HELLENIC REPUBLIC

National and Kapodistrian University of Athens

Department of Biology

Athens International Master's Programme in Neurosciences

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RESEARCH THESIS PROJECT

INVESTIGATION OF THE POTENTIAL NEUROPROTECTIVE ROLE OF A BDNF ANALOG IN PARKINSON'S DISEASE PATIENT-DERIVED NEURONS

Panagiotis Gkaravelas

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Contents

Investigation of the potential neuroprotective role of a BDNF analog in Parkinson's disease patient-derived neurons

Panagiotis Gkaravelas[1]

Summary

Parkinson's disease (PD) remains an incurable neurodegenerative disorder characterized by motor dysfunction related to the progressive loss of midbrain dopamine neurons while non-motor symptoms are also present. BNN-20 is a synthetic, BDNF-mimicking, microneurotrophin that has been shown to exhibit a pleiotropic neuroprotective effect on dopaminergic neurons of the substantia nigra pars compacta in the "weaver" mouse model of PD. Here, we assessed its potential effects in a unique human setting for the identification and interpretation of PD phenotypes. In particular, we used an induced pluripotent stem cell (iPSC)-based model of PD from patients bearing the p.A53T-α-synuclein mutation that simulates disease-relevant phenotypes, including protein aggregation, compromised neuritic growth, axonal pathology and reduced synaptic connectivity. Our findings demonstrate that BNN-20 treatment increased slightly the percentage of differentiated neurons in PD cultures and protected partially PD neurons from stress-induced cell death and their axons from fragmentation without quenching neurite outgrowth impairment or restoring expression levels of genes associated with axon guidance and synaptic function, which were significantly dysregulated in PD neurons. Further investigation is needed to identify BNN-20 as a potential neuroprotective agent.

Highlights

- BNN-20 moderately increased the percentage of differentiated neurons and ameliorated axonal pathology in PD iPSC-derived cultures.
- BNN-20 didn't rescue neurite outgrowth impairment and was ineffective in restoring expression levels of synaptic genes downregulated in PD iPSC-derived neurons.
- BNN-20 slightly protected PD neurons from stress-induced cytotoxicity.

Keywords

Microneurotrophin, BNN-20, Parkinson's disease, induced pluripotent stem cells, neural progenitor cells

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Introduction

Parkinson's disease (PD) is a complex progressive neurodegenerative disorder and the most common movement disorder in the world affecting about 1% of adults older than 60 years (Samii, Nutt and Ransom, 2004). PD is characterized by selective loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) coupled with intracytoplasmic inclusions known as Lewy bodies composed mainly of α-synuclein (αSyn) (Braak and Braak, 2000; Lees, Hardy and Revesz, 2009). The cardinal motor features of PD are resting tremor, rigidity, bradykinesia and postural instability (Samii, Nutt and Ransom, 2004). Although motor symptoms define the disease, various non-motor symptoms including psychiatric and cognitive impairments, autonomic dysfunction, sensory and sleep disturbances also have negative impacts on the quality of life in PD patients (Pfeiffer, 2016). The majority of PD cases are sporadic with unknown etiology, while an approximate 10% represent familial cases (Thomas and Beal, 2007). Alpha-synuclein (αSyn) is the major sporadic PD-linked gene (Simón-Sánchez *et al.*, 2009) while point mutations (Petrucci, Ginevrino and Valente, 2016) and multiplications of the locus (Chartier-Harlin *et al.*, 2004) cause autosomal dominant forms of early onset and aggressive Parkinsonism and dementia. The best characterized mutation is G209A in the α-synuclein gene *SNCA*, first identified in families of Italian and Greek ancestry (Polymeropoulos *et al.*, 1997) and resulting in the pathological p.A53T-αSyn protein.

Research on the pharmacotherapy of PD has recently focused on compounds that can mimic the neuroprotective properties of endogenous neurotrophic factors, while penetrating the blood-brain-barrier (BBB), hence limiting neuronal death and slowing down disease exacerbation (Gravanis, Pediaditakis and Charalampopoulos, 2017). Neurotrophins such as the nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) are synthesized mainly in the CNS and play a pivotal role in the control of neuronal development, survival and regeneration. They exert their multiple neurotrophic and neuroprotective actions through binding to specific pro-survival tyrosine kinase receptors (Trk) (Arévalo and Wu, 2006). A significant member of this group that pertains to our interests is BDNF and its partner receptor TrkB, which play a vital role in the survival and maintenance of dopaminergic neurons. Previous studies have reported high expression of BDNF and TrkB receptor in dopaminergic neurons of the SN and have assessed the potential involvement of BDNF/TrkB signaling in PD (Jin, 2020b). The expression of BDNF in SN was significantly lower in PD patients compared with that in control (Parain *et al.*, 1999) and BDNF knockout mice showed reduced dopamine (DA) D3 receptor expression (Guillin *et al.*, 2001). In addition, MPTP-induced mouse models of PD exhibited significantly reduced TrkB expression compared to their respective controls (Ding *et al.*, 2011). Furthermore, aging, the primary risk factor for PD, also leads to a marked reduction in the expression of TrkB in the dopaminergic neurons of rats' SN (Parkinson, Dayas and Smith, 2015), while αSyn mutations are linked to a loss in BDNF and TrkB expression (Kohno *et al.*, 2004; Szego *et al.*, 2011). These studies suggest that the reduction in BDNF and TrkB expression could be one of the etiological factors in PD pathogenesis. It follows that neurotrophins, including BDNF, in the specific context of PD, constitute a group of highly promising putative therapeutic agents. This claim is further

supported by studies which showed that BDNF administration ameliorated the observed pathological phenotypes in PD animal models. More specifically, BDNF increased dopaminergic neurons in SN and protected dopaminergic projections to striatum in mice models of PD, when administered before inducing PD pathology (Levivier *et al.*, 1995; Nam *et al.*, 2015), while inducing BDNF expression 6 weeks after the axonal lesion in the 6-OHDA model of PD resulted in dopaminergic axon regrowth (Kim *et al.*, 2012). In addition, studies on monkeys showed that BDNF administration led to significantly less severe non-motor PD symptoms (Tsukahara *et al.*, 1995).

Neurosteroids such as dehydroepiandrosterone (DHEA) also possess important neuroprotective and neurogenic properties in vitro and in vivo (Charalampopoulos *et al.*, 2008). DHEA, as a precursor of estradiol and testosterone, represents the most abundant steroid hormone in the human body and can be synthesized de novo in the brain (Mellon, 2007). The levels of DHEA decrease with age and this decline could be associated with age-related diseases such as PD (Schumacher *et al.*, 2003). In PD patients, lower DHEA levels were correlated with higher ratings of psychopathology, poorer memory performance and more severe Parkinsonian movements (Harris, Wolkowitz and Reus, 2001). Recent studies have demonstrated that DHEA acts as a neurotrophic factor in the brain and prevents neuronal apoptosis by interacting with and activating Trk and p75NTR receptors (Lazaridis *et al.*, 2011).

The clinical use of either neurotrophins or naturally occurring neurosteroids has been hampered by neurotrophins' inability to pass the BBB and their unstable serum pharmacokinetics and bioavailability, and the neurosteroids' endocrine side effects, including hormone-dependent neoplasias (Compagnone and Mellon, 2000; Gravanis, Pediaditakis and Charalampopoulos, 2017). The synthesis of microneutrophins was an important step in the process of overcoming the aforementioned caveats. Microneutrophins are small, lipophilic, BBB-permeable synthetic derivatives of DHEA that selectively bind and activate one or more of well-known neurotrophin receptors such as TrkA (of NGF), TrkB (of BDNF) and p75NTR, mimicking the beneficial effects of growth factors.

BNN-20 is a synthetic analogue of the endogenous neurosteroid DHEA, with a chemical modification at C17, which prevents its metabolism to estrogens or androgens (Calogeropoulou *et al.*, 2009). Thus, BNN-20 is deprived of the endocrine side effects of the parent molecule and exhibits strong neuroprotective activity, exerting anti-apoptotic effects in vitro by binding with high affinity to both NGF receptors, TrkB and p75NTR (Calogeropoulou *et al.*, 2009). Botsakis et al. have reported the strong antioxidant, antiapoptotic and anti-inflammatory effect of the BNN-20 in the "weaver" mouse model, a genetic model of PD, which exhibits progressive dopaminergic neurodegeneration in the SNpc (Botsakis *et al.*, 2017). Furthermore, their data indicated that BNN-20 exerts its beneficial effects, at least in part, by binding to TrkB receptors and their downstream signaling pathways TrkB-Akt-NFκB and TrkB-ERK1/2-NFκB, leading to the increase of produced neurotrophin BDNF which in turn stimulates its natural receptor TrkB, triggering a feed forward mechanism, leading to cell survival and probably to a neurogenic process (Botsakis et al., 2017). Recently published studies confirmed the anti-neuroinflammatory and neuroprotective properties of BNN-20, leading to elevated dopamine levels and improved motor activity (Panagiotakopoulou et al., 2020) as well as its beneficial

