Title: Efficient expression of *Igf-1* from lentiviral vectors protects *in vitro* but does not mediate behavioral recovery of a Parkinsonian lesion in rats

Ngoc B. Lu-Nguyen, Martin Broadstock, and Rafael J. Yáñez-Muñoz*

School of Biological Sciences, Royal Holloway, University of London, Egham, Surrey, TW20 0EX, UK.

*Corresponding address: Rafael J. Yáñez-Muñoz, School of Biological Sciences, Royal Holloway, University of London, Egham, Surrey, TW20 0EX, UK.

Tel: +44 (0) 1784 443180, Fax: +44 (0) 1784 414224, Email: rafael.yanez@rhul.ac.uk

Short title: Lentivector-mediated IGF-1 therapy for Parkinson's

Abstract

Gene therapy approaches delivering neurotrophic factors have offered promising results in both pre-clinical and clinical trials of Parkinson's disease (PD). However, failure of glial cell line-derived neurotrophic factor in phase II clinical trials has sparked a search for other trophic factors which may retain efficacy in the clinic. Direct protein injections of one such factor, insulin-like growth factor 1 (IGF-1), in a rodent model of PD has demonstrated impressive protection of dopaminergic neurons against 6-hydroxydopamine (6-OHDA) toxicity. However, protein infusion is associated with surgical risks, pump failure and significant costs. We therefore used lentiviral vectors to deliver *Igf-1*, with a particular focus on the novel integration-deficient lentiviral vectors (IDLVs). A neuron-specific promoter, from the human synapsin 1 gene, excellent for gene expression from IDLVs, was additionally used to enhance *Igf-1* expression. An investigation of neurotrophic effects on primary rat neuronal cultures demonstrated that neurons transduced with IDLV-*Igf-1* vectors had complete protection upon withdrawal of exogenous trophic support. Striatal transduction of such vectors into 6-OHDA-lesioned rats, however, provided neither protection of dopaminergic substantia nigra neurons nor improvement of animal behavior.

Key words: IGF-1, Parkinson's disease, lentiviral vectors, IDLVs

Introduction

Parkinson's disease (PD) results from a gradual loss of neurons within the dopaminergic substantia nigra pars compacta (SNpc) and their projections to the striatum[1]. Therapeutic strategies using trophic factors have offered encouraging results in both pre-clinical and open-label clinical trials of PD[2-6]. However, administration of the most promising factor, glial cell line-derived neurotrophic factor (GDNF), was unsuccessful in double blind, phase II clinical trials[7]. Hence, the search for other neurotrophic factors has been stepped up[4-6, 8]. One such factor is insulin-like growth factor 1 (IGF-1).

IGF-1 is well known as a peripheral growth factor (secreted mainly by hepatocytes) that regulates cell metabolism and tissue remodeling[9]. In recent years, a role of IGF-1 within the central nervous system (CNS) has been identified, appearing to be both neuroprotective and neurotrophic, and important in the maintenance of adult CNS homeostasis[10, 11]. IGF-1 is also considered a pro-survival factor, upregulated in all types of brain injuries[12]. IGF-1 exerts its multiple effects through binding to IGF-1 receptors, phosphorylation of which activates multiple downstream signaling pathways, resulting in inhibition of apoptosis and regulation of both cell differentiation and cell proliferation[13-15].

The exact role of IGF-1 in PD remains unclear, with studies reporting a decrease in plasma levels associated with cognitive decline[16]. Conversely, in *post-mortem* PD samples, an increase in plasma IGF-1 levels was observed[17], suggesting that there may be a compensatory increase in IGF-1 levels due to the ongoing cell loss. Guan *et al.*[18] and Offen *et al.*[19] demonstrated IGF-1 neuroprotection against 6-hydroxydopamine (6-OHDA) toxicity in human neuroblastoma and rodent neuronal cell cultures. Other studies using intracerebral injections of IGF-1 protein, after 6-OHDA lesioning of the nigrostriatal pathway, have suggested that IGF-1 administration can prevent dopaminergic cell loss and behavioral deficits in this pre-clinical animal model of PD[18, 20, 21]. However, direct protein infusion via a surgically implanted pump has a number of disadvantages, including limited distribution, device-related side effects and activation of the immune response[7, 22]. An alternative approach,

which may overcome these disadvantages, is the use of gene therapy to deliver the gene for IGF-1 through a viral vector system.

Numerous reports have shown both efficient and stable expression of *GDNF* from lentiviral vectors (LVs) in pre-clinical animal models of PD[23-27]. We have previously demonstrated that the novel integration-deficient LVs (IDLVs) are as efficient as their standard integration-proficient counterparts (IPLVs) at transducing rodent eye and brain cells *in vivo*[28] but have enhanced biosafety due to their reduced risk of insertional mutagenesis[28, 29]. Our recent data have confirmed efficient and long-lasting *in vivo* transduction with IDLV-*hGDNF,* mediating therapeutic effects in a PD rat model[30].

In the present study, the potential neuroprotective effects of IGF-1 were investigated through IDLV-mediated expression, with IPLV-*Igf-1* vectors used as controls. To maximize the levels of transgene expression, which can be addressed by the choice of promoters specific to target cells[31-33], we initially examined transduction efficiency and cell-type specificity of LVs through expression of the gene encoding enhanced green fluorescent protein (*eGFP)*. We compared vectors driven by the cytomegalovirus promoter (CMVp), human glial fibrillary acidic protein promoter (GFAPp) and human synapsin 1 promoter (SYNp). In particular, LV-mediated *in vivo eGFP* expression from IDLV-SYNp vectors displayed efficient, long-term, and comparable transduction efficacy with their integrating counterparts. Following vector transduction in rat primary ventral mesencephalic cell cultures and 6-OHDA-lesioned rats, the neuronal specificity of LV-SYNp vectors, beneficial to enhance *Igf-1* expression[34], was ascertained. Further investigation of LV-mediated IGF-1 neuroprotection demonstrated enhanced *in vitro* survival of rat cortical neurons against withdrawal of exogenous trophic factors. *Igf-1* expression from LVs was subsequently confirmed in 6-OHDA rat model of PD although neither dopaminergic cell survival nor animal behavioral recovery was improved following vector transduction.

Materials and Methods

Lentiviral vector production and titration: Concentrated stocks of the 3rd generation lentiviral vectors, both IPLVs and IDLVs, were produced by calcium phosphate transfection in HEK-293T cells as previously described[28]. Lentiviral transfer plasmids used in this study were pRRLsc-CMVp-*eGFP*-W, pRRLsc-hGFAPp-*eGFP*-W, pRRLsc-hSYNp-*eGFP*-W, pRRLsc-CMVp-*Gdnf*-IRES-*eGFP*-W, pRRLsc-SFFVp-*eGFP*-CMVp-*Igf-1*-W, and pRRLsc-SFFVp-*eGFP*-hSYNp-*Igf-1*-W; *Gdnf* and *Igf-1* cDNAs were from rat. All vectors were self-inactivating, contained a central polypurine tract/central termination sequence (cPPT/cTS) and Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE, abbreviated as W on plasmid names), and were pseudotyped with the vesicular stomatitis virus G protein (VSV-G) envelope. Viral titres were quantified by either flow cytometry using a FACSCanto II (BD Biosciences, UK), qPCR using Rotogen (QIAgen, UK) as described [28], or by p24 ELISA kit (SAIC, Frederick, Maryland, USA) following the manufacturer's instructions.

Animals: Animal procedures were performed in accordance with the UK Animals (Scientific Procedures) Act, 1986. Sprague-Dawley rats (Charles River, UK) were maintained in a standard 12-hour light/dark cycle with free access to food and water. For primary cell cultures, E14-E15 embryos were used for ventral mesencephalic (VM) cultures whilst E17-E18 embryos were harvested for cortical neuronal cultures. For *in vivo* work, 35 male rats weighing 250-300 g at the beginning of the study were housed 2-3 rats/cage and were split randomly into experimental groups, either for 5-month eGFP experiment or 6-week IGF-1 experiment.

Preparation of primary cultures of embryonic brain cells: Rat embryos were rapidly removed and kept in ice-cold dissection buffer containing 1X Hank's balanced salt solution (Invitrogen, UK), 100 units/ml penicillin (PAA, UK), 100 µg/ml streptomycin (PAA, UK), and 100 µM ascorbic acid (Sigma Aldrich, UK). The ventral mesencephalons containing the subtantia nigra or cortices were dissected under a Wild M3Z dissecting microscope (Wild Heerbrugg, Switzerland) with illumination support by a

KL2500 LCD instrument (Schott UK Ltd., UK). Brain tissues were dissociated in 0.05% trypsin/EDTA (PAA, UK) supplemented with 0.02% DNaseI (Promega, UK) for 5-20 min at 37°C. Tissues were washed twice in culture medium prior to de-aggregation by repeated pipetting (p1000, p200, p20, and p10 pipettors). VM or cortical neuronal culture medium included neurobasal medium (Invitrogen, UK), 2% B27 (Invitrogen, UK), 0.25% GlutaMax (Invitrogen, UK), 1% FBS (PAA, UK), and 100 µM ascorbic acid (Sigma Aldrich, UK); 10 ng/ml TGF-βIII was additionally added into VM neuronal cultures. VM astroglial culture medium included DMEM (PAA, UK), 1% N2 (Invitrogen, UK) and 10% FBS. The cell suspension was then passed through a 40-100 µm cell strainer and subsequently centrifuged at 80-200 *g* for 5 min. The cell pellet was resuspended in pre-warmed culture medium. Live cells were counted by 0.2% tryphan blue staining (Sigma Aldrich, UK). For VM neuronal cultures, 100 µl cell suspension containing 10^5 live cells was plated onto a 13 mm coverslip (Thermo Scientific, UK), which was pre-coated overnight with 50 µg/ml poly-D-lysine and placed inside a well of a 24-well plate. After 30 minutes for cell adhesion, each well was carefully topped up with 500 µl culture medium. For VM astroglial cultures, 500 µl cell suspension containing 5 x 10^4 live cells/well was plated in uncoated 24-well plates and cultured at 37°C for 48 hours. The supernatant, containing clumps of unattached cells (most of those were dead neurons), was aspirated and replaced with fresh medium. For cortical neuronal cultures, a density of 3 x 10^6 live cells/well was plated in 24-well plates pre-coated with 50 µg/ml poly-D-lysine. Cell cultures were maintained by changing the medium every 1-3 days. The methods described in this section were amended from published methodologies of Pruszak *et al*. for VM neuronal cultures[35]; Takeshima *et al*. for VM astroglial cultures[36]; and Boulos *et al*. for cortical neuronal cultures[37].

LV transduction *in vitro*: Cell culture medium was changed 2 hours prior to vector transduction. Concentrated vector stocks were diluted in medium for an optimized MOI 10 (eGFP transducing units/ml) or MOI 30 (qPCR vector units/ml) and added gently to the wells; mock cells received no vector.

Trophic support withdrawal strategy: Cortical neuronal cultures had the medium changed at day 1 *in vitro,* 2 hours prior to transduction with IDLVs at MOI 30 (qPCR vector units/ml), including: CMVp-*eGFP*, CMVp-*Gdnf*-IRES-*eGFP*, SFFVp-*eGFP*-CMVp-*Igf-1*, and SFFVp-*eGFP*-SYNp-*Igf-1* vectors. Cells were maintained for 3 days before withdrawal of exogenous trophic factors. For this, the medium was aspirated; normal culture medium was added into trophic factor-supported groups whereas only neurobasal medium was added to trophic factor-free groups. Cell viability was assessed by MTT assay after an additional 3 days.

