Gene co-expression analysis of the human substantia nigra identifies BMP2 as a neurotrophic factor that can promote neurite growth in cells overexpressing wild-type or A53T α-synuclein.

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Abstract

Introduction: α-synuclein-induced degeneration of dopaminergic neurons has been proposed to be central to the early progression of Parkinson's disease. This highlights the need to identify factors that are neuroprotective or neuroregenerative against α-synuclein-induced degeneration. Due to their potent neurotrophic effects on nigrostriatal dopaminergic neurons, we hypothesized that members of the bone morphogenetic protein (BMP) family have potential to protect these cells against α-synuclein.

Methods: To identify the most relevant BMP ligands, we used unbiased gene co-expression analysis to identify all BMP family members having a significant positive correlation with five markers of dopaminergic neurons in the human substantia nigra (SN).We then tested the ability of lead BMPs to promote neurite growth in SH-SY5Y cells and in primary cultures of ventral mesencephalon (VM) dopaminergic neurons, treated with either 6-OHDA or MPP^+, or overexpressing wild-type or A53T α-synuclein.

Results: Only the expression of BMP2 was found to be significantly correlated with multiple dopaminergic markers in the SN. We found that BMP2 treatment promoted neurite growth in SH-SY5Y cells and in dopaminergic neurons. Moreover, BMP2 treatment promoted neurite growth in both SH-SY5Y cells and VM neurons, treated with the neurotoxins 6-OHDA or MPP^+. Furthermore, BMP2 promoted neurite growth in cells overexpressing wild-type or A53T-α-synuclein.

Conclusion: These findings are important given that clinical trials of two neurotrophic factors, GDNF and neurturin, have failed to meet their primary endpoints. Our findings are a key first step in rationalising the further study of BMP2 as a potential neurotrophic factor in α-synuclein-based translational models of Parkinson's disease.

Key words: Parkinson's; α-synuclein; degeneration; neurotrophic factor; axon growth.

Introduction

The degeneration of midbrain dopaminergic (mDA) neurons leading to the loss of striatal innervation is central to the progression of Parkinson's disease (PD) (1). α-synuclein has been linked to PD since a number of mutations, such as the A30P (2) or A53T mutation in *SNCA* (3), as well as triplications (4) or duplications (5) of *SNCA*, are associated with autosomal dominant PD, while *SNCA* is also a susceptibility gene for sporadic PD (6). In addition, α-synuclein is the main component of Lewy bodies and Lewy neurites, which are pathological hallmarks of PD (7). Overexpression of α-synuclein in individual cultured mDA neurons has been shown to reduce neurite growth (8). This is also supported by the reduced connectivity and spine formation (9), and decreased neurite length and axonal degeneration (10), found in iPSC-derived neurons with *SNCA* triplication. Moreover iPSC-derived mDA neurons carrying the A53T mutation also develop neurite degeneration (11). These studies show that α-synuclein can lead to axonal and somal degeneration and highlight the need to identify factors that can promote mDA neurite growth in neurons carrying an α-synuclein load.

Bone morphogenetic proteins (BMPs) are a group of 15 structurally-related proteins that are members of the transforming growth factor (TGF)-β superfamily of ligands (12). The BMP receptors (BMPRs) (BMPR2 with BMPR1A or BMPR1B) are expressed in embryonic and adult rat mDA neurons (13). Adult male BMPR2 dominant-negative mice display an almost complete loss (~90%) of striatal innervation, with reduced locomotor activity (14). Previous studies have shown that BMP treatment increases mDA neuron number in midbrain cultures *in vitro* (15, 16). Additionally, BMP treatment, or overexpression of a constitutively-active BMPR1B, promotes neurite growth in SH-SY5Y cells (17), and in primary cultures of mDA neurons through the canonical Smad signalling pathway (12, 13, 17, 18). Moreover, knockdown of a negative regulator of BMP-Smad signalling during development promotes mDA neurite growth and leads to dopaminergic hyperinnervation of the striatum (19). However, the expression profiles of individual BMPs in mDA neurons are unknown, as is information on the effects of BMPs on neurite growth in cells overexpressing α-synuclein.

Here we show that of all BMPs, only transcripts for BMP2 and the BMPRs are co-expressed with transcripts for multiple markers of mDA neurons in the human SN. Additionally, we show that BMP2 promotes neurite growth and protects against neurotoxin-induced and wild-type- and A53T-α-synuclein-induced neurite degeneration in SH-SY5Y cells and in primary cultures of mDA neurons. These findings are an important step in rationalizing the further study of BMP2 in neurotrophic factor therapy for PD.

Materials and Methods

Gene expression analysis of the human SN

Human SN gene expression data from healthy controls (GSE:60863) (20) and age-matched control and PD patients (GSE:49036) (21) were analysed separately using the R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl). Pearson correlation analysis with a false discovery rate (FDR) multiple testing correction was used to identify all genes that were co-expressed with five dopaminergic neuronal markers: *ALDH1A1*, *NR4A2*, *KCNJ6*, *TH* and *LMX1B* in the SN, and four markers enriched in Purkinje cells in the cerebellum: *PDE1A1*, *CBLN1*, *PDE9A*, *CALB1* (GSE:60863). All gene expression data are presented as log2 expression values. Differences in expression were determined using one-way ANOVA with *post hoc* Tukey's test.