effect through the enhancement of dopaminergic neurogenesis in the SNpc in "weaver" mice (Mourtzi et al., 2021).
A large number of in vitro and in vivo animal models have been created for understanding the mechanisms of PD pathogenesis and assist in drug development. However, an important limitation is the extent to which these experimental models recapitulate key neuropathological features of the human disease (Tieu, 2011). During the last two decades, advances in cell reprogramming technologies have allowed the generation of induced pluripotent stem cells (iPSCs) from somatic cells of patients with PD (Torrent *et al.*, 2015), offering the opportunity to elucidate disease phenotypes, investigate the underlying mechanisms and screen for new drugs in a human setting. Towards this direction, Kouroupi et al. have created a robust iPSC-based model of PD from patients harboring the p.A53T-αSyn mutation that faithfully simulates disease-relevant phenotypes, including protein aggregates, compromised neuritic growth and αSyn- and Tau-associated axonal pathology with reduced synaptic connectivity (Kouroupi *et al.*, 2017). These neuropathological features closely resemble hallmarks previously identified in post-mortem brains of p.A53T-patients (Spira *et al.*, 2001; Kotzbauer *et al.*, 2004). Importantly, small molecules targeting αSyn reverted the degenerative phenotype under both basal and induced stress conditions, indicating a treatment strategy for PD and highlighting a platform for screening new disease-modifying agents.
Here, we aim to identify the potential beneficial effects of BNN-20 on this iPSC-based model of PD to ameliorate or even restore the identified pathological phenotypes. Our findings demonstrate that BNN-20 treatment increased moderately the percentage of differentiated neurons in PD cultures and protected partially PD neurons from stress-induced cell death and their axons from fragmentation without quenching neurite outgrowth impairment or restoring mRNA levels of genes associated with axon guidance and synaptic function found dysregulated in mutant neurons. This study shows that BNN-20 could be a potential neuroprotective agent against PD pathology, but further investigation is needed.

Methods

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Growth media, supplements and recombinant proteins		
DMEM, high glucose, pyruvate	Thermo Fisher Scientific	Cat# 41966029
Ham's F-12 Nutrient Mix	Thermo Fisher Scientific	Cat#21765029
GlutaMAX Supplement	Thermo Fisher Scientific	Cat# 35050038
HEPES (1 M)	Thermo Fisher Scientific	Cat# 15630106

B-27Plus Supplement (50X)	Thermo Fisher Scientific	Cat# A3582801
N-2 Supplement (100X)	Thermo Fisher Scientific	Cat# 17502048
FGF-Basic Human Recombinant	PEPROTECH	Cat# 100-18C-0100
Recombinant Human Sonic Hedgehog/Shh, N-Terminus Protein	R&D Systems	Cat# 1314-SH
Recombinant Human/Mouse FGF-8b Protein	R&D Systems	Cat# 423-F8
Recombinant Human BDNF Protein	R&D Systems	Cat# 248-BD
Recombinant Human GDNF Protein	R&D Systems	Cat# 212-GD
STEMdiff Neural Progenitor Medium	STEMCELL Technologies	Cat# 05833
Antibodies		
Cleaved Caspase-3 (Asp175) Antibody, rabbit polyclonal	Cell Signalling	9661S
Doublecortin Antibody (N-19), goat polyclonal	Santa Cruz	sc-8067
Purified Mouse Anti-Ki-67, Clone B56, mouse monoclonal	Beckton Dickinson	550609
Anti-MAP2 Antibody, clone AP20, mouse monoclonal	Millipore	MAB3418
Anti-MAP2 antibody, chicken polyclonal	Abcam	ab5392
Anti-Nestin, Human Antibody, rabbit polyclonal	Millipore	ABD69
Anti-NeuN Antibody, clone A60, mouse monoclonal	Chemicon	MAB377
Purified Mouse Anti-α-Synuclein Clone 42/α-Synuclein, mouse monoclonal	Beckton Dickinson	610787
Anti-Tyrosine Hydroxylase (TH) Antibody, rabbit polyclonal	Chemicon	AB152
Anti-β3-Tubulin (TUJ1) Antibody, rabbit polyclonal	Cell Signalling	5568
Chemicals and other reagents		
StemProAccutase Cell Dissociation Reagent	Thermo Fisher Scientific	Cat# A1110501

Adenosine 3',5'-cyclic monophosphate (cAMP)	Sigma-Aldrich	Cat# A9501
L-Ascorbic acid	Sigma-Aldrich	Cat# A4403
PBS, pH 7.4	Thermo Fisher Scientific	Cat# 10010015
Poly-L-ornithine hydrobromide	Sigma-Aldrich	Cat# P3655
Laminin from Engelbreth-Holm-Swarm murine sarcoma basement membrane	Sigma-Aldrich	Cat# L2020
Epoxomicin	Sigma-Aldrich	Cat# E3652
MG-132	Calbiochem	Cat# 474790
TRIzol Reagent	Thermo Fisher Scientific	Cat# 15596026
ProLong Gold antifade with DAPI	Cell Signaling	Cat# 8961
PFA	Sigma-Aldrich	Cat# P6148
RQ1 RNase-Free DNase	Promega	Cat#M6101
ImProm-II Reverse Transcriptase	Promega	Cat#A3802
SYBR Select Master Mix	Thermo Fisher Scientific	Cat# 4472918
Primers		
ABLIM3 forward: ACTACCATGCCCAGTTTGG reverse: GTACATTTCCTCTCCTTCGGTG	Kouroupi et al, PNAS 2017	N/A
DOC2B forward: ACAGATGAAGACATGATCCGC reverse: TCTTGGTGTGGTTGGGTTTC	Kouroupi et al, PNAS 2017	N/A
FAB7 forward: AGGACTCTCAGCACATTCAAG reverse: CTTTGCCATCCCATTTCTGTATG	Kouroupi et al, PNAS 2017	N/A
GAPDH forward: CCTCTGACTTCAACAGCGACAC reverse:AGCCAAATTCGTTGTCATACCAG	Kouroupi et al, PNAS 2017	N/A

HPCA forward: CCTTCAGCATGTATGACCTGG reverse: TTTCTCAGTCCTCTTTTCCGG	Kouroupi et al, PNAS 2017	N/A
MAP2 forward: GAGAATGGGATCAACGGAGA reverse: CTGCTACAGCCTCAGCAGTG	Kouroupi et al, PNAS 2017	N/A
NESTIN forward: TCCAGGAACGGAAAATCAAG reverse: GCCTCCTCATCCCCTACTTC	Kouroupi et al, PNAS 2017	N/A
SLITRK4 forward: GTCCTTGAGGAGCAAACAGC reverse: CTGCCTCCATGCCTATTGAT	Kouroupi et al, PNAS 2017	N/A
SV2C forward: GTGTTTGTCATCGTCTGTGC reverse: GGCTCTCATGTTGGTGTCAT	Kouroupi et al, PNAS 2017	N/A
SYN3 forward: CTACTCCGTCTACAACTTCTGC reverse: GCTGTGACCATTGGCTTATG	Kouroupi et al, PNAS 2017	N/A
TH forward: TGTCTGAGGAGCCTGAGATTCG reverse: GCTTGTCCTTGGCGTCACTG	Kouroupi et al, PNAS 2017	N/A
TRKB forward: ACAGTCAGCTCAAGCCAGACAC reverse: GTCCTGCTCAGGACAGAGGTTA	Dedoni et al, Journal of Pharmacology and Experimental Therapeutics 2019	N/A
βIII tubulin (TUJ1) forward: CATTCTGGTGGACCTGGAAC reverse: CCTCCGTGTAGTGACCCTTG	N/A	N/A
Plasticware		
Cell Culture/Petri dishes	Thermo Fisher Scientific	Cat# 430166

Cell-Culture Treated 6-well plates	Thermo Fisher Scientific	Cat# 140675
Cell-Culture Treated 24-well plates	Thermo Fisher Scientific	Cat# 3524
MicroAmp™ Fast Optical 96-Well Reaction Plates	Thermo Fisher Scientific	Cat# 4346907
MicroAmp™ Optical Adhesive Films	Thermo Fisher Scientific	Cat# 4360954
Equipment		
Confocal microscope	LEICA Microsystems	LEICA Sp8
UV Spectrophotometer	Thermo Fisher Scientific	NanoDrop™ 2000/2000c
Thermal Cycler	BIO-RAD	DNAEngine
Real-time PCR system	Applied Biosystem/ Roche	AB: ViiA™ 7 #4453543/ Roche: #05815916001
Software and Algorithms		
ImageJ	NIH	https://imagej.nih.gov/ij/
Prism	Graphpad	https://www.graphpad.com/scientific-software/prism/

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human induced-pluripotent stem cells (iPSCs) and iPSC-derived neural progenitor cells (NPCs). Unaffected (Control, CTR) and familial PD iPSC lines carrying the G209A (p.A53T) *SNCA* mutation were generated and fully characterized by Kouroupi et al. (Kouroupi *et al.*, 2017).

