doi:10.1093/brain/awaa368 BRAIN 2021: 144; 1–6 | e15

BRAIN
A JOURNAL OF NEUROLOGY

LETTER TO THE EDITOR

BMP5/7 protect dopaminergic neurons in an α-synuclein mouse model of Parkinson's disease

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Whone *et al.* (2019) recently reported a randomized clinical trial of intermittent intraputamenal glial cell line-derived neurotrophic factor (GDNF) in Parkinson's disease. Whone *et al.* achieved an important milestone by demonstrating that convection-enhanced delivery is an efficient and safe way for long-term intraparenchymal drug application. GDNF was well tolerated and caused a significant increase in ^{18}F-DOPA uptake. However, the study failed to reach its clinical end points.

Neurotrophic factors are prime candidates for the development of disease-modifying therapies for Parkinson's disease, due to their properties to restore and maintain the functional integrity of dopaminergic (DA) neurons that are dysfunctional but not lost (Kirkeby and Barker, 2019). Based on the efficacy of several neurotrophic factors to protect the degeneration of DA neurons in toxin-based Parkinson's disease animal models, some of these proteins have been tested in clinical studies. Particularly, GDNF and neurturin (NTN), both closely related members of the TGF-β protein superfamily, have been evaluated (Kirkeby and Barker, 2019). During the past 20 years, since the first clinical applications of neurotrophic factors, great efforts have been made to optimize dosage, delivery, and the choice of the appropriate patient group. In more recent clinical trials, the application of GDNF and NTN appeared to be safe. However, the efficacy data remain disappointing, since, to date, advanced clinical trials have not been able to show clinical benefit (Kirkeby and Barker, 2019). This has raised concerns about whether neurotrophic factors are indeed able to rescue the dopaminergic nigrostriatal pathway in patients with Parkinson's disease.

The accumulation of the protein α-synuclein in rare genetic forms and in the much more common sporadic forms of Parkinson's disease, together with preclinical studies, indicates that α-synuclein plays a central role in the aetiology and pathophysiology of Parkinson's disease (Brás *et al.*, 2020). In contrast to toxin-induced DA neurodegeneration, GDNF does not protect substantia nigra neurons against α-synuclein-induced DA neurodegeneration in a rat Parkinson's disease model (Decressac *et al.*, 2012). As a

