Growth differentiation factor 5 exerts neuroprotection in an α-synuclein rat model of Parkinson's disease.

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The concept of neurotrophic factor therapy holds significant promise as a disease-modifying therapy for Parkinson's disease (Paul and Sullivan, 2019). Whone et al. (2019) have recently reported the results of a randomized, double-blind, placebo-controlled trial of intermittent intraputamenal convection-enhanced delivery of glial cell line-derived neurotrophic factor (GDNF) in Parkinson's disease patients (Whone et al., 2019). Despite an extensive body of excellent work and significant improvements to dosing and delivery, this trial failed to reach its primary endpoint. This outcome is largely consistent with an earlier randomized, placebo-controlled trial of GDNF in Parkinson's disease (Lang et al., 2006). Pre-clinical studies have shown that delivery of adeno associated virus (AAV)-GDNF to either the striatum or substantia nigra failed to protect dopaminergic neurons or their terminals in a rat model of Parkinson's disease generated through viral-mediated overexpression of human wild-type α-synuclein (AAV-αSyn) (Decressac et al., 2011) or A30P mutant α-synuclein (LV-αSyn) (Lo Bianco et al., 2004). This has been suggested to be due to αSyn-induced downregulation of *Nurr1* and *Ret* in the midbrain, measured at 2 weeks post-surgery (Decressac et al., 2012). Similar αSyn-induced downregulation of *Ret* was reported in the substantia nigra of Parkinson's disease patients (Decressac et al. 2012). This may explain the lack of clinical efficacy of GDNF, as the Ret receptor has been reported to be critical for the dopaminergic neurotrophic effects of GDNF *in vivo* (Drinkut et al., 2016). However, others found that transgenic overexpression of human α-synuclein in mice, or intranigral administration of AAV-αSyn in rats, did not downregulate *Nurr1* or tyrosine hydroxylase (*Th*) expression after 8 weeks (Su et al., 2017). Therefore, the effect of α-synuclein on Ret expression remains an open and important question.

More generally, the lack of clinical efficacy of GDNF has raised the larger question of whether neurotrophic factor delivery as a therapeutic approach remains viable, or if this approach requires further testing and evaluation in clinical settings (Whone et al., 2019). Another consideration is that if Ret receptors are downregulated or not functioning optimally in the Parkinson's disease brain, then Ret-independent neurotrophic factors may have therapeutic benefit, assuming that α-synuclein does not also affect the expression of their key signalling

mediators. Thus, a critical requirement to justify the development of Ret-independent neurotrophic factor therapy, is that these factors must display therapeutic benefit in the α-synuclein model of PD, which more closely mimics the disease pathology than other rodent models. Our aims in the present study were three-fold. First, we investigated whether α-synuclein overexpression reduced *Ret* expression in the rat substantia nigra *in vivo*. Second, we examined if a Ret-independent neurotrophic factor, growth differentiation factor 5 / bone morphogenetic protein 14 (GDF5/BMP14, hereafter called GDF5), could protect dopaminergic neurons and their axon terminals in an AAV-αSyn rat model of Parkinson's disease, similar to that used in the Decressac et al. (2011) GDNF study. Like GDNF, GDF5 is a member of the transforming growth factor (TGF)-β protein superfamily (Hegarty et al., 2014b), and it exerts neuroprotective effects similar to those of GDNF in neurotoxin models of Parkinson's disease (Sullivan et al., 1997, Sullivan et al., 1998). GDF5 signals through a receptor complex consisting of bone morphogenetic protein receptor (BMPR)2, BMPR1B and the transcription factor, Smad1 (Hegarty et al., 2013, Hegarty et al., 2014a). Third, we investigated whether α-synuclein overexpression affected the expression of these three key signalling mediators of GDF5's neurotrophic actions.

Adult Sprague-Dawley rats received unilateral intranigral injection of either AAV2/6-αSyn (wild-type human α-synuclein under the control of synaptophysin promoter), or the corresponding null control vector, and were sacrificed 20 weeks later (Supplementary material). AAV-αSyn induced expression of wild-type human α-synuclein throughout the midbrain and in axonal terminals throughout the ipsilateral striatum (Fig. 1A). Additionally, there was strong pSer-129-α-synuclein expression in the ipsilateral substantia nigra, within cell bodies and processes (Fig. 1B). Intranigral AAV-αSyn led to significant reductions in the expression of transcripts for *Th* (Fig, 1C) and *dopamine transporter protein* (*Dat)* (Fig. 1D), but not *NeuN* (Fig. 1E). AAV-αSyn also induced a significant reduction in *Ret* expression (Fig. 1F), but no change in the expression of *Bmpr2, Bmpr1b* and *Smad 1,* all of which are required for the dopaminergic neurotrophic effects of GDF5 (Fig. 1G). Given these findings, and since previous studies have shown that LV- or AAV-GDNF did not protect nigrostriatal neurons in the AAV-αSyn rat model (Decressac et al. 2011), we next investigated whether AAV-GDF5 could protect nigrostriatal neurons against α-synuclein-induced toxicity, in this Parkinson's disease model.