MTT assay: Cell viability was evaluated by using a colorimetric assay employing MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma Aldrich, UK). A final concentration of 0.5 mg/ml MTT was added to cell cultures and subsequently incubated at 37°C for 3 hours. Formazan crystals produced in this reaction were solubilized in dimethyl sulfoxide (VWR, UK). The optical cell density in each well was then measured by spectrometric analysis at $OD_{570,}$ using Gen5 data analysis software (BioTek, UK).

Immunocytochemistry: For characterization of VM cell cultures, cells were gently washed in ice-cold 1X PBS and then fixed in 4% PFA for 10-15 min. Fixed cells were washed twice in 1X PBS, permeabilized in 1X PBS-T (1X PBS containing 0.25% Triton-X 100) for 10 min, and incubated in 1% BSA blocking buffer (in 1X PBS-T) for 30 min. Primary antibodies were separately added and incubated at 4°C overnight. The antibody was either mouse anti-NeuN (1:500, Millipore, UK) or rabbit anti-Tuj1 (1:3000, Sigma, UK) for neurons; rabbit anti-GFAP (1:500, DAKO, UK) for astrocytes; rabbit anti-Iba1 (1:500, WAKO, UK) for microglia; rabbit anti-TH (1:1000, Millipore, UK) for dopaminergic neurons; rabbit anti-VGLUT1 (1:500, Synaptic systems, Germany) for glutamatergic neurons; or rabbit anti-GAD67 (1:50, Synaptic systems, Germany) for GABAergic neurons. After 3 washes in 1X PBS, cells were incubated with the corresponding secondary antibodies diluted in 1% BSA blocking buffer at room temperature, for an hour, in the dark. The antibody (1:500, Invitrogen, UK) was either goat anti-rabbit AlexaFluor555 or goat anti-mouse AlexaFluor555. After another 3 washes in 1X PBS, cells were

incubated with 1 µg/ml 4,6-diamidino-2-phenylindole (DAPI) for 15 min, in the dark. Three washes in 1X PBS were then performed. Images of cells were subsequently captured and counted. Cells incubated with only primary or secondary antibody were used as negative controls for the staining. For estimation of the percentage of eGFP-producing cells in primary cell cultures transduced with LV-*eGFP* vectors, cells were washed in ice-cold 1X PBS and fixed in 4% PFA for 10-15 min. After 3 washes in 1X PBS, cells were incubated with 1 µg/ml DAPI for 15 min to stain nuclei. Another 3 washes in 1X PBS were performed prior to cell capture.

Intrastriatal injection of LVs: Animals were kept under isoflurane-induced anesthesia (5% in 100% O_2 for induction and 2.5% in 100% O_2 for maintenance). The nose bar was set at -3.3 mm. All injections were made using a 25 µl Hamilton syringe held within an automated UltraMiniPump III (World Precision Instruments); injection rate was 0.5 µl/min. Two burr-holes were made into the skull above the right striatum with an Ideal Micro-drill (Harvard Apparatus, UK). Equivalent vector titres (5 µl/hole, 10^9 qPCR vector copies/ml or $2x10^4$ ng p24/ml) were injected following stereotactic coordinates: (1) AP = +1.8 mm, ML = -2.5 mm relative to bregma and DV = -5.0 mm relative to dura; (2) AP = 0.0 mm, ML = -3.5 mm relative to bregma and DV = -5.0 mm relative to dura. The needle was left in place for 3 min before being retracted. In the eGFP experiment, animal groups included IPLV-SYNp-*eGFP* (*n* =5) and IDLV-SYNp-*eGFP* (*n* = 5). In the IGF-1 experiment, animal groups (*n* = 5 per group) were: (1) mock (0.9% sterile saline); (2) IPLV-CMVp-*Igf-1*; (3) IDLV-CMVp-*Igf-1*; (4) IPLV-SYNp-*Igf-1*; and (5) IDLV-SYNp-*Igf-1*.

6-OHDA lesioning: Two weeks post-vector transduction, animals were injected with a combined solution of pargyline (5 mg/kg, *i.p*) and desipramine (25 mg/kg, *i.p*). Thirty minutes later, 6-OHDA dissolved in a solution containing 0.9% saline and 0.02% ascorbic acid was injected into the same positions of vector administration (2 µl/site, 2.5 µg/ µl). All reagents were purchased from Sigma Aldrich, UK.

Drug-induced rotational asymmetry tests: In the IGF-1 experiment, animal behavior was tested weekly, from one week after 6-OHDA lesioning for a period of 4 weeks. Amphetamine-induced rotational tests were carried out first, followed by apomorphine tests 3 days later. In each test, animals were acclimatized in 40 cm-diameter bowls for 30 min prior to injection with amphetamine (5 mg/kg, *i.p*; Sigma Aldrich, UK) or apomorphine (1 mg/kg, *i.p*; Tocris, UK). Net rotational asymmetry was assessed for 90 min post-injection as one whole head to tail revolution, with contralateral and ipsilateral turns being scored separately.

IGF-1/GDNF detection by ELISA: In the *in vitro* experiment, cortical neuronal cultures were collected at days 3 and 6 post-transduction with IDLV-*Igf-1* or -*Gdnf* vectors. In the *in vivo* experiment, rats receiving IPLV- or IDLV-*Igf-1* vectors, or mock-treated animals were sacrificed with CO_2 followed by cervical dislocation. The striata were dissected and snap-frozen in liquid nitrogen. Tissue was homogenized in complete lysis buffer (Roche, UK) and centrifuged at 14000 *g*, 4°C for 15 min. Levels of IGF-1 or GDNF that were produced in either cell cultures or rat striata were determined with a rat IGF-1 ELISA kit (R&D System, UK) or a human GDNF ELISA kit (Promega, UK) following the manufacturers' instructions.

Immunohistochemistry: Rats were sacrificed as described above. Whole brains were fixed in 4% PFA for 3 days at 4°C. Coronal striata containing sections from the eGFP experiment or nigra containing sections from the IGF-1 experiment were sliced on a vibrating microtome (Campden Instruments, UK) at 50 µm thickness and kept in ice-cold 1X PBS. Sections were subsequently incubated in 1% BSA blocking buffer (Sigma Aldrich, UK) for an hour. Striata containing sections were incubated with primary antibody, either mouse anti-NeuN (1:500, Millipore, UK), rabbit anti-GFAP (1:500, DAKO, UK) or rabbit anti-Iba1 (1:500, WAKO, UK) whilst SN containing sections were incubated with rabbit anti-TH (1:1000, Millipore, UK) overnight at 4°C, followed by three washes in 1X PBS. Sections were then incubated with goat anti-mouse or goat anti-rabbit AlexaFluor555 antibody (1:500, Invitrogen, UK) as

appropriate for 1 hour, in the dark. After three washes in 1X PBS, sections were incubated with 1 µg/ml DAPI (Sigma Aldrich, UK) for 15 min and mounted onto SuperFrost slides.

Image capture: Both cultured cells and brain sections were visualized under an inverted fluorescence Axio Observer D1 microscope. Images were taken with an AxioCam combined with AxioVision software. Equipment and software were purchased from Carl Zeiss, UK.

Cell counting: Since cultured cells dispersed unevenly, 9 fields/well were selected manually at the same positions in all wells to avoid bias and for more accurate statistical analysis. An image was captured from each field and the cell numbers were counted manually using ImageJ software (National Institutes of Health, Maryland, US). $DAPI^+$ cell nuclei were scored to obtain the total cell number. The DAPI channel was subsequently merged with another fluorescence channel to score the total number of a specific cell type. Averages of cell numbers from the 9 fields were then used to obtain the total and marker-positive cell numbers within each well. For brain sections containing the SN, overlapping images from each section were captured and stitched automatically with AxioVision software to create a mosaic image of the section. According to the Rat Brain Atlas (Paxinos and Watson, 2007), two hemispheres of the SNpc were identified on each section. Only TH^+ cells within these regions were counted (using ImageJ software), separately between the left and right hemispheres. Cell counts from approximately thirty SNpc-containing serial sections were subsequently averaged to obtain the total TH^+ cell number per section of the SNpc in the corresponding brain hemisphere.

Measurement of eGFP intensity: For cultured cells, 9 fields/well of 24-well plates were selected and each field was individually captured as described above. The intensity of eGFP in each field was scored by AxioVision software. An average of intensity from the 9 fields was then taken and expressed as arbitrary units (a.u.)/μm^2. For brain sections containing the striatum, following image capture the area of the striatum on each section was identified according to the Rat Brain Atlas

(Paxinos and Watson, 2007). The intensity of eGFP within this area was quantified with AxioVision software and expressed as a.u./μm^2. The values from approximately 60 striatal sections from each brain were then averaged to obtain the value for the corresponding brain.

Statistical analysis: Using Prism 5 software (GraphPad, San Diego, California, USA), data were analyzed and shown as the means ± S.E.M, with error bars representing the S.E.M. *In vitro* experiments were done in triplicate. Comparisons of statistical significance were assessed by one- or two-way ANOVA followed by *post-hoc* tests. Significance levels were set at $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Results

Lentiviral transduction efficiency and cell-type specificity in rat primary ventral mesencephalic (VM) cell cultures

We initially investigated transduction efficiency and transcriptional targeting of LVs through *eGFP* expression in rat primary VM cell cultures. IPLV- and IDLV-*eGFP* vectors driven by CMVp, GFAPp, or SYNp promoters were used to transduce the VM neuronal or astroglial cultures at an optimized MOI 10 (**Figure S1**). The number of *eGFP*-expressing cells and eGFP intensity were evaluated 3 days post-vector transduction. The types of cells expressing *eGFP* were studied through fluorescence co-localization with TH (for dopaminergic neurons), NeuN/Tuj1 cells (for total neurons) or GFAP (for total astrocytes).

In cultures containing 60% neurons (including 4% TH^+ neurons) and 13% astrocytes (as characterized in **Figure S2a**), we identified a highly significant reduction in the percentage of cells expressing *eGFP* from LV-GFAPp compared to LV-CMVp and LV-SYNp vectors, regardless of vector integration proficiency (**Figure 1a**); and up to two-fold difference in eGFP intensity between IPLVs and IDLVs (**Figure 1b**). Transduction efficiency of IPLV-SYNp and IPLV-CMVp vectors was comparable, but the percentage of *eGFP*-expressing cells following transduction with IDLV-SYNp vectors was almost double the percentage observed in cells transduced with IDLV-CMVp vectors. Representative images are shown in **Figure 1c**. We also quantitated the percentage of dopaminergic neurons (TH^+) expressing *eGFP*. Despite no significant difference in the total TH^+ cell number between groups (p = 0.06, **Figure 1d**), 60% TH^+ cells were transduced by either IPLV-CMVp or IPLV-SYNp vectors (**Figure 1e**). The expression of *eGFP* was moderate in TH^+ cells transduced with IPLV-CMVp or IPLV-SYNp whilst no *eGFP* expression in TH^+ cells was observed following IPLV-GFAPp vector transduction (**Figure 1f**).