METHOD DETAILS

Culture of human iPSC-derived neural progenitor cells (NPCs). iPSC-derived NPCs were derived from iPSCs as previously described (Kouroupi *et al.*, 2017), following a neural induction protocol based on dual-SMAD inhibition (Chambers *et al.*, 2009). NPCs were either expanded in STEMdiff Neural Progenitor Medium (STEMCELL Technologies) or differentiated into neurons. Half of the medium was replaced every other day.

N**euronal differentiation of human iPSC-derived NPCs.** A schematic summary of the differentiation procedure and the timeline of analysis is shown in Figure 1A. NPCs were dissociated using accutase (StemProAccutase;Thermo Fisher Scientific) and re-plated onto glass coverslips coated with poly-L-ornithine (PLO; 20 μg/ml in water;

Sigma-Aldrich)/ laminin (5 μg/ml in PBS; Sigma-Aldrich) at a density of 50,000 cells/ cm2. For spontaneous differentiation, NPCs were cultured in DMEM:F12 (Thermo Fisher Scientific)/ B27 plus (Thermo Fisher Scientific) / N2 (Thermo Fisher Scientific)-medium for 10-15 days in vitro (DIV) and then were either analyzed by immunocytochemistry or harvested for RNA extraction and quantitative PCR (qPCR) (Figure 1A). For directed neuronal differentiation, NPCs were cultured in DMEM:F12/ B27 plus/N2-medium supplemented with 200 ng/ml human recombinant sonic hedgehog (SHH, R&D Systems), 100 ng/ml murine recombinant fibroblast growth factor 8b (FGF-8b, R&D Systems), 10 ng/ml human basic FGF (bFGF, Peprotech) and 200 μM ascorbic acid (AA, Sigma-Aldrich) for 8 DIV, followed by a cocktail consisting of 20 ng/ml brain-derived neurotrophic factor (BDNF, R&D Systems), 10 ng/ml glial cell-derived neurotrophic factor (GDNF, R&D Systems), 200 μM AA and 0.5 mM cyclic AMP (cAMP, Sigma-Aldrich) for at least 2 weeks (Kouroupi *et al.*, 2017). Half of the medium was replaced every other day. Cells were re-plated onto PLO/laminin-coated coverslips for cytotoxicity assay.

BNN-20 treatment. Microneurotrophin BNN-20 (provided by Dr Ilias Kazanis, University of Patras, Greece) was resuspended in pure ethanol at a 10^{-2}M concentration. This solution was then diluted in sterile PBS (10^{-4}) and finally, in the cell culture medium at a final concentration of 10^{-7}M. Control and PD cells were cultured in the presence of BNN-20 for up to 2 weeks as described in Figure 1A.

Immunofluorescence staining. Cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) for 20 min at room temperature. Samples were blocked with 0.1% Triton X-100 (Sigma-Aldrich) and 5% donkey serum in PBS for 30 min and were subsequently incubated with primary antibodies: anti-Ki67 (1/200; Beckton Dickinson. 550609), anti-NESTIN (1/200; Merck-Millipore, ABD69), anti-PAX6 (1/100; DSHB, AB 528427), anti-doublecortin (DCX, 1/100; Santa Cruz, sc-8067), anti- βIII-tubulin (TUJ1, 1/1000; Cell Signalling, 5568), anti-MAP2 (1/200; Millipore, MAB3418), anti-MAP2 (1/1000; Abcam,ab5392),anti-NeuN (1/100; Millipore, MAB377), anti-tyrosine hydroxylase (TH, 1/500; Millipore, AB152), anti-α-synuclein (αSYN; 1/500; BD Biosciences, 610787) and anti-cleaved caspase-3 (cCASP3, 1/400; Cell Signaling, 9661S) at 4 °C overnight in PBS containing 0.02% TritonX-100 and 1% donkey serum. After washing with PBS to remove unbound primary antibody, appropriate secondary antibodies (Molecular Probes, Thermo Fisher Scientific) conjugated to AlexaFluor 488 (green) or 546 (red) were added for at least 1 h at room temperature. Coverslips were mounted with ProLong Gold antifade reagent with DAPI (Cell Signaling) and images were acquired using a Leica SP8 confocal microscope (LEICA Microsystems) and analyzed using ImageJ software (NIH).

RNA isolation, cDNA Synthesis and qPCR. Total RNA was extracted from cell pellets using TRIzol Reagent (Thermo Fisher Scientific). RNA concentration was measured on NanoDrop One Spectrophotometer (Thermo Fisher Scientific). Following digestion with DNase I (Promega), 1-1,5 μg of total RNA was used for first-strand cDNA synthesis with the ImProm-II Reverse Transcription System (Promega) following the manufacturer's protocol. Quantitative PCR analyses were carried out in a Light

Cycler96 (Roche) Real time PCR detection system using SYBR Select Master Mix (Thermo Fisher Scientific) according to manufacturer's instructions. All primers used are listed in Key Resources Table. Results were analyzed using the comparative CT method. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used in comparisons of gene expression data.

Axon degeneration index. Analysis of axonal degeneration was performed by immunostaining for βIII-tubulin (TUJ1) in iPSC-derived control and PD neuronal cultures. The cultures were either untreated or treated with BNN-20. The number of TUJ1+ spots in fragmented axons was counted manually (ImageJ) on ten randomly selected fields and the ratio between the number of spots and the total TUJ1+ staining area (ImageJ) was defined as axon degeneration index.

Neurite analysis. Neurite analysis was performed on either DsRed-labelled or TH+ iPSC-derived dopaminergic neurons at 25 DIV. A lentiviral vector expressing the red fluorescent protein (DsRed) under the control of the human synapsin 1 promoter (LV.SYN1.DsRed) was used for the transduction of human cells in order to facilitate imaging of single neurons for morphological analysis (Kouroupi *et al.*, 2017). Transduction with LV.SYN1.DsRed was performed at least 7 days before the assay to depict and count branching neurites from single neurons. The number of neurites extending from the soma of at least 100 single DsRed-labelled neurons per sample was determined. Neurite length was estimated by manually tracing the length of all neurites on DsRed-labelled neurons using the NeuronJ plugin of ImageJ (NIH). For morphological analysis of iPSC-derived dopaminergic neurons, differentiated cells were immunostained for TH and at least 50 TH+ neurons were assessed for the number of neurites and the total neurite length.

Cytotoxicity Assay. Control and PD iPSC-derived neurons were generated using directed neuronal differentiation protocol as described above. After replating onto PLO/laminin-coated coverslips, cells were pre-treated with BNN-20 for 24 h and then were subjected to stress by adding the proteasome inhibitors epoxomicin (1 μM, Sigma-Aldrich) and MG-132 (10 μM, Calbiochem) for 24 h.

Statistics. Most experiments were replicated at least three times and data from parallel cultures were acquired. All data represent mean±standard error of mean (SEM). Statistical analysis was performed using Graphpad Prism. Comparisons between two independent groups were performed using Student's t-test. Kruskal-Wallis with Dunn's post hoc test was applied for multiple group comparisons. Probability values less than 0.05 ($p < 0.05$) were considered significant.

Results

Characterization and differentiation of human iPSC-derived NPCs

Human NPCs were derived from iPSCs of an unaffected individual and a PD patient carrying the *SNCA* G209A (p.A53T) mutation, as previously described (Kouroupi *et al.*, 2017). iPSC-derived NPCs were cultured in expansion medium and analyzed by immunocytochemistry and RT-qPCR (Figure 1A, B, D). Specifically, the majority of iPSC-derived NPCs, either control or PD, expressed Nestin (80-90%; Figure 1B, D) and PAX6 (>70%; Figure 1B, D). Both CTR and PD NPC cultures were also immunostained for Ki67 (almost 45%) to assess the proliferation and doublecortin (DCX; 15-20%), a marker for young neurons (neuroblasts), with no significant differences between them.

Subsequently, cells were differentiated spontaneously into βIII-tubulin+ (TUJ1+) neurons for almost two weeks (Figure 1A). Immunofluorescence revealed that α-synuclein (αSyn) was present in the soma and neurites of both PD and control neurons, though more cells were strongly positive for αSyn in PD cultures already at 25 DIV (Figure 1C). In agreement, quantification of αSyn mRNA by RT-qPCR revealed elevated levels in PD cultures compared to control ($p<0.05$; Figure 1E).

Before assessing the effect of BNN-20 on control and PD iPSC-derived neuronal cultures, we examined the expression of the TrkB neurotrophin receptor, through which BNN-20 has been shown to act in vivo (Botsakis et al., 2017). TrkB mRNA expression has been verified in both control and PD iPSC-derived neurons by RT-qPCR (Figure 1F).

