 (100 ng /ml)
4	GDF5 (100 ng /ml)	Yes	Medium
5	Medium	Yes	GDF5 (100 ng /ml)
6	GDF5 (100 ng /ml)	No	GDF5 (100 ng /ml)

Figure 5B


0.4
0.3
0.2
0.1
0.0
Mean absorbance value at 570 nm
*
&
&
&
&
Group 1
Group 2
Group 3
Group 4
Group 5
Group 6
Treatment group