We modelled our approach on the study by Decressac *et al.* (2011), using a similar protocol with some modifications. Adult rats received stereotaxic unilateral intranigral injection of α-synuclein vector (AAV2/6-αSyn) and concomitantly either control vector (AAV2/5-Cont) or vector carrying the human *GDF5* transgene (AAV2/5-GDF5) (Fig. 2A). Rats were sacrificed 20 weeks later for analysis of transgene expression and nigrostriatal integrity. There were no differences in α-synuclein expression levels between control and GDF5-treated animals in the striatum (Fig. 2B, C) or substantia nigra (Fig. 2D). AAV-GDF5 induced expression of human GDF5 within dopaminergic neurons in the substantia nigra (Fig. 2E). To evaluate the neurodegeneration induced by overexpression of human wild-type α-synuclein and the potential neuroprotective effect of AAV-GDF5, we measured the numbers of TH-positive and DAT-positive neurons in the ipsilateral and contralateral substantia nigra. We found that, at 20 weeks after injection of AAV-αSyn, there was ~36% loss of TH-positive (Fig. 2F, G) and ~30% loss of DAT-positive (Fig. 2H) neurons, compared to the intact side. AAV-mediated delivery

of GDF5 to the substantia nigra prevented this loss of dopamine neurons ($P < 0.01$ compared to AAV-Cont group) (Fig. 2G, H).

We next investigated whether nigral GDF5 overexpression could prevent the loss of striatal dopaminergic terminals, which is known to occur in this model (Decressac et al. 2011). Quantitative analysis of TH-positive striatal fibre innervation revealed that nigral overexpression of α-synuclein induced ~45% reduction (Fig. 2I, J). In contrast, nigral overexpression of GDF5 by AAV-GDF5 had a protective effect on striatal dopaminergic terminal density ($P < 0.01$ compared to AAV-Cont group) (Fig. 2I, J). Because of this significant protection of axonal terminals in the striatum, we repeated the experiment using a second cohort of animals to determine if this protection of axonal terminals was translated into maintenance of striatal dopamine levels. Animals received intranigral injection of AAV-αSyn and concomitantly either AAV-Cont or AAV-GDF5. Analysis of striatal dopamine levels using HPLC after 20 weeks revealed that nigral overexpression of α-synuclein induced ~45% reduction in striatal dopamine levels (Fig. 2K). AAV-mediated nigral overexpression of GDF5 had a protective effect on striatal dopamine levels ($P < 0.05$ compared to AAV-Cont group) (Fig. 2K). Collectively, these data show that intranigral injection of AAV-GDF5 protected dopaminergic neurons and their terminals, maintaining striatal dopamine levels in the AAV-αSyn rat model of Parkinson's disease.

The first implication of these findings is that, in agreement with Decressac et al. (2012), we found that viral-mediated overexpression of α-synuclein led to a significant reduction in *Ret* expression in the adult rat substantia nigra at 20 weeks post-surgery. However, it is important to note that the reduction of 25% in our study was observed at 20 weeks, in contrast to the much greater (~65%) reduction reported by Decressac et al. after 2 weeks. As we found that ~75% of *Ret* expression remained in the α-synuclein-overexpressing brain, these data suggest that the signalling capacity of GDNF may be retained, at least partially, in these animals. This is consistent with the significant putamenal increase in ^{18}F-DOPA uptake in GDNF-treated patients at Week 40 reported in the Whone et al. study (Whone et al., 2019). The key determinant of clinical efficacy of GDNF may therefore be the level of α-synuclein burden in the host brain. High levels of α-synuclein may lead to an early and strong downregulation of *Ret*, similar to that reported in the Decressac et al. study (2012), whereas modest α-synuclein levels may lead - in the short term - to minimal reductions in *Ret*, as observed by Su et al. (2017) after 8 weeks, but more substantial impairment of *Ret* expression in the longer term, as seen in our study.

The second key finding of our study is that the Ret-independent neurotrophic factor, GDF5, exerted neuroprotective effects in the AAV-αSyn rat model, in which GDNF was not effective (Decressac et al., 2011). Moreover, and unlike Ret, the expression of GDF5's receptors and downstream transcription factor SMAD1 was not affected by α-synuclein overexpression, at least at the 20-week time point examined. The observed neuroprotective effects of GDF5 in this preclinical model support the theory that Ret-independent neurotrophic factors may have therapeutic benefit in Parkinson's disease and are worthy of further exploration. Further evidence that supports this proposal comes from an independent study by Vitic et al. (2020). They show that lentiviral delivery of BMP5/7 prevented A53T-a-synuclein-induced loss of dopamine neurons, motor impairment and associated gliosis in a mouse model of Parkinson's

disease. They also demonstrated that loss of BMP/SMAD signalling led to the accumulation of a-synuclein (Vitic et al., 2020).This does not mean that Ret-dependent neurotrophic factors such as GDNF are not worthy of further testing (in fact our findings on Ret suggest that they are); on the contrary, collectively these data suggest that neurotrophic factor therapy remains a viable therapeutic approach for protecting dopaminergic neurons against the effects of α-synuclein in Parkinson's disease.