In astroglial cultures containing 36% astrocytes and 1% neurons (as characterized in **Figure S2b**), the cell-type specificity of LV-SYNp vectors was significantly different of that observed with the other vectors in both the total number of cells expressing *eGFP* and eGFP intensity. Significantly higher transduction efficiency of IPLVs compared to IDLVs was observed only in eGFP intensity from CMVp-*eGFP* vectors (**Figures 2a, b**). Representative images are shown in **Figure 2c**. We subsequently quantified specific cell types expressing *eGFP* in the transduced astroglial cultures, with a focus on astrocytes and neurons. Although there was no statistically significant difference in the number of total astrocytes (p = 0.14) or neurons (p = 0.15) between cultures (**Figure S3**), the percentage of *eGFP*-expressing cells from the various promoters used was significantly different (**Figures 2d, e**). *eGFP* was preferentially expressed in astrocytes ($GFAP^+$) following LV-GFAPp vector transduction, whereas LV-SYNp vectors mediated *eGFP* expression in the majority of neurons ($Tuj1^+$). Illustrative images are shown in **Figures 2f, g**.

Hence, our *in vitro* data demonstrated that the cell-type specificity of *eGFP* expression was promoter-type dependent, with expression induced predominantly in neurons, in astrocytes, or in both neurons and astrocytes following transduction with LV-SYNp, LV-GFAPp or LV-CMVp vectors, respectively. The results from quantifying eGFP intensity moreover indicated higher transduction efficiency of IPLVs compared to IDLVs in most cases.

***In vivo* neuronal specificity of LV-SYNp vectors**

In a bid to determine whether the neuronal specificity of LV-SYNp vectors was maintained *in vivo*, in particular in the PD animal model, IPLV- or IDLV-SYNp-*eGFP* vectors were injected into the right striatum of rats, followed by 6-OHDA lesioning two weeks later. Brains were harvested 5 months post vector injection. Successful transduction with widespread *eGFP* expression within the striatum was demonstrated (**Figures 3a, b**). No *eGFP* expression was detected in the non-injected striatal hemispheres (**Figure 3c**); however, there was anterograde presence of eGFP in the SNpr, from the

striatal injection site (**Figure 3d**). Quantification of levels of eGFP intensity showed no significant differences in transduction efficacy between IPLV- and IDLV-SYNp vectors (p = 0.12, **Figure 3e**). Further cell identification by morphology and marker expression confirmed that the majority of neurons were transduced; no significant astrocyte or microglia transduction was noticed (**Figures 3f, g**). These results are consistent with the *in vitro* findings, confirming the neuronal specificity of LV-SYNp vectors. The data in particular demonstrated comparable transduction efficiency of IDLVs with their counterparts in the post-mitotic CNS environment.

IGF-1 protective effects on rat primary cortical neuronal cultures

Following the confirmation of LV transduction efficiency and cell-type specificity (through *eGFP* expression *in vitro* and *in vivo*), we investigated the neuroprotective effect of IGF-1 using trophic factor withdrawal on primary rat cortical neuronal cultures. Since neurons have been reported as the main source of brain IGF-1[34], IDLVs expressing *Igf-1* were driven by SYNp or CMVp to enhance *Igf-1* expression. IDLVs carrying either CMVp-*eGFP*, CMVp-*Gdnf*-IRES-*eGFP*, SFFVp-*eGFP*-CMVp-*Igf-1*, or SFFVp-*eGFP*-SYNp-*Igf-1* were added to cell cultures 24 hours after cell plating. The culture medium was replaced with normal cortical neuronal medium (trophic factor-supported cultures) or with medium from which supplements had been withdrawn (trophic factor-free cultures), 3 days post vector transduction. Cell viability was determined by MTT assay 3 days afterwards and the results are shown in **Figure 4a**. Transduction efficiency was assessed by eGFP fluorescence (**Figure 4b**). Survival of cells in trophic factor-supported cultures was not significantly different between groups (p = 0.08). However, withdrawal of exogenous trophic factors led to the death of 20-30% of total cells in the non-transduced group (mock) or CMVp-*eGFP*-transduced group (negative control). Such reduction was significantly different (p < 0.001) when compared with IDLV-*Igf-1* or -*Gdnf* groups. Indeed, transduction with IDLV-*Gdnf* vectors rescued more than 90% cells in trophic factor-free cultures although a significant difference (p = 0.02) in the survival of Gdnf-treated cells between trophic-supported and trophic factor-free cultures was still observed. LV-*Igf-1* vector transduction provided

complete protection, with similar cell survival regardless of trophic support in the cultures transduced with either CMVp-*Igf-1* (p = 0.77) or SYNp-*Igf-1* (p = 0.75) vectors. Moreover, IDLVs expressing *Igf-1* displayed similar transduction efficiency (p = 0.23) regardless of promoter type (CMVp or SYNp), **Figures 4a-c**.

Using commercially available ELISA kits, we measured the level of IGF-1 **(Figure 4c)** and GDNF **(Figure 4d)** produced in the transduced neuronal cultures. Transgenic production of IGF-1 was efficient and independent of trophic support and vector promoter (4.3 - 5.5 ng/ml at day 3, and 5.8 - 6.0 ng/ml at day 6). GDNF production was similarly efficient at 3 days post-transduction (~5.6 ng/ml), but seemed slightly reduced at day 6 (~4.7 ng/ml) and even lower in the absence of trophic support (~3.7 ng/ml), matching the culture viability as shown by MTT assay (**Figure 4a**).

Our results indicated efficient transgene expression from IDLVs in transduced primary neuronal cultures. A clear neuroprotective effect of IGF-1 on cultured neurons was observed, more potent than that observed with GDNF in parallel experiments, encouraging further testing of *in vivo* IGF-1 neuroprotection.

Lack of IGF-1 protective effects on dopaminergic neurons of 6-OHDA-lesioned rats

We subsequently investigated LV-*Igf-1* transduction in a 6-OHDA rat model of PD. IPLVs or IDLVs expressing *Igf-1* were injected into the right striatum of rats, which then received a partial unilateral lesion with 6-OHDA two weeks after vector transduction. The amount of IGF-1 produced in the striatum was determined by ELISA 6 weeks post-vector administration (**Figure 5a**) showing no significant differences in the levels of IGF-1 in the contralateral hemispheres between groups (p = 0.92). As expected, the levels in LV-*Igf-1*-injected hemispheres were significantly higher than in contralateral hemispheres and mock animals, regardless of vector integration proficiency or promoter type ($p < 0.001$). Differences in efficacy between vectors were noticed, with IPLVs being

more efficient than IDLVs, and SYNp promoter being particularly efficient in the IPLV configuration. IPLVs boosted IGF-1 levels 5 to 7-fold, while IDLVs provided about 2.5-fold increases (**Figures 5a, b**).

We further examined the potential neuroprotective effects of IGF-1 on dopaminergic neurons by quantifying the total number of dopaminergic (TH^+) cells remaining in the SNpc post 6-OHDA lesioning. Following intrastriatal injection of 6-OHDA, a depletion of 50-60% of dopaminergic cell bodies was detected in the ipsilateral hemispheres of mock-treated animals, as expected (**Figure 5c**). The increased levels of IGF-1 did not appear to affect dopaminergic cell survival, as there was no significant difference in the number of TH^+ cells remaining in the ipsilateral hemisphere between *Igf-1*-treated and mock-treated groups ($p = 0.66$, **Figure 5c**). Furthermore, the ratios of remaining TH^+ cell number in the ipsilateral and contralateral hemispheres were not statistically different between groups ($p = 0.69$, **Figure 5d**). Illustrative images are shown in **Figures 5e, f**.

The potential of IGF-1 exerting beneficial behavioral effects in PD rats was also examined. **Figure 6a** shows a weight chart and an experimental schedule of this behavioral experiment, in which amphetamine- or apomorphine-induced rotations were scored. Animals gained weight as expected, with a transient reduction following 6-OHDA lesioning and no statistical differences between groups. Net rotational asymmetry scores induced by amphetamine or apomorphine are summarized in **Figures 6b, c**, respectively. There was no statistical difference between groups in response to either amphetamine ($p = 0.20$) or apomorphine ($p = 0.88$). We further analyzed whether small differences might occur during the tests by comparing the number of rotations between groups at 10-min intervals. Neither amphetamine (**Figures 6d-g**) nor apomorphine (**Figures 6h, i**) mediated any significant difference. These *in vivo* results did not succeed in showing an effect of transgenic IGF-1 on dopaminergic neuron survival or behavioral recovery of 6-OHDA-lesioned rats, despite increased levels of IGF-1.

Discussion

LVs can transduce both dividing and non-dividing CNS cells, with stable long-term expression of the transgene[38]. The development of IDLVs offers significantly reduced risk of insertional mutagenesis while maintaining similar transduction efficiency to the classical IPLVs in a range of cell types[28, 29]. We have already demonstrated neuroprotection mediated by IDLV-*hGDNF* in the 6-OHDA rodent model of PD[30], supporting the feasibility of the use of IDLVs for *Igf-1* delivery in the present study. Our work demonstrates that (1) LV-SYNp vectors provided efficient, long-lived and neuron-specific expression of transgene (through *eGFP* expression), with IDLVs as efficient as IPLVs; (2) Delivery of IDLV-*Igf-1* vectors to primary cortical neuronal cells *in vitro* provided complete neuroprotection; (3) LV-mediated *Igf-1* expression in 6-OHDA-lesioned rats, however, did not improve survival of dopaminergic cells or behavior of treated animals.

For many years PD was considered a pure motor disorder with symptoms attributed to the loss of SNpc dopaminergic neurons and subsequent depletion of striatal dopamine. However, recent studies have indicated that the impairment affects other areas of the brain leading to a variety of non-motor symptoms[39]. One such symptom is cognitive dysfunction, occurring in one-third of PD patients[40]. Whilst the underlying pathology has not been elucidated, impaired pathways connecting the basal ganglia and the frontal cortex, which may subsequently cause a degeneration of cortical neurons, have been suggested to contribute to this decline[39]. IGF-1, with its mitogenic properties exerted through the PI3K/Akt pathway, improved survival of rat cortical neurons[41, 42]. Our *in vitro* data confirm the protective effects of IGF-1 on these neurons, although we obtained effective protection at much lower levels of IGF-1 and by transduction with IDLVs rather than direct administration of IGF-1 protein.

In normal CNS tissue, IGF-1 is secreted by neurons[34], unlike other neurotrophic factors produced by glia, and acts upon nearby IGF-1 receptors in an autocrine manner. The use of neuronal-specific

promoters, such as SYNp, to drive *Igf-1* expression is therefore rational[31]. Indeed, SYNp has been extensively used to target expression of transgenes to neurons *in vitro* and *in vivo*[31, 43]. As shown, IPLV- and IDLV-SYNp-*eGFP* vectors transduced predominantly neurons in both primary cell cultures and PD rat striata. Importantly, the *in vivo* data, consistent with other studies[43, 44], demonstrated that striatal administration of vectors driven by SYNp led to eGFP detection in the SN, possibly due to anterograde axonal trafficking. At present, it is unclear whether the vectors or transgene-induced proteins are transported. However, an involvement of endocytosis likely explains the trans-synaptic transfer from neuron to neuron[45]. This appears as an advantage in PD therapy since a transgene can be administered into the striatum but provide therapeutic effects to both the striatum and the SN. Technically, intrastriatal injection of vectors is simpler and safer than intranigral injection[46] although a possible higher dose (volume) of vectors is required for intrastriatal injection.