Cell culture

SH-SY5Y cells and embryonic day (E) 14 rat VM were cultured as previously described (13), under license with full ethical approval. Cultures were treated with 50ng/ml recombinant human (rh)BMP2 (Gibco), 6-OHDA (0-15µM) (Sigma), or MPP^+ (0-1mM) (Sigma), unless otherwise indicated. For transfection experiments, SH-SY5Y cells and E14 VM cultures were transfected with 0.5-1µg of plasmid DNA using the TransIT-X2® Dynamic Delivery System (Mirus Bio) according to the manufacturer's instructions. SH-SY5Y cells were seeded at a density of $2.5x10^4$ cells per well and allowed to reach ≥80% confluency prior to transfection. E14 VM cultures were seeded at a density of $1.5x10^5$ cells per well. The TransIT-X2:DNA complex was prepared according to the manufacturer's specifications using serum-free medium, TransIT-X2 reagent and 0.5-1µg/µl stock of control EGFP plasmid or EGFP-α-synuclein-Wild Type (WT) or EGFP-α-synuclein-A53T plasmids. These were a kind gift from David Rubinsztein (Addgene plasmid #40823) (22). The transfection efficiency for SH-SY5Y cells and E14 VM cells was 31% and 3%, respectively.

Western Blotting

Western blotting was carried out as previously described (19). Cells were lysed in RIPA buffer and insoluble debris was removed via centrifugation. 28µg of protein was run on a 12% SDS-PAGE gel and transferred onto polyvinyl difluoride (PVDF) membranes. The membranes were incubated with primary antibodies against α-synuclein (1:5000, Merck Millipore) and GAPDH (1:1000, SCBT) overnight shaking at 4°C, then washed, incubated with horseradish

peroxidase-labelled anti-mouse IgG (1:5000, Invitrogen), washed and developed using the Pierce™ ECL kit (Thermo Scientific).

Immunocytochemistry

SH-SY5Y cells were fixed in 4% paraformaldehyde (PFA; Sigma) and E14 VM cultures were fixed in ice-cold methanol or 4% PFA for 15 min at room temperature (RT). Cells were washed three times for 5 min per wash in 0.02% TritonX-100 (Sigma) in 10mM phosphate buffered saline (PBS-T), before being blocked in 5% bovine serum albumin (BSA) in 10mM PBS-T for 1 h at RT, then incubated overnight at 4°C in tyrosine hydroxylase (TH) (1:2000, Merck Millipore; Catalogue number: 36-008) or α-synuclein (SNCA) (1:2000, Merck Millipore; Catalogue number: AB152) diluted in 1% BSA in 10mM PBS-T. Following three 5-min washes in PBS-T, cells were incubated in the appropriate Alexa Fluor 488- or 594- conjugated secondary antibodies (1:500; Invitrogen) diluted in 1% BSA in 10mM PBS-T, for 2 h. Following washes in PBS-T, cells were counterstained with DAPI (1:3000; Sigma) and imaged using an Olympus IX71.

Assessment of cellular morphology

To assess cellular morphology, the lengths of 5 randomly-selected neurites were measured in no less than 15 randomly-selected fields per N, resulting in at least 225 neurites analysed per experiment. To assess somal area, the somal sizes of 5 randomly-selected cells were measured in 15 randomly-selected fields per N, resulting in 225 cells analysed per experiment. To determine the number of TH positive (TH^+) cells, TH and DAPI counts were completed in 15 randomly-selected fields and the numbers of TH^+ cells were expressed as percentages of the total DAPI-stained cells. All experiments were repeated at least 3 times and all analyses were performed using Image J software.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6 (©2018 GraphPad Software, CA USA). Unpaired student's *t*-test or one-way ANOVA with *post hoc* Dunnett's or Tukey's test were used as indicated to determine significant differences between groups. Results were deemed to be significant when $p < 0.05$. All cell data are presented as mean ± SEM.

Results

Co-expression analysis of the human SN identifies an association between BMPs/BMPRs and markers of mDA neurons.

We first conducted an unbiased co-expression analysis to identify all genes that were co-expressed with five markers of mDA neurons in the human SN: *ALDH1A1*, *NR4A2/Nurr1*, *KCNJ6/Girk2*, *TH* and *LMX1B* (Fig. 1a, b) (20). These analyses revealed significant positive correlations only for specific ligands and receptors of the BMP subfamily, namely *BMP2*, *BMPR2*, *BMPR1A* and *BMPR1B*, from the lists of co-expressed genes (Fig. 1b -f). While all of the BMPRs were positively correlated with four markers of Purkinje neurons in the human cerebellum (*PDE1A, CBLN1, PDE9A, CALB1*), *BMP2* was not (Fig. 1g). *BMP2* was also expressed at significantly higher levels in the SN than in the putamen or cerebellum (Fig. 1h). We next analysed gene expression data from age- and gender-matched samples in GSE:49036 (21) to examine *BMP2* and *BMPR* expression in the SN of patients at distinct stages of PD. These analyses showed a significant downregulation of *BMP2* in the SN of PD patients at Braak stage 5/6 compared to controls (Fig. 1i), while no significant differences were observed for *BMPR1B* (data not shown) or *BMPR2* (Fig. 1j). These data suggest that BMP2-BMPR signalling may play a functional role in mDA neurons in the human SN.