CONCANNON, R. M., OKINE, B. N., FINN, D. P. & DOWD, E. 2015. Differential upregulation of the cannabinoid CB2 receptor in neurotoxic and inflammation-driven rat models of Parkinson's disease. *Experimental Neurology*, 269**,** 133-141.

DECRESSAC, M., KADKHODAEI, B., MATTSSON, B., LAGUNA, A., PERLMANN, T. & BJORKLUND, A. 2012. alpha-Synuclein-induced down-regulation of Nurr1 disrupts GDNF signaling in nigral dopamine neurons. *Sci Transl Med*, 4**,** 163ra156.

DECRESSAC, M., ULUSOY, A., MATTSSON, B., GEORGIEVSKA, B., ROMERO-RAMOS, M., KIRIK, D. & BJORKLUND, A. 2011. GDNF fails to exert neuroprotection in a rat alpha-synuclein model of Parkinson's disease. *Brain*, 134**,** 2302-11.

DRINKUT, A., TILLACK, K., MEKA, D. P., SCHULZ, J. B., KUGLER, S. & KRAMER, E. R. 2016. Ret is essential to mediate GDNF's neuroprotective and neuroregenerative effect in a Parkinson disease mouse model. *Cell Death Dis*, 7**,** e2359.

HEGARTY, S. V., COLLINS, L. M., GAVIN, A. M., ROCHE, S. L., WYATT, S. L., SULLIVAN, A. M. & O'KEEFFE, G. W. 2014a. Canonical BMP-Smad signalling promotes neurite growth in rat midbrain dopaminergic neurons. *Neuromolecular Med*, 16**,** 473-89.

HEGARTY, S. V., SULLIVAN, A. M. & O'KEEFFE, G. W. 2013. BMP2 and GDF5 induce neuronal differentiation through a Smad dependant pathway in a model of human midbrain dopaminergic neurons. *Mol Cell Neurosci*, 56**,** 263-71.

HEGARTY, S. V., SULLIVAN, A. M. & O'KEEFFE, G. W. 2014b. Roles for the TGFbeta Superfamily in the Development and Survival of Midbrain Dopaminergic Neurons. *Mol Neurobiol*.

LANG, A. E., GILL, S., PATEL, N. K., LOZANO, A., NUTT, J. G., PENN, R., BROOKS, D. J., HOTTON, G., MORO, E., HEYWOOD, P., BRODSKY, M. A., BURCHIEL, K., KELLY, P., DALVI, A., SCOTT, B., STACY, M., TURNER, D., WOOTEN, V. G., ELIAS, W. J., LAWS, E. R., DHAWAN, V., STOESSL, A. J., MATCHAM, J., COFFEY, R. J. & TRAUB, M. 2006. Randomized controlled trial of intraputamenal glial cell line-derived neurotrophic factor infusion in Parkinson disease. *Ann Neurol*, 59**,** 459-66.

LO BIANCO, C., DEGLON, N., PRALONG, W. & AEBISCHER, P. 2004. Lentiviral nigral delivery of GDNF does not prevent neurodegeneration in a genetic rat model of Parkinson's disease. *Neurobiol Dis*, 17**,** 283-9.

PAUL, G. & SULLIVAN, A. M. 2019. Trophic factors for Parkinson's disease: Where are we and where do we go from here? *Eur J Neurosci*, 49**,** 440-452.

SU, X., FISCHER, D. L., LI, X., BANKIEWICZ, K., SORTWELL, C. E. & FEDEROFF, H. J. 2017. Alpha-Synuclein mRNA Is Not Increased in Sporadic PD and Alpha-Synuclein Accumulation Does Not Block GDNF Signaling in Parkinson's Disease and Disease Models. *Mol Ther*, 25**,** 2231-2235.

SULLIVAN, A. M., OPACKA-JUFFRY, J., HOTTEN, G., POHL, J. & BLUNT, S. B. 1997. Growth/differentiation factor 5 protects nigrostriatal dopaminergic neurones in a rat model of Parkinson's disease. *Neurosci Lett*, 233**,** 73-6.