Advance access publication November 30, 2020

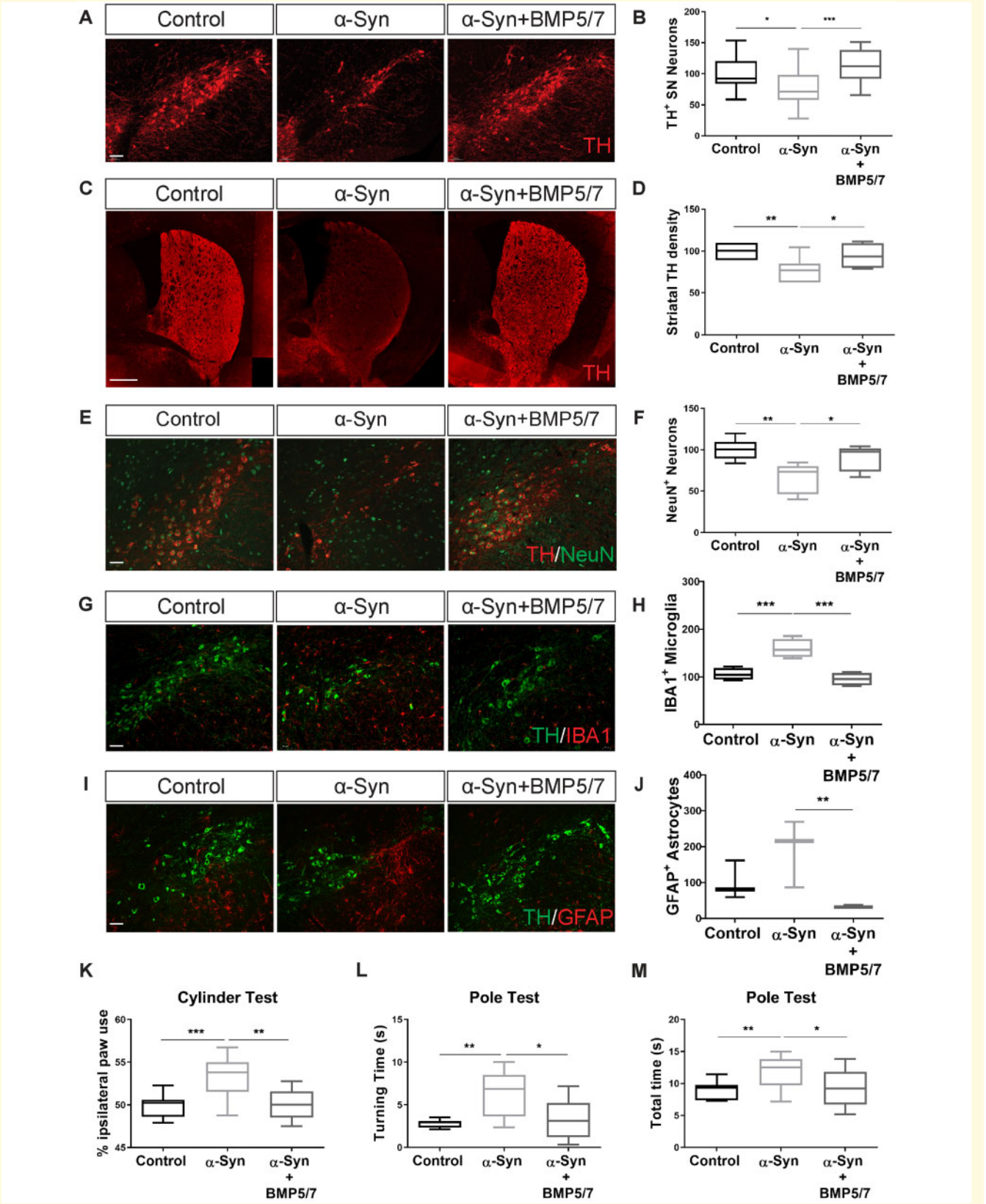

Figure 1 BMP5/7 prevent A53T α-synuclein-induced substantia nigra neuronal loss, gliosis and ameliorate associated motor impairment in a Parkinson's disease mouse model. (**A**, **B** and **E**–**J**) Representative micrographs and corresponding quantification of the substantia nigra (SN) injected with EGFP expressing vector (control), substantia nigra injected with A53T α-synuclein expressing vector (α-syn) and substantia nigra injected with A53T α-synuclein expressing vector together with striatal BMP5/7 expressing vector (α-syn + BMP5/7). (**A** and **B**) The number of TH^+ neurons was decreased by A53T α-synuclein overexpression ($P = 0.0178$). BMP5/7 treatment prevents the decrease

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possible explanation, the downregulation of the GDNF receptor Ret by excess cellular concentrations of α-synuclein was suggested (Decressac *et al.*, 2012). These results raise the question as to whether neurotrophic factors have the potential to slow or halt α-synuclein-induced neurodegeneration.

Bone morphogenetic proteins (BMPs), as GDNF and NTN, are members of the TGF-β protein superfamily. Activated BMP receptor signalling is transduced in the cytoplasm by SMAD1, 5 and 8 and inhibited by SMAD6 and 7. Recently, we discovered that BMP5, together with BMP7 (BMP5/7), are essential regulators of the development of DA neurons *in vivo* and identified SMAD1 as an essential BMP signalling component that controls the differentiation of DA progenitors, particularly to substantia nigra neurons (Jovanovic *et al.*, 2018). Moreover, we found that BMP5/7 robustly increase the maturation of human induced pluripotent and neural stem cells to DA neurons (Jovanovic *et al.*, 2018). BMP5/7 are not only essential for the development of DA neurons, but BMP7 also shows beneficial effects in a toxin-based Parkinson's disease animal model (Zuch *et al.*, 2004). However, it is unknown whether BMPs can protect substantia nigra neurons against α-synuclein-induced neurotoxicity *in vivo*. Moreover, the role of the BMP/SMAD signalling pathway in α-synuclein homeostasis and aggregation *in vivo* is unknown.

The aim of this study was to test the hypothesis that BMP5/7 protect DA neurons against α-synuclein induced neurotoxicity, and if verified, to provide insights into the mechanism of action of BMP5/7.