BMP2 promotes neurite growth in 6-OHDA- and MPP^{+-}-treated, and WT-α-synuclein- and A53T-α-synuclein-overexpressing, SH-SY5Y cells.

We next determined that 72h treatment with 1mM MPP^+ or 15µM 6-OHDA had a significant detrimental effect on neurite length in SH-SY5Y cells (Fig. 2a, b). To determine if rhBMP2 treatment could promote neurite growth in cells treated with 6-OHDA or MPP^+, SH-SH5Y cells were treated with 50 ng/ml rhBMP2 and cultured with or without either neurotoxin for 72h. Here, rhBMP2 promoted neurite growth in cells treated with either 6-OHDA or MPP^+, both of which had a detrimental effect on neurite growth in SH-SY5Y cells (Fig. 2c, d). To investigate if rhBMP2 could promote neurite growth in cells with a PD-causing WT- or A53T-α-synuclein overload, SH-SH5Y cells were transfected with a control-GFP, a WT-α-synuclein-GFP or an A53T-α-synuclein-GFP overexpression plasmid. Western blotting and immunocytochemistry revealed strong expression of α-synuclein in cells transfected with α-synuclein-GFP (Fig. 2e, f). Quantification of GFP-positive neurites showed that both WT- and A53T-α-synuclein overexpression induced decreases in neurite length compared to controls (*p*

< 0.01) (Fig. 2g, h). However, rhBMP2 treatment was found to promote neurite growth in both WT- and A53T-α-synuclein-overexpressing cells (Fig. 2g, h).

rhBMP2 protects cultured midbrain dopaminergic neurons against 6-OHDA and MPP^+.
As SH-SY5Y cells are catecholaminergic and do not recapitulate all the features of mDA neurons, we next examined the effects of rhBMP2 in primary cultures from E14 rat VM. We first determined that 72h treatment with 5μM MPP^+ or 6-OHDA had a significant detrimental effect on TH^+ mDA neurite length (Fig. 3a, b). rhBMP2 treatment induced significant increases in mDA neurite growth, even in those cultures treated with 6-OHDA or MPP^+ (Fig. 3c, d). We then examined the effect of MPP^+ and 6-OHDA on somal area and found a reduction in somal size which was not seen in cultures treated with rhBMP2 (Fig. 3e). We also assessed cell viability and found that both neurotoxins caused a significant decrease in the number of TH^+ neurons, which was partially rescued by rhBMP2 (Fig 3f, h), while the total cell number remained unaffected (Fig. 3g), indicating a partial neuroprotective effect of rhBMP2. These data show that rhBMP2 can promote neurite growth and partially protect primary mDA neurons against 6-OHDA- and MPP^+-induced degeneration.

rhBMP2 promotes neurite growth in WT-α-synuclein- and A53T-α-synuclein-overexpressing cultured midbrain dopaminergic neurons.
To investigate if rhBMP2 could also promote neurite growth in mDA neurons overexpressing WT- or A53T-α-synuclein, E14 rat VM cultures were transfected with both WT-α-synuclein-GFP and A53T-α-synuclein-GFP overexpression plasmids, or with a control-GFP plasmid. Immunocytochemistry (Fig. 4a) and subsequent quantification of α-synuclein staining (given the transfection efficiency was not sufficient for western blotting) showed that E14 VM cultures transfected with either the WT-α-synuclein-GFP or A53T-α-synuclein-GFP plasmid showed strong expression of α-synuclein compared to cells transfected with the control plasmid (Fig. 4b) . While the overexpression of WT-α-synuclein-GFP or A53T-α-synuclein had no effect on somal area compared to the control-GFP group (Fig. 4c), both WT-α-synuclein-GFP and A53T-α-synuclein-GFP resulted in a significant decrease in neurite length compared to controls ($p < 0.001$) (Fig. 4d, e). In contrast, WT-α-synuclein-GFP or A53T-α-synuclein-GFP expressing cells that were also treated with rhBMP2 were not significantly different from the controls. These data show that WT- or A53T-α-synuclein reduced neurite

growth without affecting somal area, and that rhBMP2 can promote neurite growth in cultured mDA neurons overexpressing WT- or A53T-α-synuclein.

Discussion

In the present study, we have shown that the expression of BMP2 and its receptors, BMPR1A, BMPR1B and BMPR2, is positively correlated with five key markers of dopaminergic function in the human SN, suggesting a functional relationship between the BMP pathway and mDA neurons. We have also shown that BMP2 is highly expressed in the SN compared to the putamen or indeed the cerebellum, an area known to be particularly responsive to BMPs (23). In agreement with this, previous studies have shown the expression of transcripts for BMP2, BMPR1B and BMPR2 receptors in the rat midbrain from E12, a period where neurotrophic support is critical, prior to target innervation (13). Furthermore, we have identified that BMP2 expression is significantly downregulated in the SN in PD patients at Braak stage 5/6, while there are no significant changes in the expression of its receptors. The findings of these studies suggest that endogenous BMP signalling, perhaps mediated by BMP2, may promote mDA neuronal survival and/or maintain mDA axonal integrity in the adult brain.