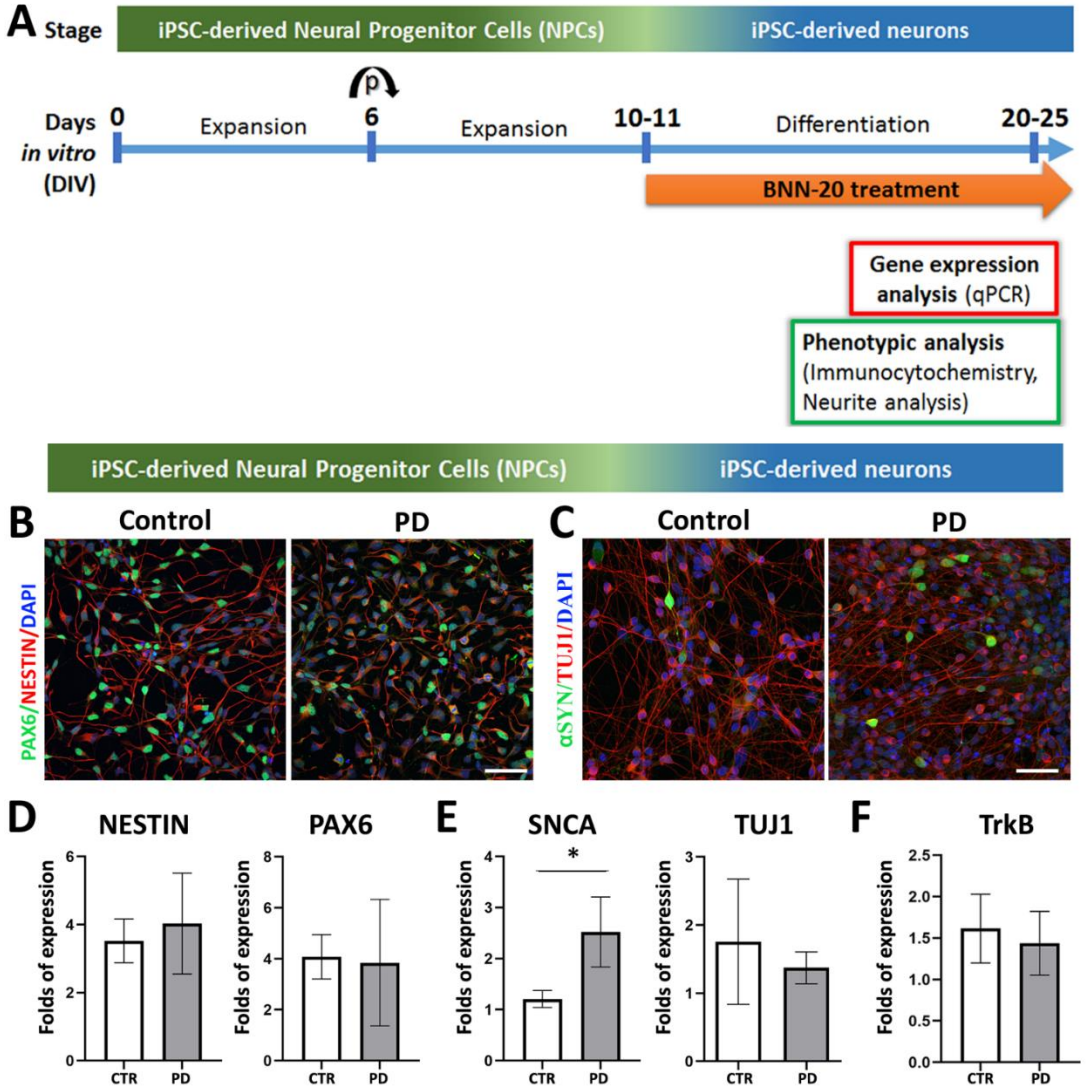

Figure 1. Human iPSC-derived NPC characterization and spontaneous neuronal differentiation. (A) Schematic drawing of the protocol used for human iPSC-derived NPC culture and differentiation and timeline of analysis. (B) Immunostaining of control and PD iPSC-derived NPCs for PAX6 (green) and Nestin (red). Cell nuclei are counterstained with DAPI (blue). Scale bar, 50 µm. (C) Immunostaining for α-synuclein (αSyn; green) and βIII-tubulin (TUJ1; red) in control and PD iPSC-derived neurons at 25 DIV. Cell nuclei are counterstained with DAPI (blue). Scale bar, 50 µm. (D-F) RT-qPCR analysis of Nestin (D), PAX6 (D), SNCA (E), TUJ1 (E) and TrkB (F) mRNA expression normalized to GAPDH levels. Data are represented as mean ± SEM (n = 3).

Slightly increased neurons in BNN-20-treated PD cultures

The first experiments that were conducted, pertaining to the investigation of the potential therapeutic benefits of BNN-20, were aiming at the assessment of the effect of BNN-20 treatment on the proliferation of human iPSC-derived NPCs, cultured in either expansion conditions or upon spontaneous neuronal differentiation. To this end, immunofluorescence analysis was performed using anti-Ki67, a marker of proliferation. Both control and PD iPSC-derived NPCs cultured in expansion medium, showed increased proliferation without any significant differences between them or

after BNN-20 treatment (Ki67+ cells; Control 42.06%; Control-BNN-20 46.92%; PD 38.46%; PD-BNN-20 43.3%; n=1). Under spontaneous neuronal differentiation conditions, Ki67+ cells were reduced significantly in all conditions examined. Upon BNN-20 treatment, Ki67+ cells were slightly fewer both in control and PD cultures (Control 24.68±8.71%; Control-BNN-20 20.77±3.25%; PD 24.06±5.72%; PD-BNN-20 18.41±0.11%; n=2). Therefore, the addition of BNN-20 did not affect significantly the proliferation of iPSC-derived NPCs.

To assess the effect of BNN-20 on the differentiation of iPSC-derived NPCs into neurons, we cultured control and PD iPSC-derived NPCs under spontaneous neuronal differentiation conditions with or without BNN-20 for up to 15 DIV (Figure 1A). Differentiated neurons were identified by immunostaining against neuronal markers βIII-tubulin (TUJ1), MAP2 and NeuN (Figure 2A, B and C, E). RT-qPCR confirmed mRNA expression of the neuronal markers TUJ1 (Figure 2D) and MAP2 (Figure 2F). Although qPCR analysis of TUJ1 and MAP2 expression didn't show any substantial difference between control and PD cultures or between BNN-20-treated and untreated cells (Figure 2D and F), moderate increase in TUJ1+ and MAP2+ neurons has been revealed by immunofluorescence analysis in PD cultures after BNN-20 treatment compared to the untreated PD cultures (Figure 2A, C; TUJ1+; Control 23.66±2.82%; Control-BNN-20 27.54±1.52%; PD 17.86±1.80%; PD-BNN-20 27.56±2.87%; n=3; Figure 2B, E; MAP2+; Control 27.18±5.46%; Control-BNN-20 26.86±2.62%; PD 20.84±2.09%; PD-BNN-20 26.19±1.91%; n=2). It is worth noting that TUJ1+ and MAP2+ cells were shown to be slightly fewer in PD cultures compared to control, whereas BNN-20 addition seemed to quench this difference (Figure 2C and E). The percentage of NeuN+ neurons were extremely low in all conditions examined (data not shown), so we didn't proceed to quantification analysis. Overall, we have seen slightly increased number of TUJ1+ and MAP2+ neurons in PD iPSC-derived cultures after BNN-20 addition for almost two weeks.