First, we tested the potential of BMP5/7 to protect against α-synuclein-induced neurotoxicity. To this end, we used an established viral vector-based mouse model in which the human α-synuclein, containing the A53T mutation, was expressed by adeno-associated virus serotype 1/2 (AAV1/2) vectors under the control of a CMV/CBA promoter (Ip *et al.*, 2017). We injected 8–12-week-old C57BL/6 male mice unilaterally into the right substantia nigra and studied animals for 12 weeks following the injection. As controls, we used mice injected with enhanced green fluorescent protein (EGFP) expressing vectors that showed a wide expression of EGFP in the ventral midbrain and in the striatum (Supplementary Fig. 1A).

Expression of A53T α-synuclein caused significant loss of DA neurons, as visualized by tyrosine hydroxylase (TH^+) and NeuN immunolabelling when compared to control animals expressing EGFP. Simultaneous striatal injection of lentiviral vectors expressing BMP5/7 (pHAGE-CMV-BMP5 together with pHAGE-CMV-BMP7) fully prevented the loss of TH^+ neurons as well as $NeuN^+$ cells that were induced by α-synuclein overexpression (Fig. 1A, B, E and F). BMP5/7 also prevented the reduction of DA projections into the striatum in the Parkinson's disease model, as measured by striatal optical density of TH immunofluorescence (Fig. 1C and D).

Loss of DA neurons following the accumulation of α-synuclein in Parkinson's disease has been associated with gliosis characterized by activated microglia and reactive astrocytes (Refolo and Stefanova, 2019). To determine if the neuroprotective effects of BMP5/7 are associated with reduced gliosis in the substantia nigra, we studied IBA1 expression as a microglia marker and GFAP as a marker for activated astrocytes. In A53T α-synuclein treated animals, the number of IBA1-positive cells was significantly increased. BMP5/7 treatment led to a significant reduction of IBA1-positive cell numbers, which was similar to control levels (Fig. 1G and H) and reduced morphological signs of activation (Supplementary Fig. 1B). Moreover, BMP5/7 treatment significantly reduced the number of GFAP-positive astrocytes (Fig. 1I and J).

To assess whether BMP5/7 could ameliorate motor impairments caused by A53T α-synuclein overexpression in the substantia nigra (Ip *et al.*, 2017), we used the Cylinder and Pole Tests, two standard paradigms used to assess motor deficiencies caused by substantia nigra lesion. BMP5/7 could fully prevent unilateral paw use in the Cylinder Test (Fig. 1K). In the Pole Test, BMP5/7 could significantly ameliorate the turning time and the total time (Fig. 1L and M).

Next, we aimed to gain insights into the molecular mechanisms mediating the therapeutic effects of BMP5/7. To this end, we studied their potential to modulate α-synuclein accumulation. A53T α-synuclein treated mice showed a significant increase of cells double-positive for TH and α-synuclein

Figure 1 Continued
in the number of TH^+ neurons ($P = 0.0017$) (Control, $n = 7$; α-syn, $n = 18$; α-syn + BMP5/7, $n = 5$). (**C** and **D**) The immunofluorescence signal of striatal TH^+ fibres is reduced following A53T α-synuclein overexpression ($P = 0.0081$) and increased by adding BMP5/7 ($P = 0.0289$) (Control, $n = 6$; α-syn, $n = 7$; α-syn + BMP5/7, $n = 5$). (**E** and **F**) The total neuronal number as assessed by $NeuN^+$ cells is decreased by A53T α-synuclein overexpression ($P = 0.0010$) and is increased after BMP5/7 treatment ($P = 0.0391$) ($n = 3$ per group). (**G** and **H**) α-Synuclein overexpression leads to an increase in the number of $IBA1^+$ microglia cells ($P = 0.0010$), which is prevented by BMP5/7 treatment ($P = 0.0003$). (**I** and **J**) The number of $GFAP^+$ activated astroglia cells show a trend towards an increase after α-synuclein overexpression ($P = 0.1005$). BMP5/7 significantly reduce the number of $GFAP^+$ cells ($P = 0.0092$) ($n = 3$ per group). (**K**) α-Synuclein overexpression leads to motor impairments as indicated by the increase in the percentage of ipsilateral paw use in the Cylinder Test ($P = 0.0004$), which is ameliorated by BMP5/7 treatment ($P = 0.0032$). (**L** and **M**) α-Synuclein overexpression causes motor impairments also in the Pole Test as indicated by increased turning time ($P = 0.0017$) and total time ($P = 0.0089$). BMP5/7 significantly reduced the turning time ($P = 0.0115$) and the total time in the Pole Test ($P = 0.0374$) (Control, $n = 7$; α-syn, $n = 18$; α-syn + BMP5/7, $n = 5$). Scale bar in **A** = 100 μm; **C** = 500 μm; **E**, **G**, and **I** = 50 μm. The data in graphs represent the mean ± standard error of the mean (SEM). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ Fisher's LSD *post hoc* test, after significant one-way ANOVA.