Using SH-SY5Y cells as an initial cellular model, we examined the functional role of BMP2 and found that rhBMP2 exerts a concentration-dependent effect on neurite growth (data not shown). In support of this, a number of previous studies have shown that rhBMP2 can promote survival and growth in rodent neurons (for review see ref (12)). In addition, we also found that rhBMP2 promoted neurite growth against the neuritotoxic effects of two well-established dopaminergic neurotoxins, MPP^+ and 6-OHDA. We then found that the overexpression of WT- and A53T-α-synuclein-GFP in SH-SY5Y cells caused a significant decrease in neurite length, which was not seen when co-treated with rhBMP2. However, while SH-SY5Y cells are widely-used as an *in vitro* model of mDA neurons, they are ultimately catecholaminergic cells and thus do not replicate all of the features of mDA neurons. To address this, we used primary cultures of E14 rat VM and found that rhBMP2 treatment protected mDA neurons against MPP^+- and 6-OHDA-induced axonal degeneration. Furthermore, we have shown that MPP^+ causes a reduction to cell soma size, and that both MPP^+ and 6-OHDA reduce numbers of TH^+ neurons, which is partially protected by rhBMP2. In support of our findings, rhBMP2-treatment has also been shown to increase the survival of transplanted E14 VM mDA neurons and to decrease motor deficits, in 6-OHDA-lesioned rat models of PD (24).

Collectively, these data show that rhBMP2 can protect mDA neurons against neurotoxin-induced injury and thus may have significant potential as a neurotrophic factor for use in PD therapy.

While MPP^+ and 6-OHDA are very useful neurotoxins for modelling PD, they do not mimic the α-synuclein accumulation that is central to disease progression (25). We therefore overexpressed WT- and A53T-α-synuclein in E14 VM cultures and found a decrease in neurite length, in agreement with a previous study using E14 rat mDA neurons (8). Here, we found that rhBMP2 could promote neurite growth against this α-synuclein-induced axonal degeneration. In addition, while both WT- and A53T- α-synuclein had significant effects on neurite length, neither WT- or A53T-α-synuclein had an effect on somal size, which is in agreement with a previous study (8). This is significant as it is possible that the degenerative aetiology of PD may occur at both somal and axon levels (26). Indeed, there have been several studies describing axonal degeneration using induced pluripotent stem cell (iPSC)-derived mDA neurons isolated from patients with PD. Specifically, iPSCs derived from PD patients with *SNCA* triplications displayed decreased neuronal differentiation, activity and neurite outgrowth compared to control iPSCs (27), as well as atypical neurite length and axonal fragmentation (10). Additionally, it has also been shown that PD patient iPSC-derived mDA neurons display phenotypic characteristics of PD, including Lewy-like neurites comprised of pathological α-synuclein, protein aggregation, atypical neurite outgrowth, and fragmented axons containing swollen varicosities of α-synuclein or Tau, and have a reduced capability to form synapses (11). These data agree with previous *in vivo* studies showing that transgenic mice expressing human α-synuclein develop synaptic dysfunction in striatal DA terminals and display an age-dependent reduction in DA release (28, 29). Therefore, given this mounting evidence in support of the role of α-synuclein in axonal degeneration in PD, it is important to identify neurotrophic factors that can protect against α-synuclein-induced axonal degeneration.

In summary, we report that BMP2 is co-expressed with mDA neuronal markers in the human SN and is downregulated in PD, and that treatment with rhBMP2 can promote mDA neurite growth and protect against neurotoxin- and α-synuclein-induced degeneration. These findings are important given that clinical trials of two dopaminergic neurotrophic factors, GDNF and neurturin, in PD patients have failed to meet their primary endpoints (30). In light of this, the findings in our study are an important first step in rationalising the further study of rhBMP2 as a potential neuroprotective therapy using α-synuclein-based translational models of PD.

Conflict of interest statement

The authors report no conflicts of interest or financial disclosures

Author contributions

SG performed the experiments, analysed the data and co-wrote the manuscript. LC, AS and GOK co-wrote the manuscript. LC, SG and GOK designed the experiments. LC, AS and GOK supervised the study.