The most likely explanation for the failure of transgenic *Igf-1* to mediate *in vivo* neuroprotection in our study is the insufficient levels of IGF-1 achieved. IGF-1 was previously used at 30-150 ng/ml in embryonic mesencephalic neuronal cultures[8, 47], while we obtained ~5 ng/ml IGF-1 by transgenic expression *in vitro*, but still achieved complete neuroprotection. However, *in vivo* neuroprotection in 6-OHDA-lesioned rats has been shown with continuous intracerebroventricular IGF-1 infusion for a week at 100 µg/ml (10 µg in total)[21], which is many orders of magnitude higher than the 15-35 pg/mg IGF-1 obtained in the current study. Ebert *et al.*[48] have previously reported the protective effects of IGF-1 on 6-OHDA-lesioned rats, which were achieved following striatal transplantation of neural progenitor cells expressing LV-*IGF-1*. The authors did not show at which levels IGF-1 provided such protection, and it could not be ruled out that other factors provided by the progenitor cells were involved. Indeed, in some systems progenitor cells can improve both cell survival and animal behavior in PD models against both 6-OHDA and MPTP toxicity[49, 50]. It is also noteworthy that to obtain similar protection on dopaminergic cell survival, previous investigators have used purified IGF-1 at concentrations about 30 times higher than that of GDNF[8, 51]. This is probably necessary due to the half-life of IGF-1 being less than 10 min whereas GDNF can last for 6-8 days[52, 53]. Since the doses

of *Igf-1* expressing vectors used in this study are similar to those used previously for delivery of other neurotrophic factor (i.e., GDNF) with demonstrated therapeutic benefits[30], insufficient *in vivo* expression of *Igf-1* as presented here is an obvious challenge for the application of IGF-1 gene therapy in PD. A vector dosing study should be carried out to determine the doses of *Igf-1*-expressing vectors that can mediate neuroprotection. The use of more concentrated vector stocks, stronger transcription regulation sequences, codon-optimization of the transgene and stabilization of the transgenic IGF-1 protein should be explored to develop an *Igf-1* vector of demonstrated pre-clinical efficacy.

In summary, our studies provide evidence for efficient transgenic delivery by IDLVs in PD and support their use in the CNS as a safer delivery system. Our data, moreover, confirm neuroprotection by IGF-1 on cultured cortical neurons, resulting from efficient IDLV-*Igf-1* delivery. However, IGF-1-mediated protection through transgenic *Igf-1* expression was unsuccessful in 6-OHDA-lesioned rats. Further studies are required to develop an effective *Igf-1* delivery system and to ascertain the benefit of IGF-1 gene therapy for PD treatment.

Acknowledgements

The authors thank the EU FP7 program (project NEUGENE: grant agreement n. 222925), Royal Holloway, University of London and University of Medicine and Pharmacy at Ho Chi Minh city, Vietnam for financial support. Author contributions: R.J.Y.-M. conceived and directed the project; R.J.Y.-M. and M.B. designed the experiments; N.-B.L.-N. and M.B. performed all experiments; N.-B.L.-N., M.B. and R.J.Y.-M. analyzed the data; N.-B.L.-N., M.B. and R.J.Y.-M. wrote the manuscript.

Author Disclosure Statement

No competing financial interests exist.

References

1. Chen, Q, He, Y, and Yang, K (2005). Gene therapy for Parkinson's disease: progress and challenges. *Current gene therapy* **5**: 71-80.
2. Gill, SS, Patel, NK, Hotton, GR, O'Sullivan, K, McCarter, R, Bunnage, M, *et al.* (2003). Direct brain infusion of glial cell line-derived neurotrophic factor in Parkinson disease. *Nature medicine* **9**: 589-595.
3. Marks, WJ, Jr., Ostrem, JL, Verhagen, L, Starr, PA, Larson, PS, Bakay, RA, *et al.* (2008). Safety and tolerability of intraputaminal delivery of CERE-120 (adeno-associated virus serotype 2-neurturin) to patients with idiopathic Parkinson's disease: an open-label, phase I trial. *Lancet Neurol* **7**: 400-408.
4. Sun, M, Kong, L, Wang, X, Lu, XG, Gao, Q, and Geller, AI (2005). Comparison of the capability of GDNF, BDNF, or both, to protect nigrostriatal neurons in a rat model of Parkinson's disease. *Brain Res* **1052**: 119-129.
5. Lindholm, P, Voutilainen, MH, Lauren, J, Peranen, J, Leppanen, VM, Andressoo, JO, *et al.* (2007). Novel neurotrophic factor CDNF protects and rescues midbrain dopamine neurons in vivo. *Nature* **448**: 73-77.
6. Voutilainen, MH, Back, S, Porsti, E, Toppinen, L, Lindgren, L, Lindholm, P, *et al.* (2009). Mesencephalic astrocyte-derived neurotrophic factor is neurorestorative in rat model of Parkinson's disease. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **29**: 9651-9659.
7. Lang, AE, Gill, S, Patel, NK, Lozano, A, Nutt, JG, Penn, R, *et al.* (2006). Randomized controlled trial of intraputamenal glial cell line-derived neurotrophic factor infusion in Parkinson disease. *Annals of neurology* **59**: 459-466.
8. Knusel, B, Michel, PP, Schwaber, JS, and Hefti, F (1990). Selective and nonselective stimulation of central cholinergic and dopaminergic development in vitro by nerve growth factor, basic fibroblast growth factor, epidermal growth factor, insulin and the insulin-like growth factors I and II. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **10**: 558-570.
9. Duan, C, and Xu, Q (2005). Roles of insulin-like growth factor (IGF) binding proteins in regulating IGF actions. *Gen Comp Endocrinol* **142**: 44-52.
10. Fernandez, S, Fernandez, AM, Lopez-Lopez, C, and Torres-Aleman, I (2007). Emerging roles of insulin-like growth factor-I in the adult brain. *Growth Horm IGF Res* **17**: 89-95.
11. Russo, VC, Gluckman, PD, Feldman, EL, and Werther, GA (2005). The insulin-like growth factor system and its pleiotropic functions in brain. *Endocr Rev* **26**: 916-943.
12. Fernandez, AM, and Torres-Aleman, I (2012). The many faces of insulin-like peptide signalling in the brain. *Nature reviews Neuroscience* **13**: 225-239.
13. Avila-Gomez, IC, Velez-Pardo, C, and Jimenez-Del-Rio, M (2010). Effects of insulin-like growth factor-1 on rotenone-induced apoptosis in human lymphocyte cells. *Basic Clin Pharmacol Toxicol* **106**: 53-61.
14. Sun, X, Huang, L, Zhang, M, Sun, S, and Wu, Y (2010). Insulin like growth factor-1 prevents 1-mentyl-4-phenylphyridinium-induced apoptosis in PC12 cells through activation of glycogen synthase kinase-3beta. *Toxicology* **271**: 5-12.

15. Wang, L, Yang, HJ, Xia, YY, and Feng, ZW (2010). Insulin-like growth factor 1 protects human neuroblastoma cells SH-EP1 against MPP+-induced apoptosis by AKT/GSK-3beta/JNK signaling. *Apoptosis* **15**: 1470-1479.
16. Landi, F, Capoluongo, E, Russo, A, Onder, G, Cesari, M, Lulli, P, *et al.* (2007). Free insulin-like growth factor-I and cognitive function in older persons living in community. *Growth Horm IGF Res* **17**: 58-66.
17. Tong, M, Dong, M, and de la Monte, SM (2009). Brain insulin-like growth factor and neurotrophin resistance in Parkinson's disease and dementia with Lewy bodies: potential role of manganese neurotoxicity. *J Alzheimers Dis* **16**: 585-599.
18. Guan, J, Krishnamurthi, R, Waldvogel, HJ, Faull, RL, Clark, R, and Gluckman, P (2000). N-terminal tripeptide of IGF-1 (GPE) prevents the loss of TH positive neurons after 6-OHDA induced nigral lesion in rats. *Brain Res* **859**: 286-292.
19. Offen, D, Shtaif, B, Hadad, D, Weizman, A, Melamed, E, and Gil-Ad, I (2001). Protective effect of insulin-like-growth-factor-1 against dopamine-induced neurotoxicity in human and rodent neuronal cultures: possible implications for Parkinson's disease. *Neurosci Lett* **316**: 129-132.
20. Quesada, A, Lee, BY, and Micevych, PE (2008). PI3 kinase/Akt activation mediates estrogen and IGF-1 nigral DA neuronal neuroprotection against a unilateral rat model of Parkinson's disease. *Dev Neurobiol* **68**: 632-644.
21. Quesada, A, and Micevych, PE (2004). Estrogen interacts with the IGF-1 system to protect nigrostriatal dopamine and maintain motoric behavior after 6-hydroxdopamine lesions. *Journal of neuroscience research* **75**: 107-116.
22. Salvatore, MF, Ai, Y, Fischer, B, Zhang, AM, Grondin, RC, Zhang, Z, *et al.* (2006). Point source concentration of GDNF may explain failure of phase II clinical trial. *Experimental neurology* **202**: 497-505.
23. Dowd, E, Monville, C, Torres, EM, Wong, LF, Azzouz, M, Mazarakis, ND, *et al.* (2005). Lentivector-mediated delivery of GDNF protects complex motor functions relevant to human Parkinsonism in a rat lesion model. *The European journal of neuroscience* **22**: 2587-2595.
24. Emborg, ME, Moirano, J, Raschke, J, Bondarenko, V, Zufferey, R, Peng, S, *et al.* (2009). Response of aged parkinsonian monkeys to in vivo gene transfer of GDNF. *Neurobiology of disease* **36**: 303-311.
25. Georgievska, B, Kirik, D, and Bjorklund, A (2004). Overexpression of glial cell line-derived neurotrophic factor using a lentiviral vector induces time- and dose-dependent downregulation of tyrosine hydroxylase in the intact nigrostriatal dopamine system. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **24**: 6437-6445.
26. Kordower, JH, Emborg, ME, Bloch, J, Ma, SY, Chu, Y, Leventhal, L, *et al.* (2000). Neurodegeneration prevented by lentiviral vector delivery of GDNF in primate models of Parkinson's disease. *Science* **290**: 767-773.
27. Palfi, S, Leventhal, L, Chu, Y, Ma, SY, Emborg, M, Bakay, R, *et al.* (2002). Lentivirally delivered glial cell line-derived neurotrophic factor increases the number of striatal dopaminergic neurons in primate models of nigrostriatal degeneration. *The*