SULLIVAN, A. M., POHL, J. & BLUNT, S. B. 1998. Growth/differentiation factor 5 and glial cell line-derived neurotrophic factor enhance survival and function of dopaminergic grafts in a rat model of Parkinson's disease. *Eur J Neurosci*, 10**,** 3681-8.

VITIC, Z., SAFORY, H., JOVANOVIC, V. M., SARUSI, Y., STAVSKY, A., KAHN, J., KUZMINA, A., TOKER, L., GITLER, D., TAUBE, R., FRIEDEL, R. H., ENGELENDER, S. & BRODSKI, C. 2020. BMP5/7 protect dopaminergic neurons in an α -synuclein mouse model of Parkinson's disease. *Brain*.

WHONE, A., LUZ, M., BOCA, M., WOOLLEY, M., MOONEY, L., DHARIA, S., BROADFOOT, J., CRONIN, D., SCHROERS, C., BARUA, N. U., LONGPRE, L., BARCLAY, C. L., BOIKO, C., JOHNSON, G. A., FIBIGER, H. C., HARRISON, R., LEWIS, O., PRITCHARD, G., HOWELL, M., IRVING, C., JOHNSON, D., KINCH, S., MARSHALL, C., LAWRENCE, A. D., BLINDER, S., SOSSI, V., STOESSL, A. J., SKINNER, P., MOHR, E. & GILL, S. S. 2019. Randomized trial of intermittent intraputamenal glial cell line-derived neurotrophic factor in Parkinson's disease. *Brain,* 142**,** 512-525.
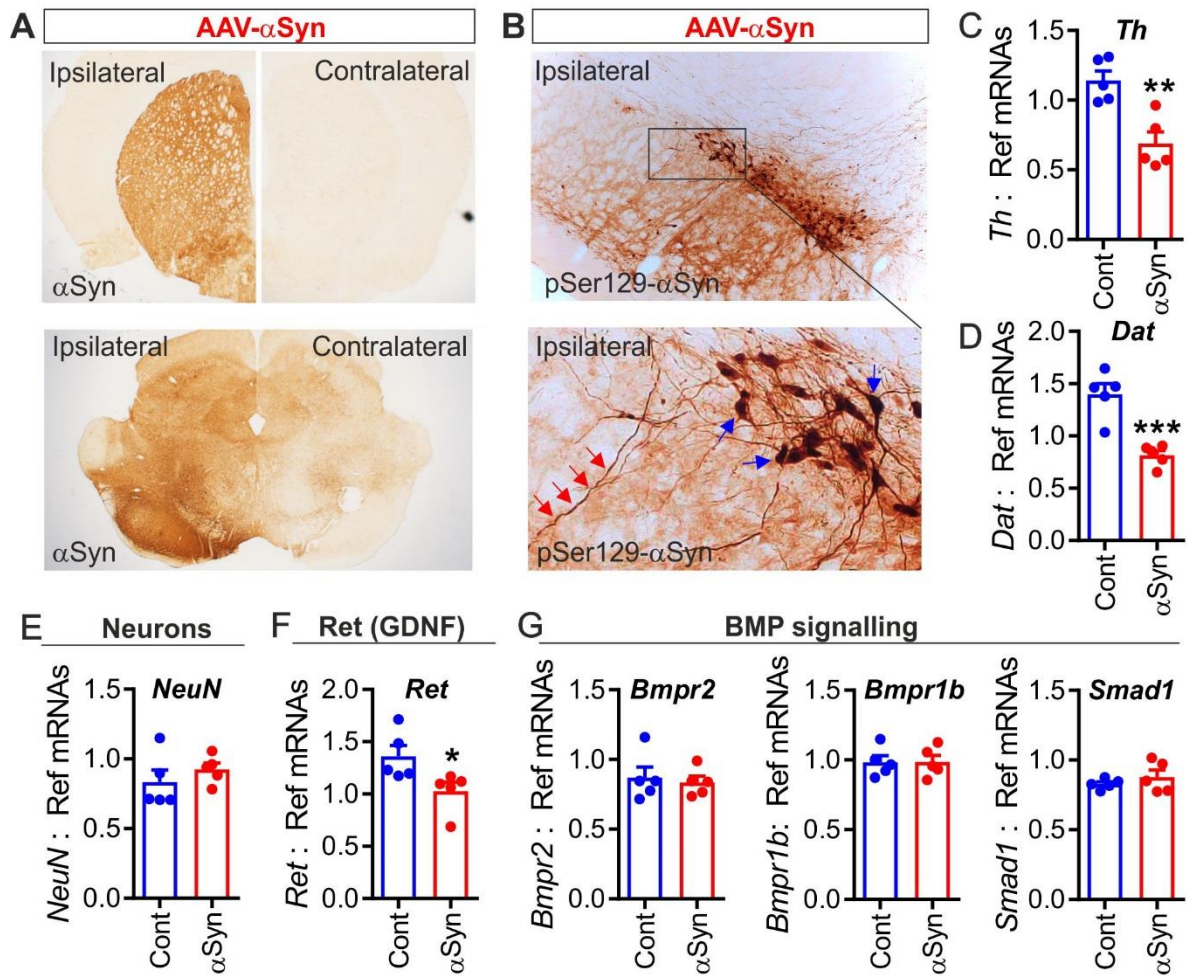
FIGURES

Figure 1: α-synuclein downregulates *Ret* but not key mediators of BMP signalling *in vivo*.