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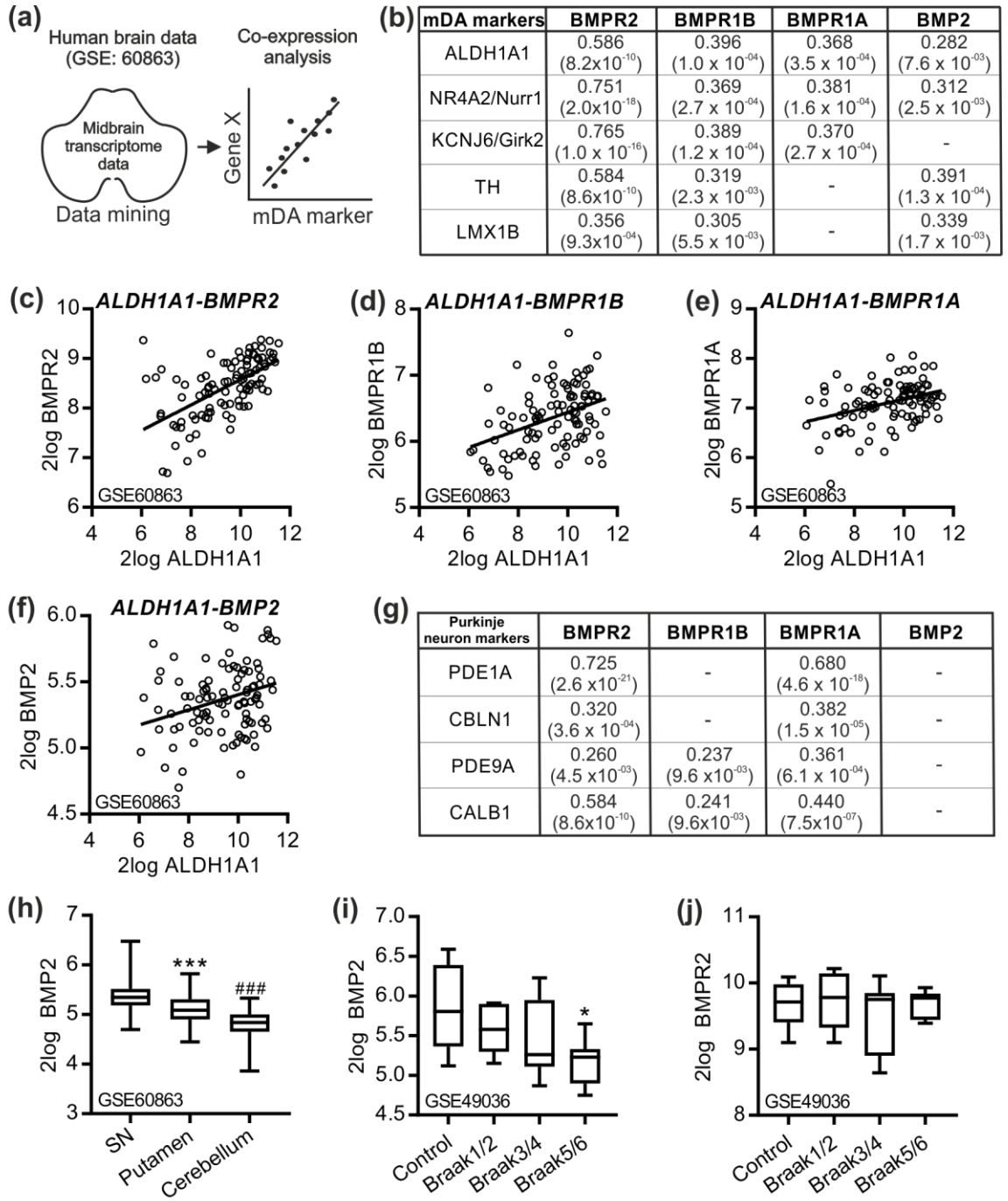
Figures and Figure Legends



mDA markers	BMPR2	BMPR1B	BMPR1A	BMP2
ALDH1A1	0.586 ($8.2x10^{-10}$)	0.396 (1.0×10^{-04})	0.368 (3.5×10^{-04})	0.282 (7.6×10^{-03})
NR4A2/Nurr1	0.751 ($2.0x10^{-18}$)	0.369 (2.7×10^{-04})	0.381 (1.6×10^{-04})	0.312 (2.5×10^{-03})
KCNJ6/Girk2	0.765 (1.0×10^{-16})	0.389 (1.2×10^{-04})	0.370 (2.7×10^{-04})	-
TH	0.584 ($8.6x10^{-10}$)	0.319 (2.3×10^{-03})	-	0.391 (1.3×10^{-04})
LMX1B	0.356 ($9.3x10^{-04}$)	0.305 (5.5×10^{-03})	-	0.339 (1.7×10^{-03})

Purkinje neuron markers	BMPR2	BMPR1B	BMPR1A	BMP2
PDE1A	0.725 ($2.6 x10^{-21}$)	-	0.680 (4.6×10^{-18})	-
CBLN1	0.320 (3.6×10^{-04})	-	0.382 (1.5×10^{-05})	-
PDE9A	0.260 ($4.5 x10^{-03}$)	0.237 ($9.6 x10^{-03}$)	0.361 (6.1×10^{-04})	-
CALB1	0.584 ($8.6x10^{-10}$)	0.241 ($9.6x10^{-03}$)	0.440 ($7.5x10^{-07}$)	-