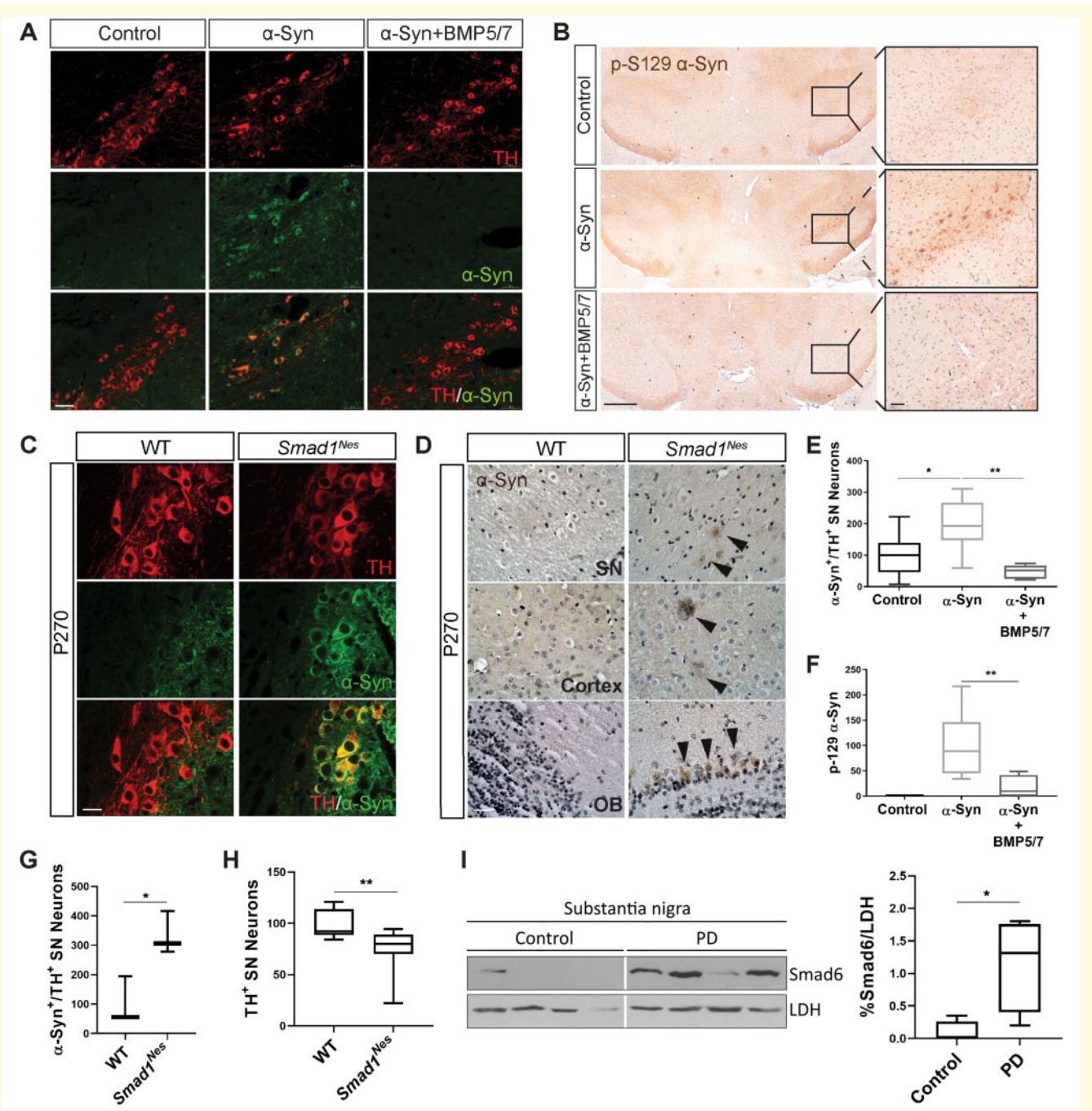

Figure 2 The BMP/SMAD signalling pathway modulates α-synuclein accumulation and is attenuated in Parkinson's disease post-mortem brains. (**A**, **B**, **E** and **F**) Representative micrographs and corresponding quantification of the substantia nigra (SN) injected with EGFP expressing vector (control), substantia nigra injected with A53T α-synuclein expressing vector (α-syn) and substantia nigra injected with A53T α-synuclein expressing vector together with striatal BMP5/7 expressing vector (α-syn + BMP5/7). (**A**) Immunofluorescence staining of TH (*top*), α-synuclein (*middle*) and double immunolabelling of substantia nigra DA neurons (*bottom*). (**E**) A53T α-synuclein overexpression leads to α-synuclein accumulation in TH^+ neurons ($P = 0.0150$), which is attenuated in BMP5/7 treated animals ($P = 0.0015$). Fisher's LSD *post hoc* test, after significant one-way ANOVA (Control, $n = 7$; α-syn, $n = 11$; α-syn + BMP5/7, $n = 5$). (**B**) Representative micrographs of immunohistochemical staining of p-S129 α-synuclein of the ventral midbrain with the injected side on the *right* and the contralateral control side on the *left*. The *right* column shows higher magnification of the injected side. (**F**) In controls, p-S129 α-synuclein-positive cells are undetected, whereas there are abundant p-S129 α-synuclein-positive cells in the injected side (set to 100 in the bar graph). The number of p-S129 α-synuclein-positive cells was significantly