Journal of neuroscience : the official journal of the Society for Neuroscience **22**: 4942-4954.
28. Yáñez-Muñoz, RJ, Balaggan, KS, MacNeil, A, Howe, SJ, Schmidt, M, Smith, AJ, *et al.* (2006). Effective gene therapy with nonintegrating lentiviral vectors. *Nat Med* **12**: 348-353.
29. Wanisch, K, and Yáñez-Muñoz, RJ (2009). Integration-deficient lentiviral vectors: a slow coming of age. *Mol Ther* **17**: 1316-1332.
30. Lu-Nguyen, NB, Broadstock, M, Schliesser, MG, Bartholomae, CC, von Kalle, C, Schmidt, M, *et al.* (2014). Transgenic Expression of Human Glial Cell Line-Derived Neurotrophic Factor from Integration-Deficient Lentiviral Vectors is Neuroprotective in a Rodent Model of Parkinson's Disease. *Hum Gene Ther* **25**: 631-641.
31. Hioki, H, Kameda, H, Nakamura, H, Okunomiya, T, Ohira, K, Nakamura, K, *et al.* (2007). Efficient gene transduction of neurons by lentivirus with enhanced neuron-specific promoters. *Gene therapy* **14**: 872-882.
32. Jakobsson, J, Ericson, C, Jansson, M, Bjork, E, and Lundberg, C (2003). Targeted transgene expression in rat brain using lentiviral vectors. *J Neurosci Res* **73**: 876-885.
33. Li, M, Husic, N, Lin, Y, Christensen, H, Malik, I, McIver, S, *et al.* (2010). Optimal promoter usage for lentiviral vector-mediated transduction of cultured central nervous system cells. *Journal of neuroscience methods* **189**: 56-64.
34. Mashayekhi, F, Mirzajani, E, Naji, M, and Azari, M (2010). Expression of insulin-like growth factor-1 and insulin-like growth factor binding proteins in the serum and cerebrospinal fluid of patients with Parkinson's disease. *J Clin Neurosci* **17**: 623-627.
35. Pruszak, J, Just, L, Isacson, O, and Nikkhah, G (2009). Isolation and culture of ventral mesencephalic precursor cells and dopaminergic neurons from rodent brains. *Curr Protoc Stem Cell Biol* **Chapter 2**: Unit 2D 5.
36. Takeshima, T, Shimoda, K, Sauve, Y, and Commissiong, JW (1994). Astrocyte-dependent and -independent phases of the development and survival of rat embryonic day 14 mesencephalic, dopaminergic neurons in culture. *Neuroscience* **60**: 809-823.
37. Boulos, S, Meloni, BP, Arthur, PG, Bojarski, C, and Knuckey, NW (2006). Assessment of CMV, RSV and SYN1 promoters and the woodchuck post-transcriptional regulatory element in adenovirus vectors for transgene expression in cortical neuronal cultures. *Brain Res* **1102**: 27-38.
38. Bartus, RT, Herzog, CD, Bishop, K, Ostrove, JM, Tuszynski, M, Kordower, JH, *et al.* (2007). Issues regarding gene therapy products for Parkinson's disease: the development of CERE-120 (AAV-NTN) as one reference point. *Parkinsonism Relat Disord* **13 Suppl 3**: S469-477.
39. Chaudhuri, KR, Healy, DG, and Schapira, AH (2006). Non-motor symptoms of Parkinson's disease: diagnosis and management. *Lancet Neurol* **5**: 235-245.
40. Lindgren, HS, and Dunnett, SB (2012). Cognitive dysfunction and depression in Parkinson's disease: what can be learned from rodent models? *The European journal of neuroscience* **35**: 1894-1907.
41. Hodge, RD, D'Ercole, AJ, and O'Kusky, JR (2007). Insulin-like growth factor-I (IGF-I) inhibits neuronal apoptosis in the developing cerebral cortex in vivo. *Int J Dev Neurosci* **25**: 233-241.

42. Mairet-Coello, G, Tury, A, and DiCicco-Bloom, E (2009). Insulin-like growth factor-1 promotes G(1)/S cell cycle progression through bidirectional regulation of cyclins and cyclin-dependent kinase inhibitors via the phosphatidylinositol 3-kinase/Akt pathway in developing rat cerebral cortex. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **29**: 775-788.
43. Drinkut, A, Tereshchenko, Y, Schulz, JB, Bahr, M, and Kugler, S (2012). Efficient gene therapy for Parkinson's disease using astrocytes as hosts for localized neurotrophic factor delivery. *Molecular therapy : the journal of the American Society of Gene Therapy* **20**: 534-543.
44. Richardson, RM, Kells, AP, Rosenbluth, KH, Salegio, EA, Fiandaca, MS, Larson, PS, *et al.* (2011). Interventional MRI-guided putaminal delivery of AAV2-GDNF for a planned clinical trial in Parkinson's disease. *Molecular therapy : the journal of the American Society of Gene Therapy* **19**: 1048-1057.
45. Desplats, P, Lee, HJ, Bae, EJ, Patrick, C, Rockenstein, E, Crews, L, *et al.* (2009). Inclusion formation and neuronal cell death through neuron-to-neuron transmission of alpha-synuclein. *Proc Natl Acad Sci U S A* **106**: 13010-13015.
46. Manfredsson, FP, Tumer, N, Erdos, B, Landa, T, Broxson, CS, Sullivan, LF, *et al.* (2009). Nigrostriatal rAAV-mediated GDNF overexpression induces robust weight loss in a rat model of age-related obesity. *Molecular therapy : the journal of the American Society of Gene Therapy* **17**: 980-991.
47. Zawada, WM, Kirschman, DL, Cohen, JJ, Heidenreich, KA, and Freed, CR (1996). Growth factors rescue embryonic dopamine neurons from programmed cell death. *Experimental neurology* **140**: 60-67.
48. Ebert, AD, Beres, AJ, Barber, AE, and Svendsen, CN (2008). Human neural progenitor cells over-expressing IGF-1 protect dopamine neurons and restore function in a rat model of Parkinson's disease. *Experimental neurology* **209**: 213-223.
49. Redmond, DE, Jr., Bjugstad, KB, Teng, YD, Ourednik, V, Ourednik, J, Wakeman, DR, *et al.* (2007). Behavioral improvement in a primate Parkinson's model is associated with multiple homeostatic effects of human neural stem cells. *Proceedings of the National Academy of Sciences of the United States of America* **104**: 12175-12180.
50. Sadan, O, Shemesh, N, Cohen, Y, Melamed, E, and Offen, D (2009). Adult neurotrophic factor-secreting stem cells: a potential novel therapy for neurodegenerative diseases. *Isr Med Assoc J* **11**: 201-204.
51. Lin, LF, Doherty, DH, Lile, JD, Bektesh, S, and Collins, F (1993). GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science* **260**: 1130-1132.
52. Cohick, WS, and Clemmons, DR (1993). The insulin-like growth factors. *Annual review of physiology* **55**: 131-153.
53. Granholm, AC, Reyland, M, Albeck, D, Sanders, L, Gerhardt, G, Hoernig, G, *et al.* (2000). Glial cell line-derived neurotrophic factor is essential for postnatal survival of midbrain dopamine neurons. *J Neurosci* **20**: 3182-3190.

Figure legends

Figure 1: LV-mediated *eGFP* expression in VM neuronal cultures. Rat primary VM neuronal cultures were transduced with IPLV- or IDLV-*eGFP* vectors at MOI 10 (eGFP transducing units), one day after cell seeding. **(a)** The percentage of total cells expressing *eGFP* and **(b)** intensity of eGFP fluorescence were evaluated 3 days post-vector transduction. **(c)** Representative images are shown; scale bars = 50 µm; nuclei were stained blue with DAPI. In cultures transduced with IPLVs, **(d)** the total TH^+ cell number per well and **(e)** percentage of TH^+ cells expressing *eGFP* were additionally quantified. Total TH^+ cell number was not significantly different between groups; however, efficient *eGFP* expression by CMVp and SYNp, and not GFAPp, was observed. **(f)** Representative images illustrate fluorescence co-localization of TH^+ and $eGFP^+$ cells. Nuclei were stained blue with DAPI; TH^+ cells were stained red; arrows indicate $TH^+/eGFP^+$ cells; scale bars = 20 µm. Statistical analysis was by two-way ANOVA (in a, b) or one-way ANOVA (in d, e) followed by Bonferroni's *post-hoc* test. Error bars represent the S.E.M; $n = 3$; $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

Figure 2: LV-mediated *eGFP* expression in VM astroglial cultures. Cell cultures were transduced with IPLVs (*n* = 3) or IDLVs (*n* = 3) at MOI 10 (eGFP transducing units) 3 days after plating the cells. **(a)** Percentage of cells expressing *eGFP* and **(b)** intensity of eGFP fluorescence were evaluated 3 days post transduction. Significant differences were observed in cell-type specificity between SYNp and other promoters whereas the difference between IPLVs and IDLVs was only significant in eGFP intensity expressed from CMVp-*eGFP* vectors. **(c)** Representative images are shown; scale bars = 50 µm; nuclei were stained blue with DAPI. **(d)** Astrocytes or **(e)** neurons expressing *eGFP* were quantified as percentages of total astrocytes or neurons, as appropriate, showing significantly different cell-type specificity of the vectors, with LV-GFAPp predominantly targeting astrocytes and LV-SYNp predominantly targeting neurons. **(f, g)** Representative images from cultures transduced with IPLVs (as an example) illustrating the types of cells transduced. **(f)** Astrocytes were identified by $GFAP^+$ staining, whereas **(g)** neurons were identified through $Tuj1^+$ staining. Nuclei were stained blue with DAPI; $GFAP^+$ or $Tuj1^+$ cells were stained red; scale bars are 50 µm. Data were analyzed by one- or two-way ANOVA followed by Bonferroni's *post-hoc* test. Error bars represent the S.E.M; $*p<0.05$, $**p<0.01$, $***p<0.001$.

Figure 3. Neuronal specificity of LV-SYNp vectors in 6-OHDA-treated rat striata. The right striata were injected with IPLV- or IDLV-SYNp-*eGFP* vectors 2 weeks before unilateral 6-OHDA lesioning at the same positions as for vector administration. Animal brains were harvested 5 months later. Representative serial striatal sections from **(a)** IPLV- and **(b)** IDLV-*eGFP*-injected animals show both vector injection site and area of vector spread. **(c)** No *eGFP* expression in the non-injected left striatal hemisphere but **(d)** anterograde eGFP presence in the corresponding SNpr (co-stained in red for TH^+ cells) was detected. **(e)** An evaluation of eGFP levels indicated no significant difference in vector transduction efficiency between IPLVs and IDLVs. **(f, g)** Representative high-magnification images from IPLV- and IDLV-*eGFP*-injected striata, respectively, are shown. Cell-type identification by morphology and marker expression demonstrated the majority of $eGFP^+$ cells were neurons ($NeuN^+$); no significant astrocyte ($GFAP^+$) or microglia ($Iba1^+$) transduction was observed. Nuclei were stained blue with DAPI; scale bar: (a-c) 2000 μm; (d) 500 μm; (f, g) 100 μm (insets, 20 μm). Data in (e) were analyzed for statistical significance by two-tailed Student's t-test; error bars represent the S.E.M.; *n* = 3 per group.

Figure 4. IGF-1 protection on rat primary embryonic cortical neurons. Rat primary cortical neuronal cultures were transduced with IDLVs expressing *Igf-1, Gdnf* (positive control), or *eGFP* (negative control) one day after cell plating. Trophic factor withdrawal was applied 3 days post-transduction and cells were cultured for an additional 3 days. **(a)** Results from an MTT assay indicated cell survival in trophic-supported cultures was not significantly different between groups, whereas in trophic-free cultures, IDLV-*Igf-1* or IDLV-*Gdnf* displayed enhanced survival. Data are shown as percentages compared to the mock (non-transduced group) in trophic-support cultures. Statistical analysis by one-way ANOVA and Dunnett's post-hoc test; error bars represent the S.E.M; *n* = 3. **(b)** Representative images illustrate efficient transduction of the cortical cell cultures through *eGFP* expression; scale bar = 50 µm. **(c, d)** ELISA results presenting levels of **(c)** IGF-1 and **(d)** GDNF produced in transduced cortical neuronal cultures at days 3 and 6 following IDLV transduction. Samples were run in duplicate with *n* = 2. Black bars: trophic-support groups, white bars: trophic-free groups.