Representative images showing **(A)** wild-type α-synuclein (αSyn) immunostaining in rat striatum and midbrain, and **(B)** pSer129-αSyn staining in substantia nigra, at 20 weeks after unilateral stereotaxic injection of AAV-αSyn vector into the substantia nigra. Blue arrows indicate dopaminergic neuronal cell bodies and red arrows indicate processes. Real-time PCR data showing expression of transcripts for **(C)** *Th* (** $t = 4.211$, d.f. = 8, $P = 0.0029$), **(D)** *Dat* (** $t = 5.28$, d.f. = 8, $P = 0.0007$) **(E)** *NeuN*, **(F)** *Ret* (** $t = 2.45$, d.f. = 8, $P = 0.0399$) and **(G)** *Bmpr2*, *Bmpr1b* and *Smad1,* key mediators of BMP signalling ($n = 5$ per group). All data are presented as mean ± SEM and analysed by Student's *t*-test.
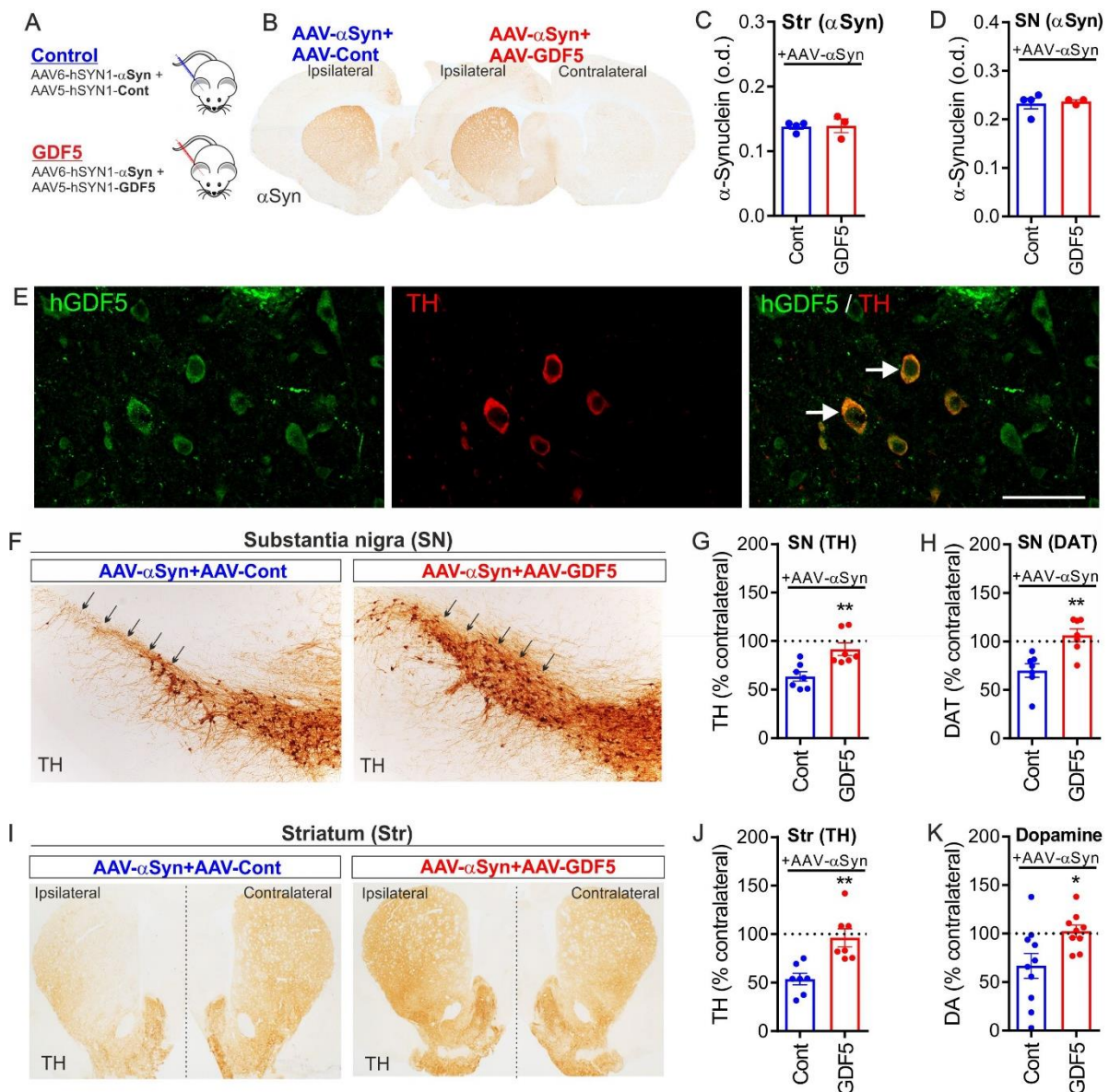
Figure 2: AAV-GDF5 maintains nigrostriatal integrity and striatal dopamine levels in the rat AAV-αSyn model of Parkinson's disease.