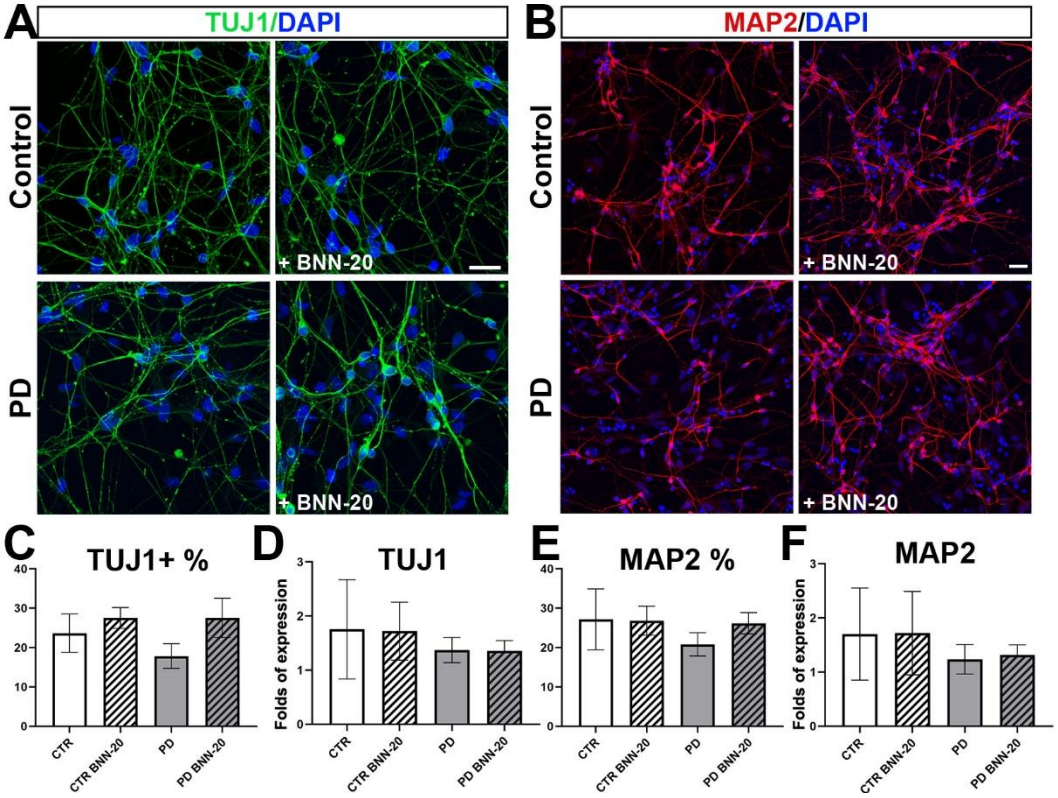

Figure 2. Slightly increased neurons in BNN-20-treated PD cultures. (A, B) Immunostaining for the neuronal markers TUJ1 (green; A) and MAP2 (red; B) in control and PD iPSC-derived neurons at 25 DIV. Cell nuclei are counterstained with DAPI (blue). Scale bars, 20 μm. (C) Graph showing the percentage of TUJ1+/ DAPI+ cells. Data are represented as mean ± SEM (n=3). (D) RT-qPCR analysis of TUJ1 mRNA expression normalized to GAPDH levels. Data are represented as mean ± SEM (n = 3). (E) Graph showing the percentage of MAP2+/ DAPI+ cells. Data are represented as mean ± SEM (n = 3). (F) RT-qPCR analysis of MAP2 mRNA expression normalized to GAPDH levels. Data are represented as mean ± SEM (n = 3).

BNN-20 ameliorated axonal pathology, but not neurite outgrowth of PD iPSC-derived neurons

Based on a previous study of our team, PD iPSC-derived neurons exhibit distinct morphological traits compared to unaffected cells, which are suggestive of extensive neuritic pathology and degeneration. More specifically, PD neuronal processes immunostained for TUJ1 appear more contorted with αSyn+ swollen varicosities and large spheroid inclusions similar to the dystrophic neurites identified in the brain of p.A53T patients (Duda *et al.*, 2002; Kouroupi *et al.*, 2017). Although these pathological phenotypes were observed after neuronal differentiation and maturation for at least four weeks (Kouroupi *et al.*, 2017), in the present study we managed to identify some of these disease-related phenotypes at earlier time point, that is at the second week of neuronal differentiation (Figure 1A). In particular, using a lentiviral vector expressing DsRed under the control of the human synapsin 1 promoter (LV.SYN1.DsRed) to label single neurons, we assessed the morphology of control and PD neurons. The neurite length of DsRed+ neurons was significantly reduced in PD neurons (Neurite length: Control 86.63±6.71 μm vs PD 46.96±17.26 μm, n=3; Figure

3A and B; Neurites from soma: Control 1.01±0.12 vs PD 0.67±0.16; Figure 3C) while TUJ1+ fragmented axons were detected quite often in PD cultures (Axon degeneration index; Control 34.33±2.51, PD 45.28±4.68; n=2; Figure 3D, E). We then examined whether BNN-20 could rescue the observed pathology. BNN-20 didn't protect from neuritic impairments, but alleviated to some extent the existence of distorted/degenerating axons (Axon degeneration index; Control 34.33±2.51, Control-BNN-20 34.27±7.38; PD 45.28±4.68, PD- BNN-20 36.14±0.52; n=2; Figure 3E).
Since Mourtzi et. al demonstrated that BNN-20 administration to "weaver" mice increased the number of dopaminergic neurons in the SNpc (Mourtzi *et al.*, 2021), we proceeded to morphological analysis of TH+ dopaminergic neurons. To this end, we measured the total neurite length and the number of neurites extending from the soma of at least 50 TH+ neurons. Although the neurite length seems to decrease in PD neurons, it wasn't improved upon BNN-20 addition (Control 169±2.99 µm; Control-BNN-20 175.23±12.72 µm, PD 127.9±7.14 µm, PD-BNN-20 114.9±7.56 µm; n=2; Figure 3F and G). Interestingly, we noticed that BNN-20 seems to slightly increase the number of neurites extending from the soma, since there was a slight shift from neurons that bear one neurite to neurons that bear at least two, with this being more evident in PD neurons (Figure 3H). This was not the case with DsRed+ neurons, which showed decreased number of neurites per soma upon BNN-20 treatment (Figure 3C). Overall, BNN-20 seems to ameliorate PD pathology, alleviating partially axonal degeneration.

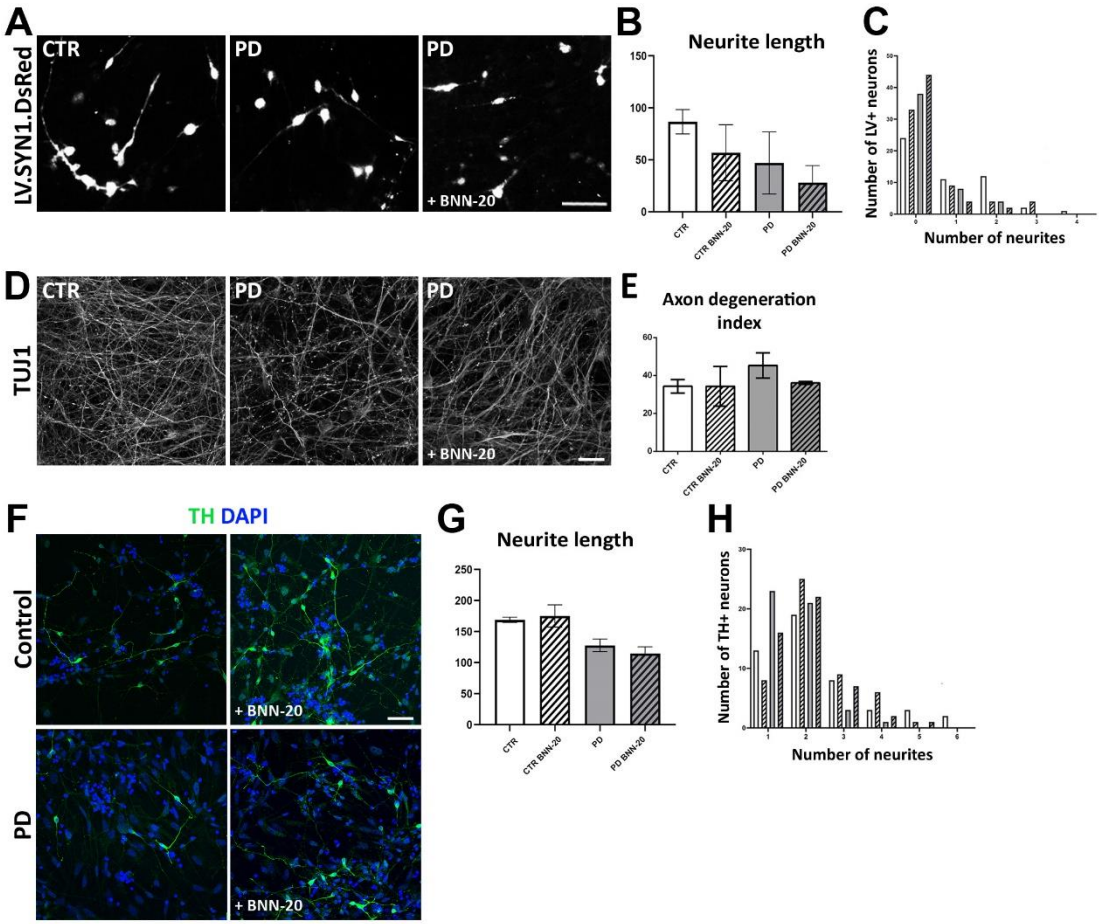

Figure 3. BNN-20 ameliorated axonal pathology, but not neurite outgrowth of PD iPSC-derived neurons. (A) Representative fluorescent images of iPSC-derived neurons transduced with LV.SYN1.DsRed. Scale bar, 50 μm. (B, C) Quantification of the total neurite length (B) and neurites extending from the soma (C) in DsRed+ cells. (D) Axonal pathology observed by TUJ1 immunostaining in PD cells at 25 DIV, is slightly improved by BNN-20 treatment. Scale bar, 20 μm. (E) Quantification of axonal degeneration by measuring the ratio of TUJ1+ spots over the total TUJ1+ area in untreated or BNN-20-treated PD iPSC-derived neurons. Data are represented as mean ± SEM (n=2). (F) Immunostaining for TH in control and PD untreated and BNN-20-treated iPSC-derived neurons at 25 DIV. Scale bar, 20 μm. (G, H) Quantification of the total neurite length (G) and neurites extending from the soma (H) in TH+ neurons.

Gene expression analysis of iPSC-derived neurons

Previous work from our team has revealed that a number of important genes which code proteins that are involved in the formation and maintenance of proper synaptic function, are dramatically downregulated in PD iPSC-derived neurons in comparison to their respective controls. Based on these results, we wanted to check the expression of selected genes at an earlier stage, almost two weeks of neuronal differentiation. To this end, gene expression analysis of control and PD neurons was conducted by RT-qPCR for SYN3, SV2C, DOC2B, SLITRK4, FABP7 and ABLIM3.