Figure 5: *Igf-1* expression in rat striata does not mediate protective effects on SNpc dopaminergic neurons. CMVp-*Igf-1* and SYNp-*Igf-1* vectors, either IPLVs or IDLVs, were injected into the right striatum of rats 2 weeks prior to 6-OHDA lesioning; mock-treated animals received 0.9% saline. **(a)** Quantification of IGF-1 production showing no significant difference in contralateral hemispheres between groups; in contrast, significant vector-mediated IGF-1 production was observed in the ipsilateral hemispheres. **(b)** Ratios of IGF-1 levels between ipsilateral and contralateral hemispheres. Data were statistically analyzed by one-way ANOVA followed by Bonferroni's *post-hoc* test; error bars represent the S.E.M; *n* = 5 per group. **(c)** Total TH^+ cell numbers (per brain section) in the SNpc were counted 6 weeks after vector transduction. **(d)** Ratio of remaining SNpc TH^+ cells between ipsilateral and contralateral hemispheres. The lesion caused a reduction to about 40-50% in all groups, with no significant difference in TH^+ cell survival observed between *Igf-1*-treated groups and mock. Statistical analysis by one-way ANOVA and Bonferroni's *post-hoc* test; error bars represent the S.E.M; *n* = 5 per group. **(e, f)** Representative images of SN sections demonstrate the depletion of TH+ cells in the ipsilateral hemisphere (right) compared to the contralateral hemisphere (left), with no difference between **(e)** mock and **(f)** *Igf-1*-treated groups; scale bar = 500 µm.

Figure 6: Drug-induced rotational tests. (a) Experimental design and weight of animals are shown. Animals had normal weight gain, with no statistical difference between groups, except for a reduction on the week after 6-OHDA lesioning. **(b, c)** Summaries of net rotational asymmetry induced by **(b)** amphetamine or **(c)** apomorphine. Following 30 min acclimation (baseline), rats were injected with the corresponding drug. Net 360º rotations were scored for 90 min. For amphetamine, ipsilateral turns were given positive values and contralateral turns were counted as negative values; for apomorphine, contralateral turns were scored as positive and ipsilateral as negative. No significant behavioral changes between animal groups were detected in either test. **(d-i)** The number of rotations per 10 minute interval was scored. **(d-g)** Amphetamine-induced rotational tests at weeks 4, 5, 6, 7, respectively; **(h, i)** apomorphine-induced rotational tests at weeks 4 and 7. No significant difference between groups was observed. Statistical analysis by two-way ANOVA and Bonferroni's *post-hoc* test; error bars represent the S.E.M; $n = 5$ per group.

Figure 1

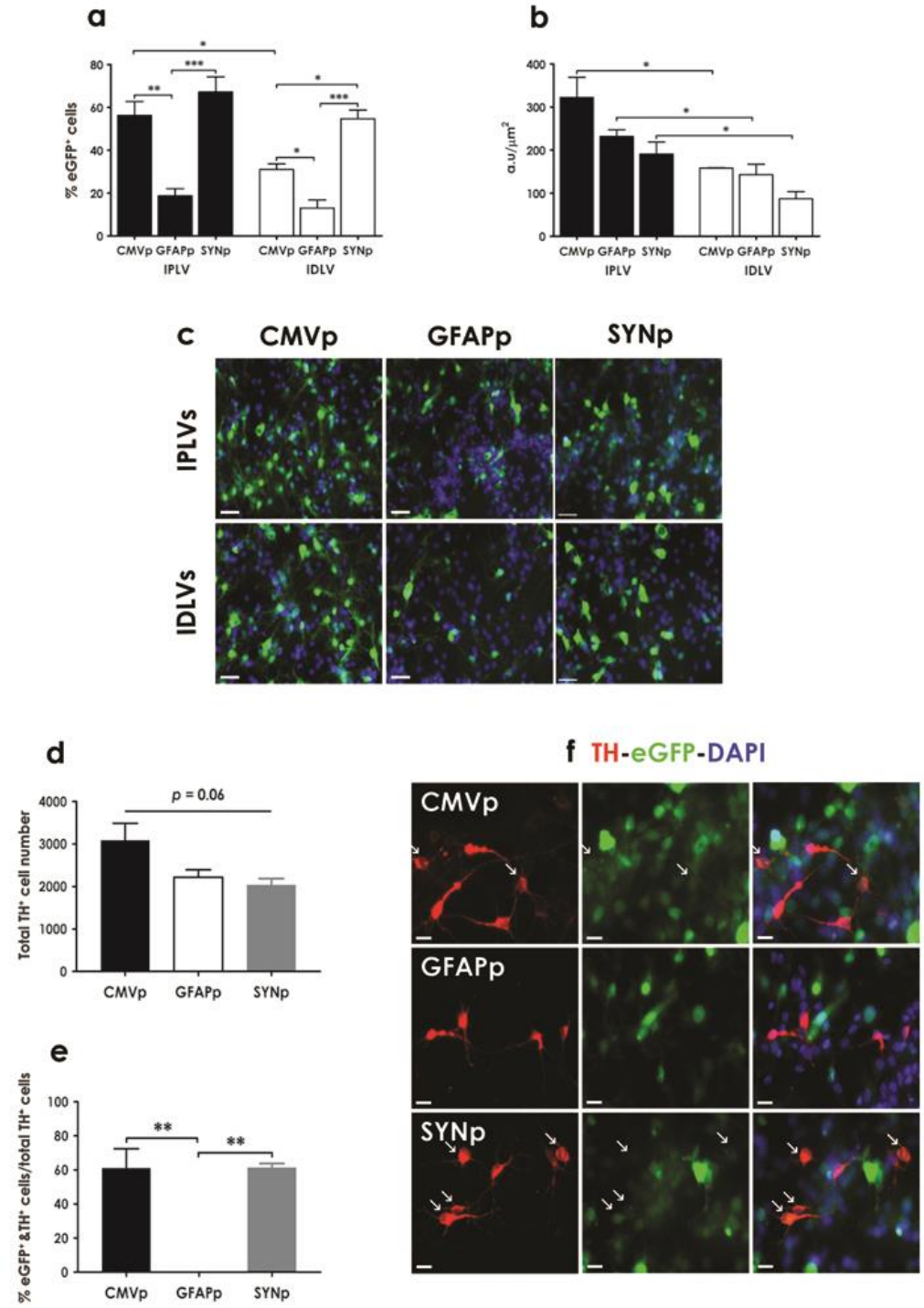

a
% eGFP+ cells
CMVp GFAPp SYNp CMVp GFAPp SYNp
IPLV
IDLV
b
a.u/μm2
CMVp GFAPp SYNp CMVp GFAPp SYNp
IPLV
IDLV
c
CMVp
GFAPp
SYNp
IPLVs
IDLVs
d
p = 0.06
Total TH+ cell number
CMVp GFAPp SYNp
e
% eGFP+ &TH+ cells/total TH+ cells
CMVp GFAPp SYNp
f TH-eGFP-DAPI
CMVp
GFAPp
SYNp

Figure 2

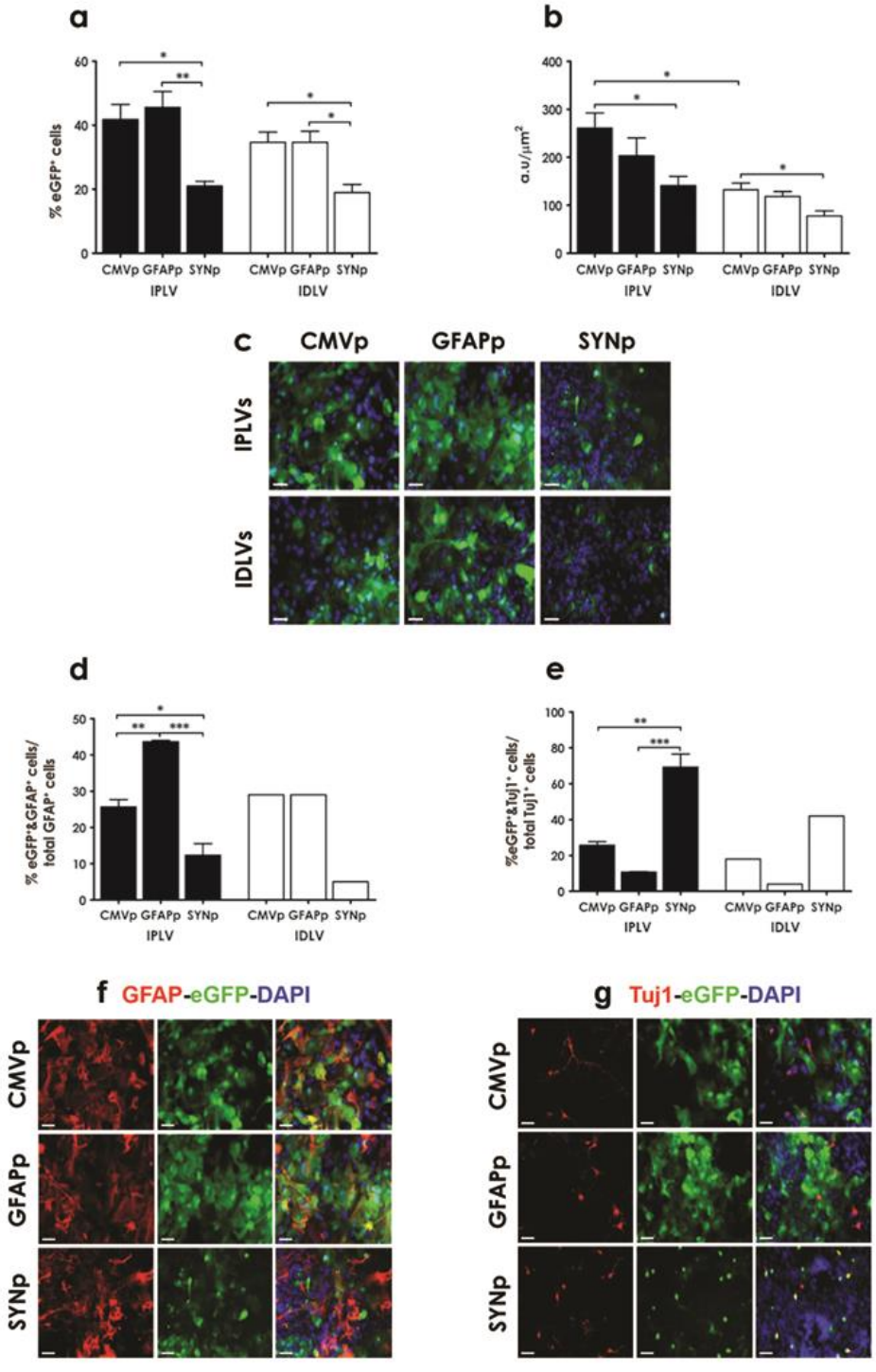
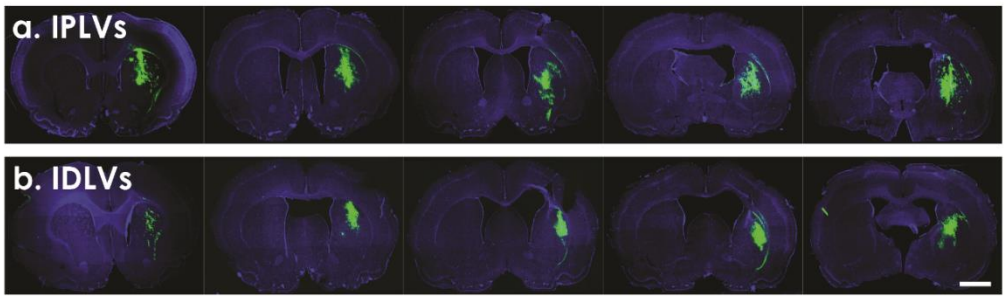
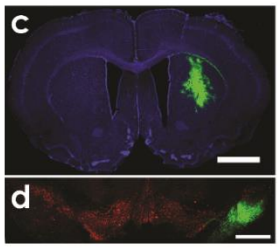
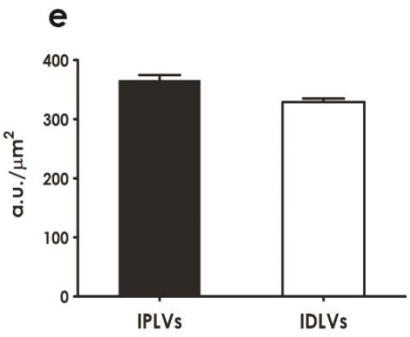

a
% eGFP+ cells
CMVp GFAPp SYNp
IPLV
CMVp GFAPp SYNp
IDLV
b
a.u/μm2
c
CMVp
GFAPp
SYNp
IPLVs
IDLVs
d
% eGFP+&GFAP+ cells/ total GFAP+ cells
e
%eGFP+&Tuj1+ cells/ total Tuj1+ cells
f GFAP-eGFP-DAPI
g Tuj1-eGFP-DAPI
CMVp
GFAPp
SYNp