Figure 1: BMP2-BMPRs are co-expressed with mDA neuron markers in the human SN. (a, b) Pearson correlation analysis showing the *r values* and false discovery rate (FDR)-corrected *p*-values (in parentheses) from the SN (n=99). (c-f) Graphs showing the correlation between *BMPR2, BMPR1B, BMPR1A, BMP2* and *ALDH1A1* expression in the SN. All data are 2log expression values. (g) Results of the Pearson correlation analysis showing the *r values* and FDR-corrected p-values (in parentheses) of the correlation of *BMPR2, BMPR1B, BMPR1A* and *BMP2* with four Purkinje neuron markers. (h) Box plot showing *BMP2* expression in the human SN (n=99), putamen (*n* = 121) and cerebellum (*n* = 121) (*** $p < 0.001$ and ### $p < 0.001$ vs. SN). (i, j) 2log expression values of (i) BMP2 and (j) BMPR2 in the SN of PD at distinct Braak stages of PD, and in age-matched controls (*$p < 0.05$ vs. Control). (h-i) One-

way ANOVA with *post-hoc* Tukey's test. Raw data was obtained from (b - h) GSE:60863 and (i, j) GSE:49036 and analysed using the R2: Genomics analysis and visualisation platform (https://hgserver1.amc.nl/cgi-bin/r2/main.cgi).

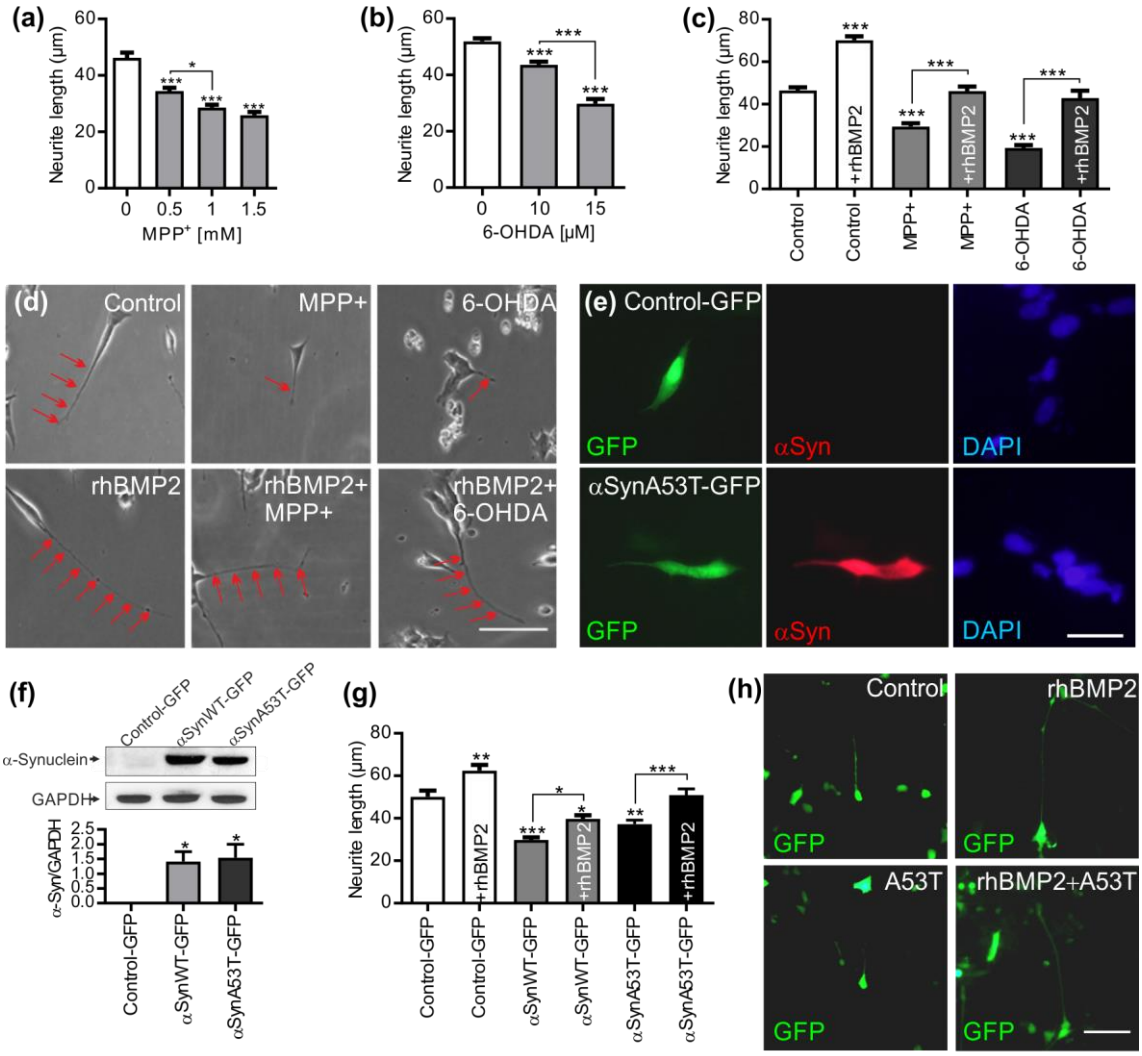


Figure 2: rhBMP2 promotes neurite growth in MPP⁺-treated, 6-OHDA-treated and A53T α-synuclein-overexpressing SH-SY5Y cells.