SYN3, SV2C and DOC2B are found in the presynaptic area, where they are involved in synaptogenesis and neurite extension, synaptic vesicle organization, spontaneous synaptic vesicle exocytosis, and regulation of neurotransmitter release, respectively. Their expression remained unchanged by PD status at this stage. On the contrary, the expression of SLITRK4, which is located at the postsynaptic membrane to act as an organizer of excitatory synapse formation, was significantly downregulated, being hardly detectable in PD cells and without affected by BNN-20. Finally, a number of genes associated with axon guidance were also perturbed in iPSC-derived PD cultures. In particular, FABP7 and ABLIM3 were downregulated in PD neurons, however BNN-20 treatment didn't restore their expression.

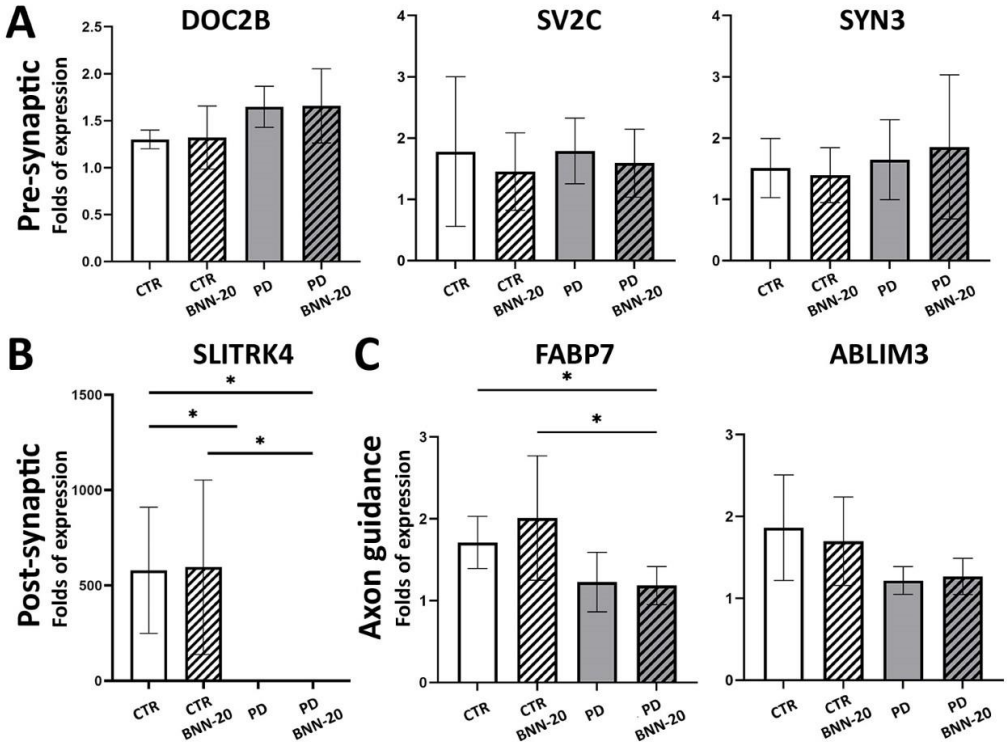

Figure 4. Gene expression analysis of iPSC-derived neurons. RT-qPCR analysis of selected genes associated with synapse formation and function (Pre-synaptic; DOC2B, SV2C, SYN3; Post-synaptic; SLITRK4) and axon guidance (ABLIM3 and FABP7). Gene expression normalized to GAPDH. Data are represented as mean ± SEM (one-way ANOVA, n = 3–4 for each condition).

Induced-stress phenotypes in iPSC-derived neurons

To investigate if BNN-20 is protective under induced stress conditions, we used the proteasome inhibitors, epoxomicin and MG-132, to accelerate neuronal degeneration and cell death. Each inhibitor was added for 24 h and induced increased cleaved caspase-3 (cCASP3) immunoreactivity and a moderate disruption of the MAP2+ network, while BNN-20 protected partially the cultures, lessening the cCASP3 immunoreactivity (Figure 5).

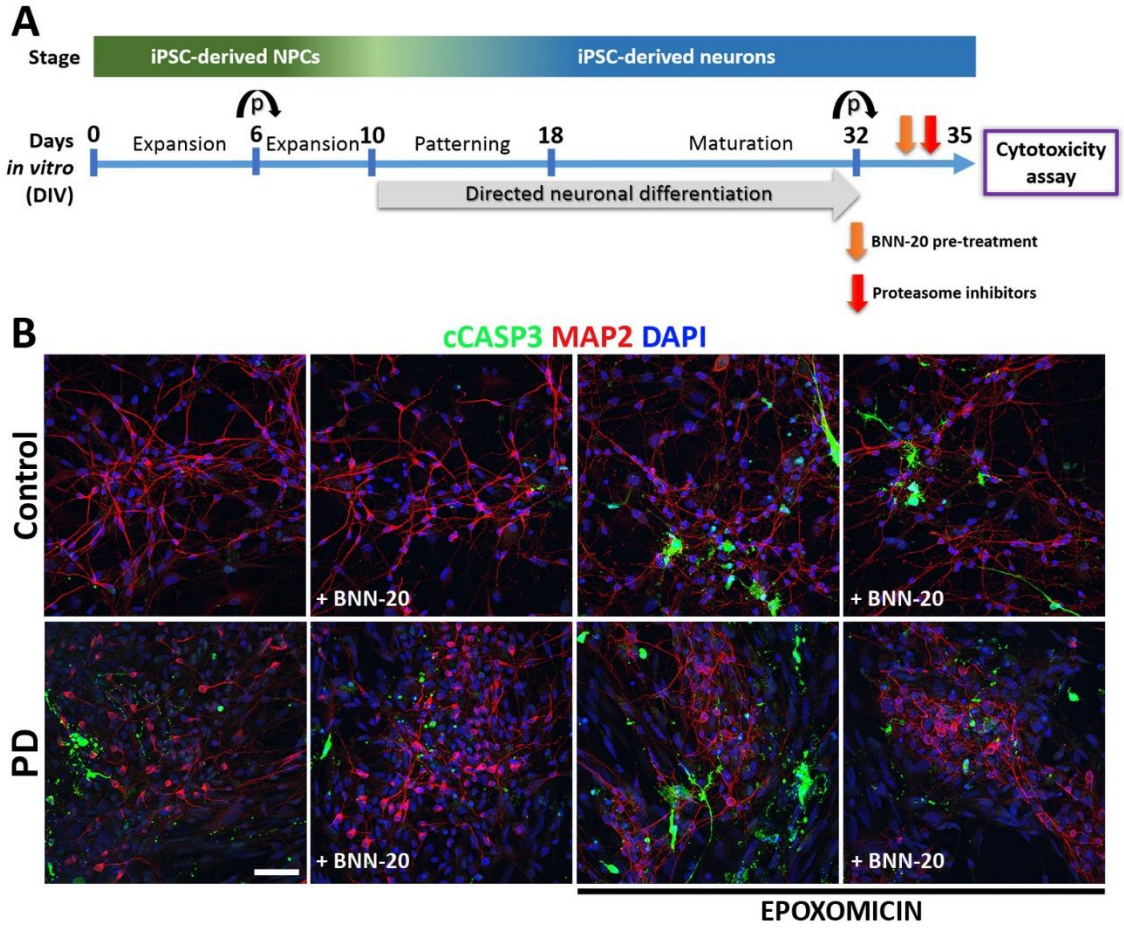

Figure 5. Cytotoxic effect of proteasome inhibition on iPSC-derived neurons. (A) Schematic drawing of the directed neuronal differentiation protocol and timeline of analysis. (B) Representative fluorescent images of control and PD iPSC-derived neurons at 35 DIV immunostained for active cleaved caspase-3 (cCASP3; green) and MAP2 (red). Cells were pre-treated with BNN-20 for 24 h and then were subjected to stress by adding the proteasome inhibitor epoxomicin (1 µM) for 24 h. Cell nuclei are counterstained with DAPI (blue). Scale bar, 50 µm.