(A) Animals received unilateral injection of AAV-αSyn with either AAV-Cont (blue) or AAV-GDF5 (red) into the substantia nigra. **(B)** Representative images of α-synuclein immunostaining in the striatum. Quantification of α-synuclein immunostaining in **(C)** striatum and **(D)** substantia nigra. **(E)** Representative photomicrographs showing expression of the human (h) GDF5 transgene (green) in TH-positive neurons (red; white arrows) in the substantia nigra at 20 weeks post-injection of AAV-GDF5. **(F)** Representative photomicrographs of TH-immunostained sections through the substantia nigra on the ipsilateral side. **(G, H)** Numbers of **(G)** TH-immunopositive (** $t = 3.46$, d.f. = 12, $P = 0.0047$) and **(H)** DAT-immunopositive neurons (** $t = 3.76$, d.f. = 12, $P = 0.0027$) in the ipsilateral SN, expressed as percentage of the intact side ($n = 7$ per group). **(I)** Representative photomicrographs of TH-immunostained sections through the ipsilateral and contralateral striatum. **(J)** Quantification of TH immunoreactivity in the ipsilateral striatum, expressed as percentage of the intact side (** $t = 3.86$, d.f. = 12, $P = 0.0023$) ($n = 7$ per group). **(K)** HPLC analysis of dopamine (DA) levels in the ipsilateral striatum, expressed as percentage of the intact side (** $t = 2.397$, d.f. = 17, $P = 0.0283$) ($n = 9$-10 per group). All data are presented as mean ± SEM and analysed by Student's t-test.

MATERIALS AND METHODS

Animal Husbandry and Study Design

80 adult female Sprague-Dawley rats were purchased from Envigo, UK, and maintained on a 12h:12h light:dark cycle with access to food and water *ad libitum*. Animals were assigned to one of the following four experimental groups: AAV-Control (n=20), AAV-α-Synuclein (n=20), AAV-GDF5 (n=20) or AAV-α-Synuclein+AAV-GDF5 (n=20). Rats were housed in groups of four in standard housing cages containing environmental enrichment. All experiments were conducted fully in accordance with the European Directive 2010/63/EU and under an authorisation granted by the Health Products Regulatory Authority Ireland (AE19130/P057).

Virus Preparation and Stereotactic Surgery

An α-Synuclein plasmid was generously donated from Dr Eilis Dowd (National University of Ireland, Galway) and Professor Deniz Kirik (Lund University, Sweden). AAV2/6-α-Synuclein, AAV2/5-GDF5, AAV2/5-Null and AAV2/6-Null viral vectors were produced by Vector Biosystems Inc, Philadelphia, USA. Briefly, AAV2 inverted terminal repeats coding for human wild-type α-synuclein or human GDF5 were packaged using AAV6 or AAV5 capsid proteins, to produce AAV2/6 and AAV2/5 viral vectors and the corresponding empty control vectors. Transgene expression was driven by synapsin-1 promoter and enhanced using woodchuck hepatitis virus post-transcriptional regulatory element (WPRE). Stereotaxic surgery was conducted under general anaesthesia induced by the inhalation agent isoflurane. Each animal was placed in a stereotactic frame, an incision was made to the scalp and a small hole was drilled into the skull. AAV-Control animals were administered 2 µL AAV2/5-Null (1.0×10^{10} vg/µL) + AAV2/6-Null (5.3×10^{9} vg/µL). AAV-α-Synuclein animals were administered 2 µL AAV2/6-SnaSW (5.2×10^{10} vg/µL) + AAV2/5-Null (1.0×10^{10} vg/µL). AAV-GDF5 animals received 2 µL AAV2/5-GDF5 (1.3×10^{10} vg/µL) + AAV2/6-Null (5.3×10^{9} vg/µL). AAV-α-Synuclein+AAV-GDF5 animals received 2 µL AAV2/6-SnaSW (5.2×10^{10} vg/µL) + AAV2/5-GDF5 (1.3×10^{10} vg/µL). All vector combinations were given unilaterally into the SN at coordinates AP -5.3, ML ± 2.0, DV -7.2 relative to bregma, at an infusion rate of 1µl/min with an additional 2 min for diffusion before withdrawal and suturing. Post-surgery, animals received the analgesic Carprofen (5 mg/kg, s.c.) and 5% glucose solution (i.p.) and were allowed to recover fully on a heating mat before being returned to their home cages.