(a, b) Total neurite length of SH-SY5Y cells following 72h treatment with either (a) MPP⁺ or (b) 6-OHDA, at the concentrations indicated. (c) Total neurite length and (d) representative photomicrographs of SH-SY5Y cells treated with 50ng/ml rhBMP2 and cultured with or without 1mM MPP⁺ or 15µM 6-OHDA for 72 h. Scale bar = 100µm. (e) Representative images of SH-SY5Y cells transfected with constructs expressing GFP (Control-GFP) or A53T-α-synuclein-GFP (α-synA53T-GFP) immunocytochemically stained for α-synuclein protein at 24h, scale bar = 10µm; quantification of this expression is shown in (f). (g) Total neurite length and (h) representative images of transfected SH-SY5Y cells expressing either control-GFP, α-synWT-GFP or α-synA53T-GFP and cultured with or without 50ng/ml rhBMP2 for 72h. Scale bar = 50µm. All data are presented as mean ± SEM from at least three experiments. (* $p < 0.05$, $p < 0.01$, $p < 0.001$ vs. control or as indicated. One-way ANOVA with Tukey's (a, b, c, g) or Dunnett's (f) *post hoc* test).

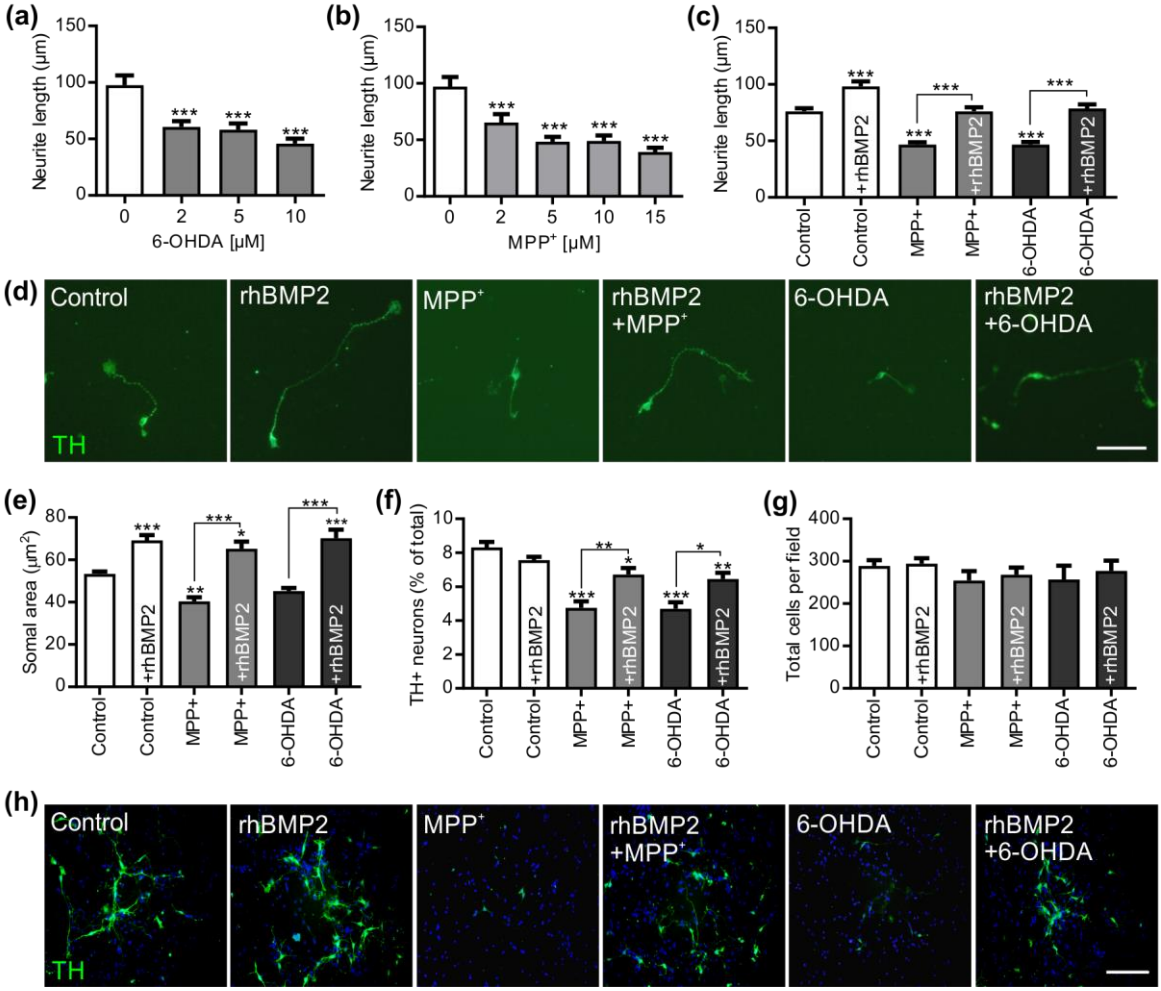


Figure 3: rhBMP2 promotes neurite growth and partially protects against mDA neuron loss in MPP⁺-treated and 6-OHDA-treated primary cultures of rat VM.
Total neurite length of DA neurons in E14 VM cultures following 72h treatment with (a) 6-OHDA or (b) MPP^+, at the concentrations indicated. (c) Total neurite length and (d) representative photomicrographs of dopaminergic neurons in E14 VM cultures treated with 50ng/ml rhBMP2 and cultured with or without 5µM MPP^+ or 5µM 