Discussion

PD is the second most common neurodegenerative disorder after Alzheimer's disease that affects millions of people worldwide creating serious socio-economic burdens, thus constituting a medical issue that needs to be tackled effectively. Until now available medication only alleviates the symptoms, but disease-modifying therapies that prevent or slow disease progression are still lacking, representing an area of unmet medical need. The discovery of new therapeutic compounds targeting PD is of outmost clinical importance. Neurotrophins are molecular cornerstones of key processes in the central nervous system, such as neurodevelopment and plasticity, constituting a group of potential therapeutic compounds. BDNF, an important member of this group, has been proposed to play a role in the emergence or the exacerbation of PD pathology (Jin, 2020a). This has drawn the attention to the therapeutic potential of this molecule, but several pharmacokinetic limitations, such

as its inability to cross the blood-brain barrier, its short half-life and potential adverse effects, restrict its use in clinical practice (Botsakis et al., 2017).
Microneutrophin BNN-20, a synthetic analogue of the endogenous neurosteroid DHEA which acts as a BDNF analog, has been proved beneficial, since it partially restored a series of pathological phenotypes in the "weaver" mouse model of PD (Botsakis et al., 2017; Panagiotakopoulou et al., 2020). Here, we explored whether BNN-20 could act beneficially in iPSC-derived NPCs and differentiated neurons generated from patients with PD carrying the p.A53T mutation, a human cell-based model that recapitulates key aspects of PD neuropathology and has been already used as a platform for testing of novel disease-modifying compounds (Kouroupi et al., 2017; Antoniou et al., 2020).
Control and PD iPSC-derived NPCs were cultured either in expansion or differentiation conditions with or without BNN-20 and we assessed their proliferation and differentiation capacity at selected time points (Figure 1A). BNN-20 treatment didn't affect the proliferation of human iPSC-derived NPCs, a finding in line with data coming from neural stem cells isolated from "weaver" mouse brains (Mourtzi et al., 2021). However, we observed a slight increase in the percentage of TUJ1+ and MAP2+ neurons in PD cultures, but not in control cultures, showing rather neuroprotection than enhanced neuronal differentiation. Botsakis et al. have shown that BNN-20 administration partially inhibits neuronal loss in the SNpc of the "weaver" mouse (Botsakis et al., 2017). However, in another study investigating the effect of BNN-20 on "weaver" mice, Mourtzi et al. suggested that there is another mechanism underlying the increase in TH+ neurons of the "weaver" SNpc, according to which BNN-20 seems to produce pro-neurogenic effects, as its administration led to increased neurogenesis in the SNpc and to a partial reversal of dopaminergic cell loss (Mourtzi et al., 2021). This wasn't confirmed in our human setting, since qPCR analysis didn't show substantial differences in gene expression of neuronal markers TUJ1 and MAP2 between BNN-20-treated and untreated cultures. Thus, BNN-20 seems to exert a neuroprotective effect in our human stem cell-based model of PD. Botsakis et al. proposed that BNN-20 protected the dopaminergic neurons via a strong anti-oxidant and anti-apoptotic effect, probably mediated through the TrkB neurotrophin receptor's PI3K-Akt-NF-κB signaling pathway (Botsakis et al., 2017). This could be further investigated in future studies that would shed light on the mechanism through which BNN-20 exerts its beneficial effects in the iPSC-derived neuronal cultures.
As previously described, our iPSC-based model of PD from patients harboring the p.A53T-αSyn mutation, faithfully simulates disease pathogenesis and uncovers novel disease-relevant phenotypes at basal conditions, including compromised neurite outgrowth and severe axonal neuropathology (Kouroupi et al., 2017). Thus, we sought to determine the effect of BNN-20 on the pathological phenotype of PD neurons. To this end, we assessed neurite outgrowth using a lentiviral vector expressing DsRed under the control of the human synapsin 1 promoter (LV.SYN1.DsRed) and labeling single neurons and in parallel cultures, TH immunostaining to trace specifically the dopaminergic neurons that mostly affected in PD. Importantly, we observed reduced DsRed+ and TH+ neurite outgrowth in the PD status even at earlier stage of differentiation (up to 25 DIV) examined in the current study. However, BNN-20 didn't rescue the observed pathology, apart from a slight increase in the number of neurites extending from the soma of TH+ neurons, which was not reproduced when we observed DsRed+ neurons. Regarding axonal degeneration, we identified previously

described TUJ1+ contorted axons and swollen varicosities in PD neurons at a lower extent at this earlier differentiation stage, with BNN-20 alleviating somehow this phenotype. BNN-20 could probably help axons partially retain their structural integrity due to its proposed anti-oxidant properties, which may enable neurons withstand PD status-induced stress.

Based on previous studies from our team which showed that the expression of genes implicated in the proper synaptic integrity and function, was significantly dysregulated (Kouroupi et al., 2017), we wanted to examine if this happens at the differentiation time point being studied here and whether BNN-20 acts beneficially. Specifically, the expression of the post-synaptic adhesion molecule SLITRK4 and the axon guidance-associated gene FABP7 was significantly downregulated in PD neurons with the expression of the former being practically nulled, whereas BNN-20 was ineffective in restoring their expression to normal levels.

Since we studied iPSC-derived neurons in a more juvenile developmental stage, where the pathological processes which underpin PD manifestations haven't fully unfolded yet, we wanted to investigate whether BNN-20 could exert its beneficial potential in a setting of accelerated neurodegeneration induced by the application of proteasome inhibitors, epoxomicin and MG-132. Our preliminary results have shown that BNN-20 was able to partially protect neurons from cell death by decreasing cleaved caspase-3 immunoreactivity. Our observations were mostly qualitative and further analysis is needed in order to verify this finding.

In conclusion, in the present study, we aimed to investigate the potential beneficial effects of the microneurotrophin BNN-20 on human iPSC-derived neuronal cultures bearing the p.A53T mutation, which reproduce a number of PD-relevant phenotypes such as axonal neuropathology and synaptic gene expression dysregulation in an earlier differentiation stage than previously described by Kouroupi et al. (Kouroupi et al., 2017). Based on our findings, BNN-20 seems to be a promising neuroprotective compound, since it ameliorates neuropathology to some extent, but further experimental evidences are needed to back up its potential clinical usefulness.

Acknowledgments

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RESEARCH PROPOSAL

BNN-20: A microneurotrophin as a potential therapeutic agent for Parkinson's disease

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized mainly by motor symptoms such as bradykinesia and resting tremor, while non-motor symptoms are also present. The underlying pathology involves the progressive loss of dopaminergic neurons in the substantia nigra of the midbrain. Current therapeutic strategies are symptomatic, since they don't address the pathological mechanisms underpinning the clinical manifestations of the disease. Therefore, there is an urgent need for the discovery of novel effective therapeutic compounds. Neurotrophins, molecules that play a vital role in the development of the central nervous system and are implicated in its maintenance, plasticity and proper function, constitute a group of potentially beneficial compounds, but possess a number of physicochemical features which make their therapeutic utilization unfeasible. BNN-20 is a synthetic microneurotrophin which has been shown to act as a BDNF analog and is deprived of such features, due to its low molecular weight and its capacity to easily pass the blood-brain barrier (BBB). Based on published data and our preliminary experiments, it exhibits a pleiotropic neuroprotective effect on dopaminergic neurons of the substantia nigra pars compacta in the "weaver" mouse model of PD and a slightly beneficial effect on PD patient-derived neurons, ameliorating pathological phenotypes in a unique human stem cell-based setting that bears disease-associated features. In this proposal we aim to complement our investigations and explore whether the use of the microneurotrophin BNN-20 is a promising therapeutic strategy for PD.

Project description

Specific aims

We aim to build upon recent animal model-based studies and our team's preliminary data coming from a human induced pluripotent stem cell (iPSC)-based in vitro model, which suggest that BNN-20 exhibits beneficial effects both in the "weaver" mouse model and in iPSC-derived neurons from PD patients, thus holding promise as a future therapeutic compound against PD. To expand upon this knowledge and further explore the efficacy of BNN-20 in restoring disease phenotypes, we will address a number of objectives which include shedding light on the mechanism through which BNN-20 exerts its actions and assessing the capacity of BNN-20 to protect neurons in terms of their functional integrity which is clearly perturbed in PD cultures.

Introduction and significance

PD is the most common movement disorder and the second most common neurodegenerative disorder after Alzheimer disease. It is clinically characterized by motor and non-motor manifestations, whereas its neuropathological hallmarks

include the progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and the formation of intracellular proteinaceous inclusions known as Lewy bodies composed mainly of α-synuclein (αSyn) (Lees, Hardy and Revesz, 2009). Alpha-synuclein (αSyn) is the major sporadic PD-linked gene (Simón-Sánchez *et al.*, 2009), while point mutations (Petrucci, Ginevrino and Valente, 2016) and multiplications of the (Chartier-Harlin *et al.*, 2004) cause autosomal dominant forms of early onset and aggressive Parkinsonism and dementia. The best characterized point mutation in the α-synuclein gene *SNCA* is G209A, first identified in families of Italian and Greek ancestry (Polymeropoulos *et al.*, 1997), which translates to a pathological form of αSyn, the p.A53T-αSyn protein.

In vitro and in vivo animal models used so far don't fully recapitulate key neuropathological features of the human disease. During the last two decades, advances in cell reprogramming technologies have allowed the generation of induced pluripotent stem cells (iPSCs) from somatic cells of patients with PD (Torrent *et al.*, 2015), enabling us to shed light on mechanisms underpinning pathological phenotypes and to perform effective drug screening in a human setting. Towards this direction, Kouroupi et al. have created a robust iPSC-based model of PD from patients bearing the p.A53T-αSyn mutation that faithfully reproduces disease-relevant phenotypes, including protein aggregates, compromised neuritic growth and αSyn- and Tau-associated axonal pathology with reduced synaptic connectivity (Kouroupi *et al.*, 2017), closely resembling neuropathological features previously observed in post-mortem brains of p.A53T-patients (Spira *et al.*, 2001), thus providing a clinically relevant platform for screening new disease-modifying agents.

