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^{o}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^{o}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).

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