6-OHDA for 72 h. Scale bar = 50µm. (e) Somal area, (f) TH^+ neurons as a percentage of total cells and (g) number of cells per field of view, in primary cultures of the E14 rat VM treated with 50ng/ml rhBMP2 and cultured with or without 5µM MPP^+ or 5µM 6-OHDA for 72h. (h) Representative photomicrographs of TH/DAPI-stained E14 VM cultures, treated as indicated. Scale bar = 50µm. All data are presented as mean ± SEM from at least three experiments. (* $p < 0.05$, $p < 0.01$, $p < 0.001$ vs. control or as indicated. One-way ANOVA with Tukey's *post hoc* test).

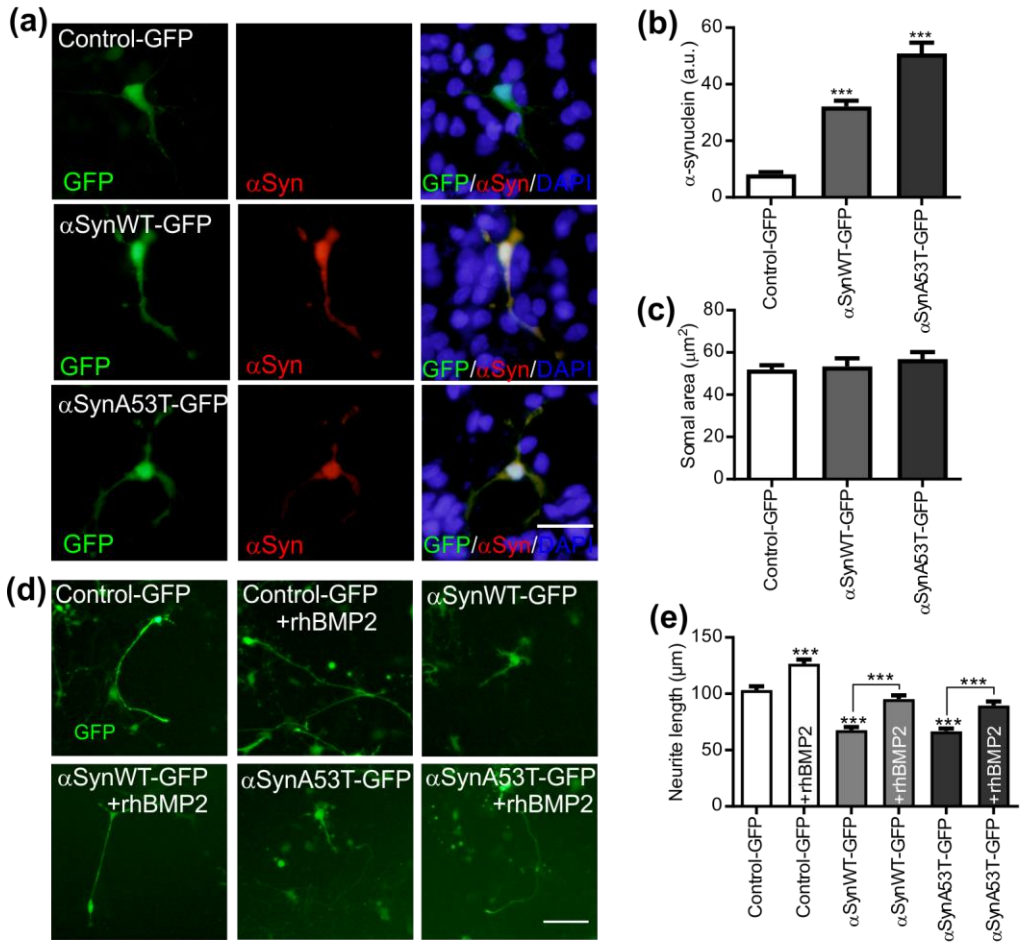


Figure 4: rhBMP2 promotes neurite growth in wild-type α-synuclein- and A53T α-synuclein-overexpressing cells in primary cultures of E14 rat VM.
(a) Representative images of primary cultures of E14 rat VM transfected with constructs expressing GFP (Control-GFP), α-synucleinWT-GFP (α-synWT-GFP) or α-synuclein A53T-GFP (α-synA53T-GFP), then immunocytochemically stained for α-synuclein at 72h. Scale bar = 10μm. Quantification of (b) α-synuclein expression and (c) cell somal area in cultures transfected with Control-GFP, α-synWT-GFP and α-synA53T-GFP. (d) Representative images (Scale bar = 50μm) and (e) quantification of total neurite length of E14 VM cultures transfected with Control-GFP, α-synWT-GFP and α-synA53T-GFP and cultured with or without 50ng/ml rhBMP2 for 72h. All data are presented as mean ± SEM from at least three experiments. (*** $p < 0.001$ vs. control or as indicated. One-way ANOVA with Tukey's *post hoc* test).