Neurotrophins such as brain-derived neurotrophic factor (BDNF), constitute a group of molecules synthesized in the central nervous system (CNS) that play a key role in the survival and maintenance of neurons. They produce their various neurotrophic and neuroprotective actions through binding to specific pro-survival tyrosine kinase (Trk) receptors (Arévalo and Wu, 2006). BDNF and its partner receptor TrkB play a vital role in the survival and maintenance of dopaminergic neurons. Animal studies which have assessed the potential involvement of BDNF/TrkB signaling in PD suggest that the reduction in BDNF and TrkB expression could be one of the etiological factors in PD pathogenesis. It follows that neurotrophins constitute a group of highly promising putative therapeutic agents in the specific context of PD.

Nevertheless, neurotrophins' inability to pass the BBB and their unstable serum pharmacokinetics and bioavailability have hindered their clinical utilization (Gravanis, Pediaditakis and Charalampopoulos, 2017). The synthesis of microneutrophins was an important step in the process of overcoming the aforementioned caveats. These molecules are small, lipophilic, BBB-permeable and selectively bind and activate one or more of well-known neurotrophin receptors such as TrkA (of NGF), TrkB (of BDNF) and p75NTR, mimicking the beneficial effects of growth factors.

BNN-20 is a synthetic microneurotrophin which exhibits strong neuroprotective activity, exerting anti-apoptotic effects in vitro by binding with high affinity to both NGF receptors, TrkB and p75NTR (Calogeropoulou *et al.*, 2009). Regarding the impact of the in vivo BNN-20 administration, Botsakis et al. have reported a strong antioxidant, antiapoptotic and anti-inflammatory effect of BNN-20 in the "weaver" mouse model, a genetic model of PD, which exhibits progressive dopaminergic neurodegeneration in the SNpc (Botsakis *et al.*, 2017). In addition, they proposed that

BNN-20 exerts its functions, at least in part, by binding to TrkB receptor and the downstream signaling pathways TrkB-Akt-NFκB and TrkB-ERK1/2-NFκB, leading to increased BDNF production which in turn stimulates its natural receptor TrkB (Botsakis *et al.*, 2017). Recent published studies confirmed the anti-neuroinflammatory and neuroprotective properties of BNN-20 (Panagiotakopoulou *et al.*, 2020) and revealed its role as a potent modulator of neurogenesis in the SNpc (Mourtzi *et al.*, 2021).
Here, we aim to identify the potential beneficial effects of BNN-20 on a human iPSC-based model of PD. This study will enable us to examine the molecular actions of BNN-20 by deciphering the signaling pathways in which it is involved and expand our knowledge regarding the effectiveness of this compound to delay or even rescue the disease-associated manifestations.

Research strategy

Our overall approach aims to explore the potential usefulness of BNN-20 as a therapeutic agent against PD pathology. Our experimental approach will be based on the molecular and functional analysis of PD patient-derived neurons upon BNN-20 treatment. The proposed experiments will be organized in two work packages (WPs):
WP1: Delineating the molecular actions of BNN-20 in a human cellular setting through gene expression and proteome analysis
WP2: Functional analysis of BNN-20-treated PD iPSC-derived neuronal cultures vis-a-vis untreated or control cells
In both WPs we will use unaffected (Control, CTR) and PD iPSC lines carrying the p.A53T-αSyn mutation, generated and characterized by Kouroupi et al. (Kouroupi *et al.*, 2017). Human iPSC-derived neural progenitors will be subjected to directed neuronal differentiation as previously described (Kouroupi *et al.*, 2017). Cells will be treated either during neuronal differentiation or in the final stage of differentiation where disease-relevant phenotypes are evident.

WP1: Delineating the molecular actions of BNN-20 in a human cellular setting through gene expression and proteome analysis
Our first task is to explore the molecular mechanism through which BNN-20 acts. To this end, we will determine mRNA and protein levels of molecules involved in the TrkB-Akt-NFκB signaling pathway by RT-qPCR and western blotting respectively. Since it has been suggested that BNN-20 induced BDNF expression through Akt signaling (Botsakis *et al.*, 2017), we will also assess BDNF levels as well as the levels of anti-apoptotic (Bcl-2) and pro-apoptotic (Bax) proteins. Further, since mitochondrial perturbations and oxidative stress have been suggested as common pathological phenotypes of the parkinsonian brain (Toulorge, Schapira and Hajj, 2016), we will determine the levels of reactive oxygen species (ROS) in our cellular model before and after BNN-20 treatment.
Our investigations will be supplemented by RNA-sequencing analysis, in order to assess the effect of BNN-20 on gene expression profile. Total cellular RNA will be extracted from BNN-20-treated and untreated control and PD samples and sequenced. RNA libraries will be prepared with the TruSeq RNA Sample Preparation Kit V2 (Illumina). RNA-seq reads will be quality checked using FastQC. Following pre-processing, GSNAP spliced aligner will be utilized to map the RNA-seq reads against

the reference genome (GRCh37/hg19 genome assembly). Differential gene expression analysis will be performed using DESeq. Enrichment analyses will be performed against Gene Ontology using the hypergeometric distribution, KEGG and Reactome pathway databases. All gene annotations will be derived from Ensembl.
In parallel, we will perform comparative proteomics analysis of lysates from BNN-20-treated and untreated control and PD neurons using the Electrospray Quadropole-Time of Flight Mass Spectrometer coupled to the Nano UPLC system that allows optimal identification of proteins from complex mixtures. Depending on the results, we will expand our studies on the proteome of isolated synaptosomal fractions, in order to track changes in the interactome of neuronal synapses caused by PD pathology or BNN-20 treatment.

WP2: Functional analysis of BNN-20-treated PD iPSC-derived neuronal cultures vis-a-vis untreated or control cells

In the WP2 we will perform functional analysis of BNN-20-treated PD iPSC-derived neuronal cultures vis-a-vis untreated or control cells by calcium imaging. The frequency of spontaneous calcium fluxes and the developmental stage at which they emerge can reveal the structural and functional maturity of the neuronal cultures as well as the impact of BNN-20. To this end, iPSC-derived neurons will be incubated with culture medium containing the green-colored calcium-sensitive dye Fluo-4. Cells will be excited at 488 nm with a fluorescein isothiocyanate (FITC) filter and the fluorescence signals will be recorded at 10 frames/s using a fluorescent microscope, Olympus Time lapse IX81 Cell-R with a CCD camera. The Ca2+ amplitude will be counted over a 5-min period. ImageJ software will be used for the analysis of spontaneous cytosolic Ca2+ dynamics. In each neuron showing spontaneous oscillation, calcium transients in the soma will be quantified.

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Title: Investigation of the potential neuroprotective role of a BDNF analog in Parkinson's disease patient-derived neurons
Student Name: Panagiotis Gkaravelas

Budget

BUDGET (for 2 years)		
Category		**Total in €**
Direct Costs - Personnel		
PhD Candidate		**24000**
Total Direct costs for Personnel		**24000**
Direct Costs - Other	**Justification**	
6.1.2 Consumables	Cell culture reagents, Molecular biology reagents, Immunocytochemistry reagents, Other chemicals, etc.	**25000**
6.1.3 Travel	Scientific meetings, Research collaborations	**1500**
6.1.4 Dissemination	Publications, Participation in scientific meetings & courses, Science communication public events	**1500**
6.1.5 Use and/or Access to equipment etc.	Sequencing service, etc.	**2000**
Total Direct Costs		**54000**
Indirect Costs (Institution overhead, 10%)		**6000**
Total Budget		**60000**

Title: Investigation of the potential neuroprotective role of a BDNF analog in Parkinson's disease patient-derived neurons
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PUBLICATIONS

Participation in scientific meetings

"Investigation of the potential neuroprotective role of BNN-20 in Parkinson's disease patient-derived neurons". **P. Gkaravelas**, N. Antoniou, G. Athanasopoulou, T. Mourtzi, I. Kazanis, R. Matsas, G. Kouroupi. 29th Virtual Meeting of the Hellenic Society for Neurosciences, October 8-10, 2021 (Poster presentation)

Original articles in preparation for submission

- "Early signs of molecular defects in iPSC-derived neural stems cells from patients with familial Parkinson's disease". E. Akrioti, T. Karamitros, **P. Gkaravelas**, G. Kouroupi, R. Matsas, E. Taoufik
- "Investigation of the effect of the microneurotrophin BNN-20 on Parkinson's Disease patient induced pluripotent stem cell-derived neurons and on the success of stem cell transplantation in an animal model of the disease". T. Mourtzi, N. Antoniou, **P. Gkaravelas**, C. Dimitriou, M. Anesti, R. Matsas, F. Angelatou, G. Kouroupi, I. Kazanis