reduced by BMP5/7 treatment. Unpaired two-tailed Student's *t*-test ($P = 0.0031$) ($n = 5$ per group). (**C** and **D**) Representative micrographs of the substantia nigra (in **C** and **D**) and the cortex and olfactory bulb (in **D**) of wild-type (WT) controls and of *Smad1*^{Nes} mice in which *Smad1* is excised in neurons. (**C**) Immunofluorescence of TH (*top*), α-synuclein (*middle*) and double immunolabelling of substantia nigra DA neurons (*bottom*) and (**G** and **H**) quantification. (**G** and **H**) *Smad1*^{Nes} mutants showed an increased number of TH^+/α-synuclein$^+$ cells ($P = 0.0211$) associated with a reduced number of TH^+ substantia nigra neurons ($P = 0.0086$). Unpaired two-tailed Student's *t*-test ($n = 3$ per

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immunostaining, an effect that was significantly reduced by BMP5/7 expression (Fig. 2A and E).

Importance has been attributed to α-synuclein phosphorylation at residue S129 (p-S129). Under normal conditions, only a small fraction (<4%) of α-synuclein is constitutively phosphorylated at S129 in the brain. However, in the brains of patients suffering from synucleinopathies such as Parkinson's disease, a very strong accumulation (>90%) of p-S129 is observed (Oueslati, 2016). In control mice, p-S129 α-synuclein was not detected. Mice treated with A53T α-synuclein showed a robust p-S129 α-synuclein immunostaining in the substantia nigra, which was significantly reduced by BMP5/7 treatment. (Fig. 2B and F).