Tissue Processing

Animals were sacrificed 20 weeks post-surgery by either decapitation under isoflurane anaesthesia (5% in O_2) for qRT-PCR and HPLC analyses, or transcardial perfusion-fixation under terminal pentobarbital anaesthesia (50 mg/kg) for immunohistochemical analyses. For qRT-PCR and HPLC analyses, the SN and striatum were microdissected from each brain and stored in the appropriate buffer at -20ºC until needed. For immunohistochemical analyses, brains were post-fixed in 4% paraformaldehyde for 24 h and cryoprotected in 30% sucrose solution with 0.1% sodium azide. Sections (30 µm thickness) were cut on a freezing stage sledge microtome and were used for immunohistochemistry.

Immunohistochemistry

Immunohistochemistry was carried out as previously described (Concannon et al., 2015) with minor amendments. Briefly, sections were washed for 10 min using tris-buffered saline (TBS) solution. Where appropriate, sections were quenched using 3% Hydrogen Peroxide/10% Methanol in distilled water for 5 min to remove endogenous peroxidase activity, followed by 3 x 5 min washes with TBS. Non-specific antibody binding was blocked for 1 h using 3% Goat, Horse or Rabbit serum as required diluted in TBS containing 0.02% Triton-X100 (TXTBS). Sections were incubated overnight at room temperature (RT) with primary antibody diluted in TXTBS containing 1% serum. Primary antibodies were TH (1:1000, Merck Millipore or Abcam), DAT (1:200, SCBT), GDF5 (1:100, Abcam), α-Synuclein (1:1000, Millipore), pSer129-α-Synuclein (1:1000, Abcam). Following 3 x 5-min TBS washes, sections were incubated in secondary antibody diluted in TXTBS containing 1% serum for 2 h. For immunofluorescence, Alexa Fluor 488- or 594-conjugated secondary antibodies (1:200; Invitrogen) were used. Sections were washed for 3 x 5-min using TBS and cover-slipped using fluorescent mounting media (Dako Diagnostics). For chromogen staining, biotinylated goat-anti-rabbit IgG (1:200, Jackson Immunoresearch Lab), horse-anti-mouse IgG (1:200, Vector Labs) or rabbit-anti-goat IgG (1:200, Vector Labs) secondary antibodies were used. Following 3 x 5-min TBS washes, sections were incubated in a Streptavidin–biotin–horseradish peroxidase solution (Vector Labs) for 2 h. Sections were then washed 3 x 5 min with TBS before developing with DAB (Vector Labs). The sections were dehydrated using increasing concentrations of Ethanol, cleared in Xylene and cover-slipped using DPX mounting media (BDH Chemicals). Images of the sections were taken using the Olympus BX53 Upright Microscope and the Olympus FV1000 Confocal Laser Scanning Biological Microscope.

HPLC

HPLC analysis was performed on striatal samples, as previously described (Clarke et al, 2013). Dissected striatal brain tissue was sonicated in 1 ml of chilled mobile phase, spiked with 2ng/20ul of N-Methyl 5-HT (Sigma, UK) as internal standard. The mobile phase contained 0.1M citric acid, 0.1M sodium dihydrogen phosphate, 0.01mM EDTA (Alkem/Reagecon, Cork, Ireland), 5.6mM octane-1-sulphonic acid (Sigma) and 9% (v/v) methanol (Alkem/Reagecon), and was adjusted to pH 2.8 using 4N sodium hydroxide (Alkem/Reagecon). Homogenates were then centrifuged at 14,000RPM for 20 min at 4°C and 20µl of the supernatant injected onto the HPLC system which was coupled to an electrochemical detector. A reverse-phase column (Kinetex 2.6u C18 100 x 4.6mm, Phenomenex, UK) maintained at 30°C was employed in the separation (Flow rate 0.9ml/min), the glassy carbon working electrode combined with an Ag/AgCL reference electrode (Shimdazu) was operated at +0.8V and the chromatograms generated were analysed using Class-VP 5 software (Shimadzu). Dopamine was identified by its characteristic retention time as determined by standard injections which were run at regular intervals during the sample analysis. Analyte:Internal standard peak height ratios were measured and compared with standard injections to calculate the results.