Figure 3







f. IPLV transduction

g. IDLV transduction

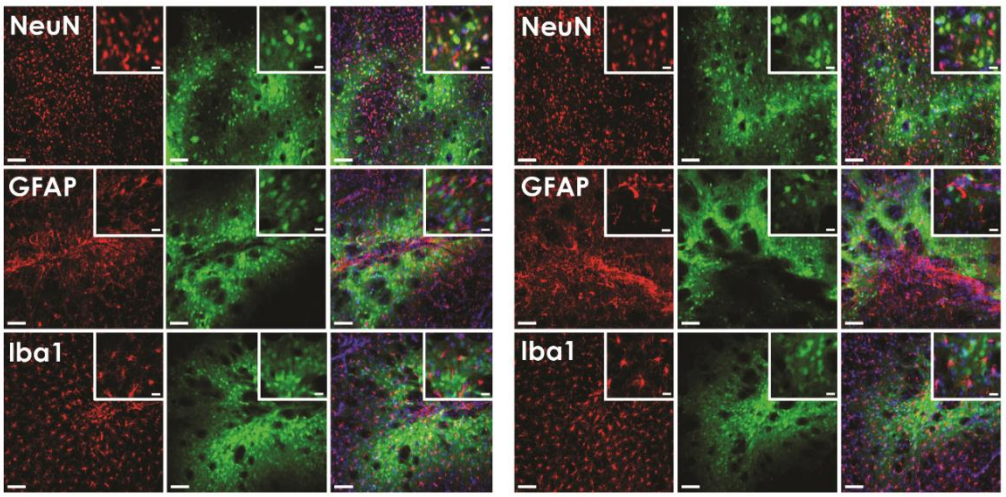


Figure 4

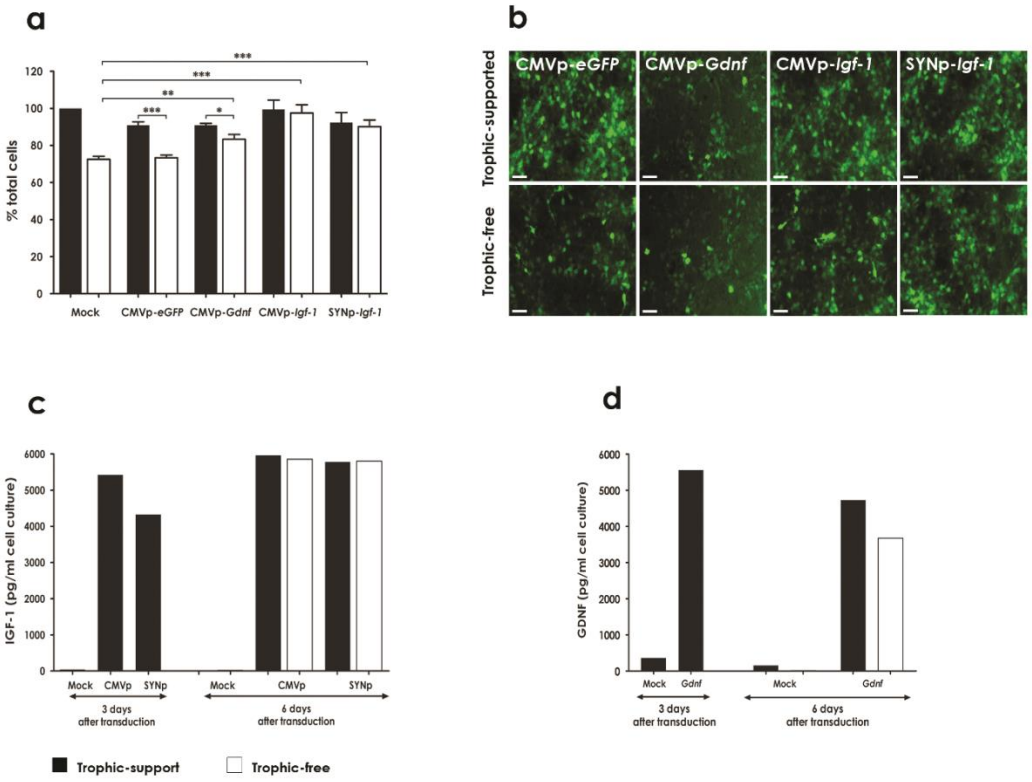

a
% total cells
Mock
CMVp-eGFP
CMVp-Gdnf
CMVp-Igf-1
SYNp-Igf-1
b
Trophic-supported
Trophic-free
CMVp-eGFP
CMVp-Gdnf
CMVp-Igf-1
SYNp-Igf-1
c
IGF-1 (pg/ml cell culture)
Mock
CMVp
SYNp
3 days after transduction
6 days after transduction
Trophic-support
Trophic-free
d
GDNF (pg/ml cell culture)
Mock
Gdnf
3 days after transduction
6 days after transduction

Figure 5

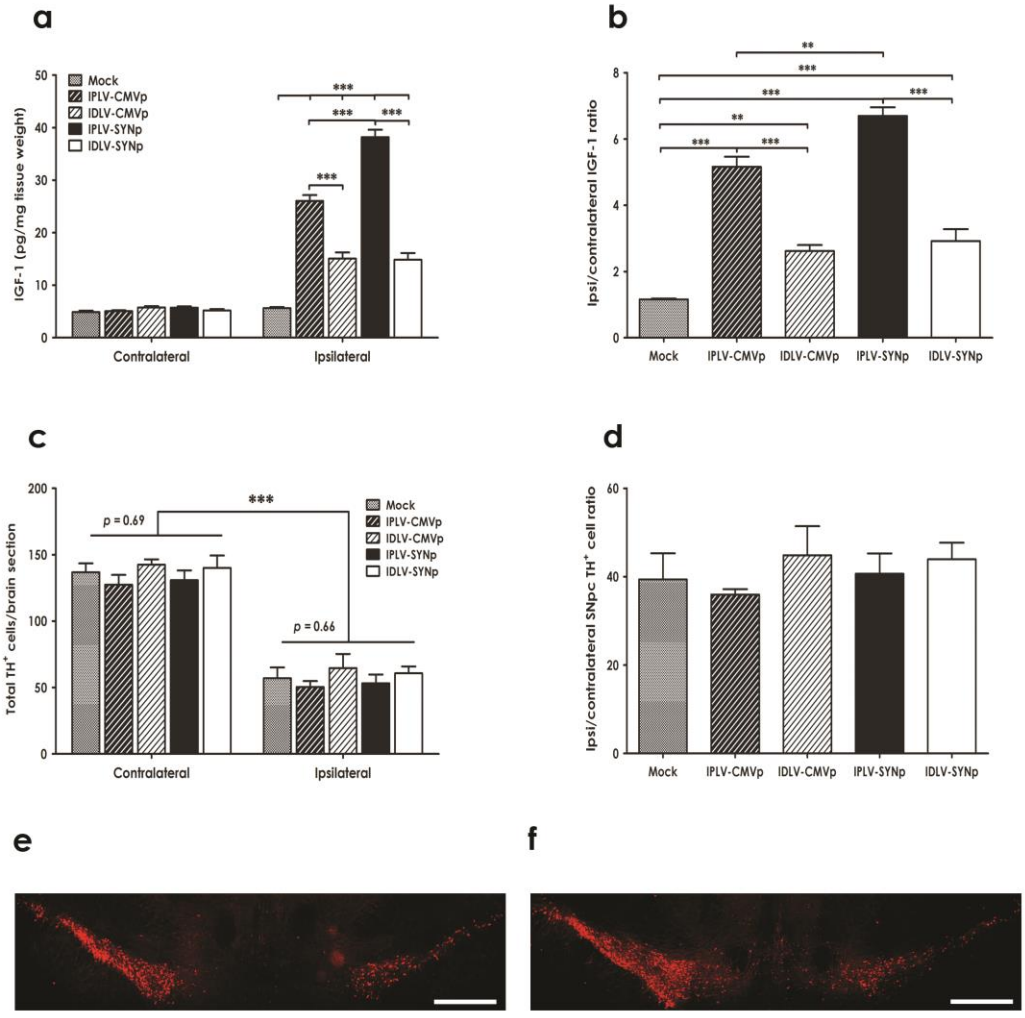
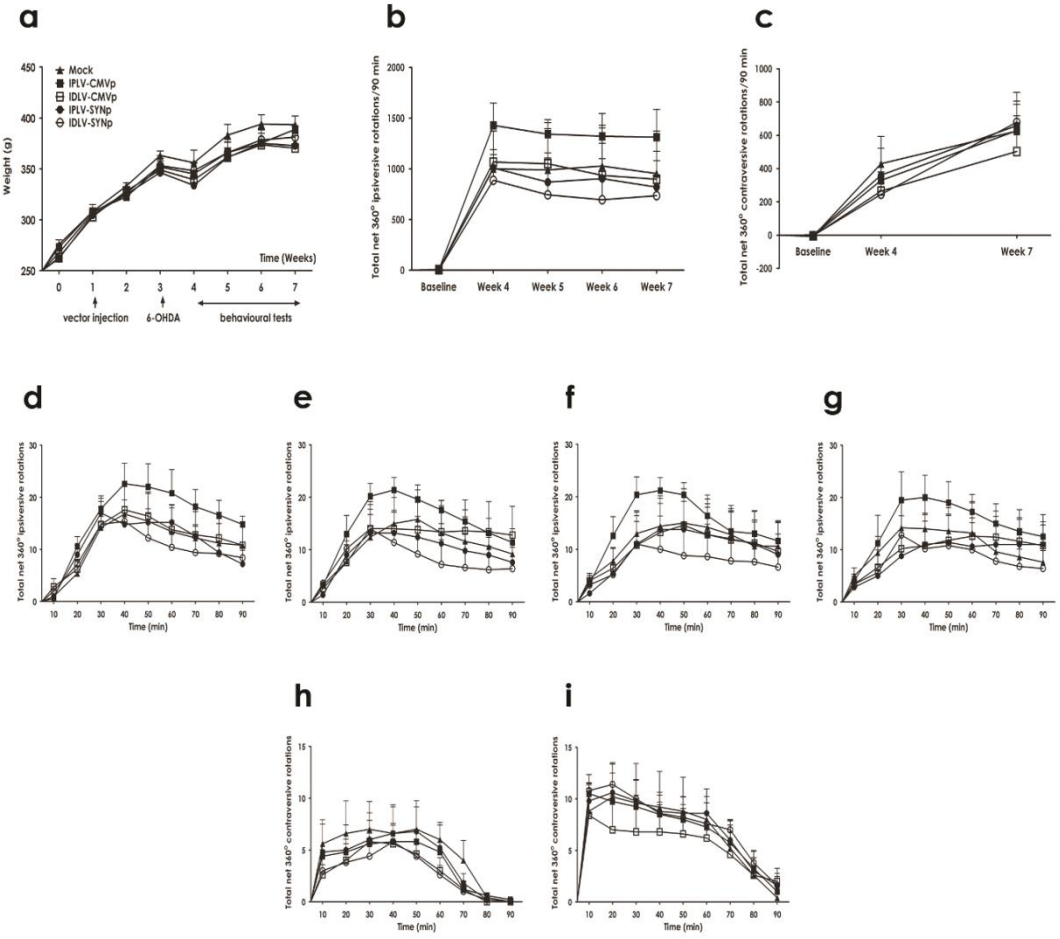
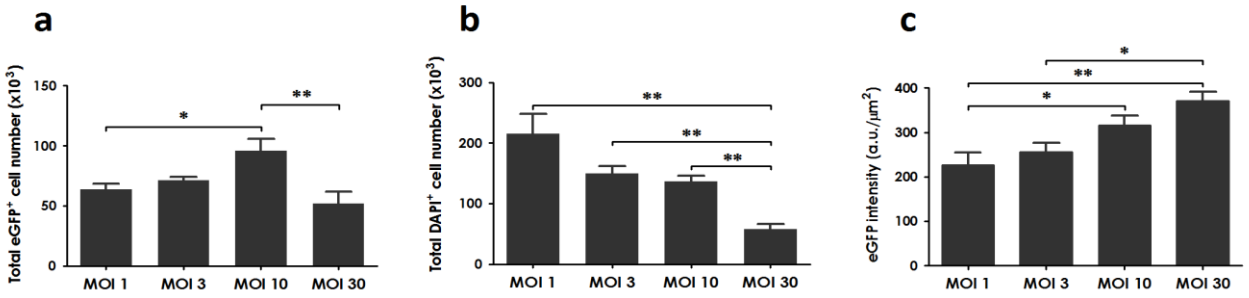