To study the effects of BMPs on α-synuclein, we complemented our BMP treatment experiments with BMP pathway inactivation experiments. To achieve this, in embryonic mouse neural stem cells, we conditionally deleted the *Smad1* gene (flanked by two *loxP* sites) by using a Nestin-Cre recombinase allele ($Smad1^{Nes}$). Thus, by removing the SMAD1 protein, which plays a critical role in the canonical BMP signal transduction cascade, we significantly attenuated intracellular BMP signalling in neurons. Previously, we reported that $Smad1^{Nes}$ mutants show a significant decrease in the number of DA neurons in embryonic and postnatal stages, predominantly in the substantia nigra (Jovanovic *et al.*, 2018). In adult $Smad1^{Nes}$ mutants, there was a significant increase in TH/α-synuclein-positive neurons compared to controls, which was associated with a reduction in DA neurons (Fig. 2C, G and H). Immunohistochemistry, using proteinase K as an epitope retrieval step that breaks down normal synuclein while sparing aggregated synuclein (Beach *et al.*, 2008), did not show any aggregates in wild-types. In contrast, $Smad1^{Nes}$ mutants exhibited α-synuclein aggregates in the substantia nigra, cortex, and olfactory bulb (Fig. 2D).

A recent meta-analysis of differentially expressed genes in the substantia nigra of idiopathic Parkinson's disease patients indicated that the BMP pathway is downregulated in Parkinson's disease (Kelly *et al.*, 2019). Moreover, in an independent study, the mRNA of the inhibitory SMAD6 was found to be upregulated in the substantia nigra of Parkinson's disease patients (Briggs *et al.*, 2015). Therefore, we investigated SMAD6 protein expression in the substantia nigra of Parkinson's disease post-mortem brains. SMAD6 protein was significantly upregulated in the substantia nigra of patients with Parkinson's disease compared to age-matched control subjects, providing further evidence of a downregulation of the BMP/SMAD pathway in Parkinson's disease (Fig. 2I).

In summary, we report that BMP5/7 prevents α-synuclein-induced loss of DA neurons, motor impairments and associated gliosis. Moreover, we demonstrate that BMP5/7 treatment significantly reduces α-synuclein accumulation. Complementarily, loss of BMP/SMAD signalling leads to the accumulation of α-synuclein, suggesting that the therapeutic mechanism of action of BMP5/7 involves the reduction of α-synuclein accumulation. Finally, we provide evidence that the inhibitory SMAD6 is upregulated in the substantia nigra of Parkinson's disease patients.

The therapeutic potential of BMPs for treating Parkinson's disease is further supported by an independent study (Goulding *et al.*, 2021). There, the authors show that growth differentiation factor 5 (BMP 14) overexpression maintains nigrostriatal integrity and striatal DA levels in an α-synuclein overexpression model of Parkinson's disease. They complement our approach, in which we used A53T overexpression in mice and lentiviral vectors for BMP treatment, by using wild-type α-synuclein in rats and AAV-delivered BMPs. This study independently corroborates our findings providing evidence for the therapeutic efficacy of BMPs.

We conclude that neurotrophic factors remain a valid approach and that dose adjustments, progress in drug delivery and testing new drug candidates, including BMPs, are promising strategies to establish a disease-modifying therapy for Parkinson's disease.

Data availability

The data that support the findings of this study are available from the corresponding author upon request.

Funding

This work was supported by the United States–Israel Binational Science Foundation (Grant 2019246 to C.B. and R.H.F) and the Israel Science Foundation (Grant 1310/19 to D.G.).

Competing interests

The authors report no competing interests.

Figure 2 Continued

group). (**D**) Immunohistochemistry visualizing aggregated α-synuclein after proteinase K treatment. In contrast to controls that show no aggregates, $Smad1^{Nes}$ mutants show α-synuclein aggregates in the substantia nigra, cortex, and olfactory bulb (OB) (n = 3 per group). (**I**) Homogenates prepared from substantia nigra of Parkinson's disease and control patients were probed with anti-SMAD6 antibody (*left*). Graph depicts SMAD6 levels normalized to LDH (*right*). Substantia nigra of four Parkinson's disease patients and four aged-matched controls (Parkinson's UK Brain Bank) were analysed in three independent experiments. Unpaired two-tailed Student's t-test (P = 0.0285). Scale bar in **A** and **B** = 50 μm; **B** (*insets*) = 500 μm; **C** and **D** = 100 μm; The data in graphs represent mean ± SEM. Two-tailed unpaired Student's t-test $^{*}P < 0.05$, $^{**}P < 0.01$. Fisher's LSD *post hoc* test, after significant one-way ANOVA $^{*}P < 0.05$, $^{**}P < 0.01$.

Supplementary material

Supplementary material is available at *Brain* online.

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