qRT-PCR

Total RNA was extracted from SN samples using the Qiagen RNeasy Universal Plus kit according to the manufacturer's instructions. Levels of *Th*, *Dat*, *NeuN*, *Ret*, *Bmpr2*, *Bmpr1b* and *Smad1* mRNAs were quantified by real-time PCR relative to a geometric mean of mRNAs for the reference genes glyceraldehyde phosphate dehydrogenase (*Gapdh*), ubiquitin C (*Ubqc*) and tata binding protein (*Tbp*). 5 µl of SN total RNA was reverse-transcribed for 1 h at 45°C using the AffinityScript kit (Agilent, Berkshire, UK) in a 25 µl reaction according to the manufacturer's instructions. 2 µl of cDNA was amplified in a 20 µl reaction volume using Brilliant III ultrafast qPCR master mix reagents (Agilent Technologies). PCR products were detected using dual-labelled (FAM/BHQ1) hybridization probes specific to each of the cDNAs (MWG/Eurofins, Ebersberg, Germany). The PCR primers were: *Th* forward: 5'-AGA GAT TGC CTT CCA GTA-3' and reverse: 5'-CCT TCA GCG TGA CAT ATA C-3'; *Dat* forward: 5′-CAC CAC CTC CAT TAA CTC-3′ and reverse: 5′-CAG GAT AGA TGATGA AGA TGA G-3′; *NeuN* forward: 5'-CAC TCT CTT GTC CGT TTG-3' and reverse: 5′-CCG ATG GTA TGA TGG TAG-3′; *Ret* forward: 5′-TGT TCT CTT CCT CCA TTT CA-3′ and reverse: 5′-AGT TCT CCA CGC AAA CTT-3′; *Bmpr2* forward: 5′-CAA CTT CAC TGA GAA CTT C-3′ and reverse: 5′-GCT AAT ACA GAA ACC GAT G-3′; *Bmpr1b* forward: 5'-AAA GGT AGC TGT GAA AGT-3' and reverse: 5′-ATG ATA GTC TGT GAT GAG G-3′; *Smad1* forward: 5'-TCG GAG GAG AGG TGT ATG-3' and reverse: 5′-GCG AAC TCT TGG TTG TTG-3′; *Gapdh* forward: 5′-TGG TCA TCA ACG GGA AAC-3′ and reverse: 5′-CCA CGA CAT ACT CAG CAC-3′; *Ubqc* forward: 5′-TTC TCA CCA CAG TAT CTA G-3′ and reverse: 5′-AGT GCA ATG AAA CTT GTT A-3′; *Tbp* forward: 5'-CAC CAA TGA CTC CTA TGA-3' and reverse: 5'-TGG GAT TAT ATT CAG CAT TTC-3'. Dual-labelled probes were: *Th*: 5'-FAM-AAG CAC GGT GAA CCA ATTCC-BHQ1-3'; *Dat*: 5'-FAM-CTT CTC CTC TGG CTT CGT CGT-BHQ1-3'; *NeuN*: 5'-FAM-ATC AGC AGC CGC ATA GAC TC-BHQ1-3'; *Ret*: 5'-FAM-CGT GTC TGT GCT GCC TGT C-BHQ1-3'; *Bmpr2*: 5'-FAM-CAC CTC CTG ATA CAA CAC CAC TC-BHQ1-3'; *Bmpr1b*: 5'-FAM-TCA CCA CGG AGG AAG CCA-BHQ1-3'; *Smad1*: 5'-FAM-CAG TGA CAG CAG CAT CTT CGT G-BHQ1-3'; *Gapdh*: 5′-FAM-CAT CAC CAT CTT CCA GGA GCG AGA-BHQ1-3; *Ubqc*: 5′-FAM-CCT TCT TGT GCT TGT TCT TGG-BHQ1-3, *Tbp*: 5'-FAM-TCC TGC CAC ACC AGC CTC TG -BHQ1-3'. Forward and reverse primers were used at a concentration of 150 nM and dual-labelled probes were used at a concentration of 200 nM. PCR was performed using the Mx3000P platform (Agilent) using the following conditions: 95°C for 3 min followed by 40 cycles of 95°C for 12 s and 60°C for 35 s. Standard curves were generated for each cDNA for every real-time PCR run, by using serial threefold dilutions of reverse-transcribed adult rat brain total RNA (Zyagen, San Diego, USA). Relative mRNA levels were quantified in SN dissected from 5 separate

animals for each experimental condition. Primer and probe sequences were designed using Beacon Designer software (Premier Biosoft, Palo Alto, USA).

CLARKE, G., GRENHAM, S., SCULLY, P., FITZGERALD, P., MOLONEY, R.D., SHANAHAN, F., DINAN, T.G., CRYAN, J.F. 2013. The microbiome-gut-brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner. *Mol Psychiatry,* 18, 666–673.

CONCANNON, R. M., OKINE, B. N., FINN, D. P. & DOWD, E. 2015. Differential upregulation of the cannabinoid CB2 receptor in neurotoxic and inflammation-driven rat models of Parkinson's disease. *Experimental Neurology,* 269**,** 133-141.