a
IGF-1 (pg/mg tissue weight)
Mock
IPLV-CMVp
IDLV-CMVp
IPLV-SYNp
IDLV-SYNp
Contralateral
Ipsilateral
b
Ipsi/contralateral IGF-1 ratio
Mock
IPLV-CMVp
IDLV-CMVp
IPLV-SYNp
IDLV-SYNp
c
Total TH+ cells/brain section
p = 0.69
p = 0.66
Contralateral
Ipsilateral
d
Ipsi/contralateral SNpc TH+ cell ratio
e
f

Figure 6


a
Mock
IPLV-CMVp
IDLV-CMVp
IPLV-SYNp
IDLV-SYNp
Weight (g)
Time (Weeks)
vector injection
6-OHDA
behavioural tests
b
Total net 360° ipsiversive rotations/90 min
Baseline
Week 4
Week 5
Week 6
Week 7
c
Total net 360° contraversive rotations/90 min
Baseline
Week 4
Week 7
d
e
f
g
Total net 360° ipsiversive rotations
Time (min)
h
i
Total net 360° contraversive rotations
Time (min)

Supplementary materials

Figure S1: Optimization of LV transduction. E14 rat primary ventral mesencephalic (VM) neuronal cultures were transduced with IPLV-CMVp-*eGFP* vectors at MOI 1, 3, 10 or 30 (eGFP transducing units/ml) one day after cell seeding. **(a)** The number of eGFP$^+$ cells, **(b)** the number of total cells (DAPI$^+$) and **(c)** the intensity of eGFP fluorescence were evaluated 3 days post-transduction. Cultures transduced at MOI 10 displayed the best results regarding the combined number of eGFP$^+$ cells and eGFP intensity. Statistical analysis by one-way ANOVA and Bonferroni's *post-hoc* test; error bars represent the S.E.M; $n = 3$; *$p<0.05$, **$p<0.01$. **(d)** Representative images are shown; scale bars = 50 µm; nuclei were stained blue with DAPI.



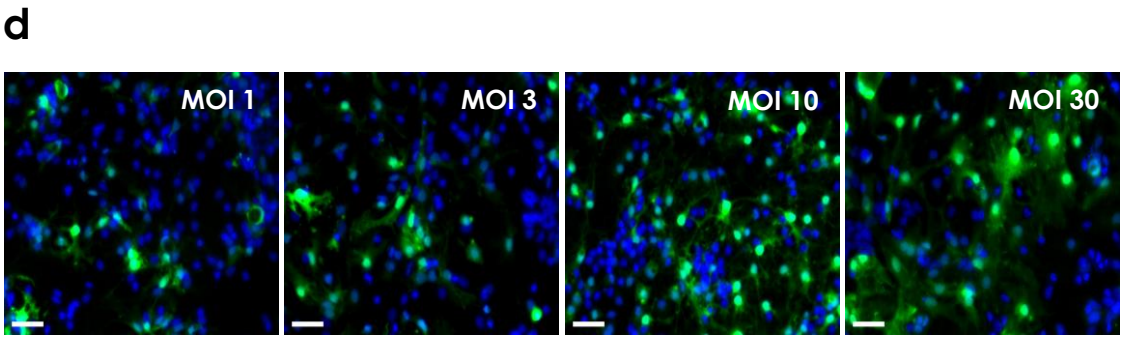
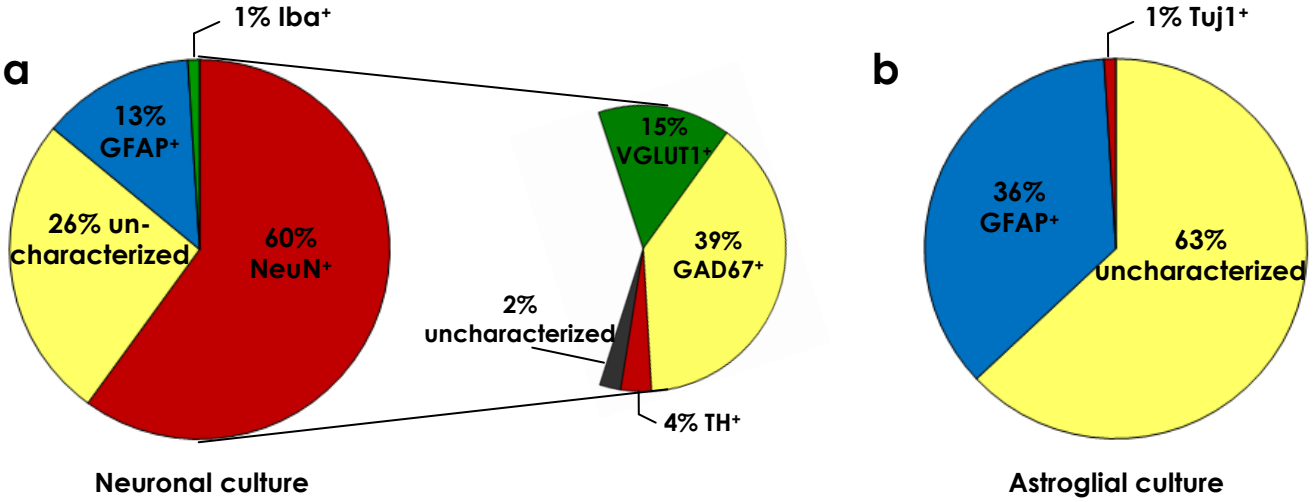


Figure S2: Composition of rat primary VM cell cultures. E14 rat VM cells were cultured under specific conditions for the growth of either neurons or glia. Cell cultures were characterised by immunocytochemistry at day 4 *in vitro* for **(a)** neuronal cultures (*n* = 3) and day 6 *in vitro* for **(b)** astroglial cultures (*n* = 2). Representative images demonstrating the composition of **(c)** neuronal and **(d)** glial cultures are shown. Scale bars = 50 µm. Nuclei were stained blue with DAPI. Red cells were positive with primary antibodies against the protein markers indicated on images: neurons are NeuN$^+$ or Tuj1$^+$; astrocytes are GFAP$^+$; microglia are Iba1$^+$; GABAergic neurons are GAD67$^+$; glutamatergic neurons are VGLUT1$^+$; and dopaminergic neurons are TH$^+$ cells. Uncharacterized cells were negative with all tested antibodies.



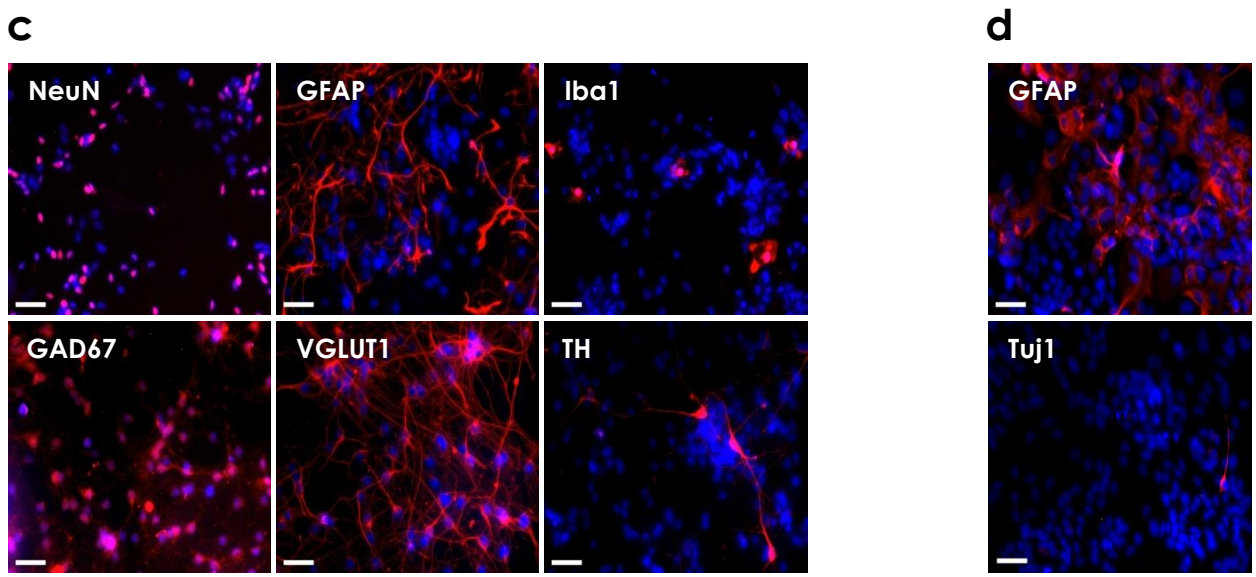


Figure S3: Quantification of astrocytes and neurons in rat VM astroglial cultures transduced with LVs. Primary rat VM astroglial cultures were transduced with IPLV-*eGFP* (*n* = 3) at MOI 10 (eGFP transducing units), 3 days after cell seeding. Total numbers of **(a)** astrocytes ($GFAP^+$) and **(b)** neurons ($Tuj1^+$) were evaluated 3 days post-vector transduction demonstrating no significant difference in cell number between groups. Data were statistically analysed by one-way ANOVA and Bonferroni's *post-hoc* test. Error bars represent the S.E.M.