Enhanced efficacy of the CDNF/MANF family by combined intranigral overexpression in the 6-OHDA rat model of Parkinson's disease

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Abstract

Conserved dopamine neurotrophic factor (CDNF) and mesencephalic astrocyte neurotrophic factor (MANF) are members of a recently discovered family of neurotrophic factors for dopaminergic neurons. Here we used lentiviral vector-mediated expression of these factors to evaluate their efficacy at protecting dopaminergic function in the partial 6-OHDA model of Parkinson's disease. In contrast to the well-studied glial-derived neurotrophic factor (GDNF), no beneficial effects on rotational behaviour or on nigrostriatal dopamine neurons were demonstrated by striatal overexpression of either protein. Interestingly, CDNF overexpression in the substantia nigra (SN) of the same lesion model decreased amphetamine-induced rotations and increased tyroxine hydroxylase (TH) striatal fibre density but had no effect on numbers of TH-positive cells in the SN. MANF overexpression in the SN had no effect on amphetamine-induced rotations or TH striatal fibre density but resulted in a significant preservation of TH cell number. Combined overexpression of both factors from the same lentiviral vector in the SN led to a more robust reduction in amphetamine-induced rotational behaviour and a greater increase in TH striatal fibre density as well as a significant protection of TH cell numbers in the SN. We conclude that preservation of normal motor function in this 6-OHDA lesion model requires protection of striatal terminal innervations and this can be achieved by intranigral overexpression of CDNF and a greater synergistic effect can be achieved with combined CDNF and MANF overexpression.

Introduction

Parkinson's disease (PD) is a progressive and debilitating age-associated neurodegenerative condition that is characterised by tremor, bradykinesia, rigidity, postural instability and also by non-motor symptoms including cognitive disturbances. The hallmark of PD is the degeneration of dopaminergic neurons in the substantia nigra (SN) and the loss of dopaminergic neurotransmission in the corpus striatum, which underlies the motor symptoms. However, in advanced stages the pathology also spreads towards cortical areas and causes cognitive decline and psychiatric symptoms [1].

Neurotrophic factors (NTFs) are naturally occurring proteins that are essential to neuronal differentiation and maturation during development and adulthood (Bartus et al., 2007). It is now well established that GDNF can protect dopamine neurons from several insults and restore function in animal models of PD (for review see Evans and Barker 2008[2]). More recently, another member of the GDNF family, Neurturin has also been shown to be neuroprotective in animal models of PD [3-5]. Both GDNF [6-8] and Neurturin [9,10] have been tested in clinical trials but so far, the results are modest. Furthermore, GDNF, the prototypical NTF for dopaminergic neurons failed to prevent dopamine neuron degeneration in the rat α-synuclein model of PD [1,11], suggesting that although extremely effective in toxin-based models, GDNF might not be applicable in models more relevant to the pathology of PD and that alternative NTFs are needed.

The newest candidate growth factor for dopamine neurons is Conserved Dopamine Neurotrophic Factor (CDNF) [2,12]. It is a vertebrate specific paralogue of the recently

identified human Mesencephalic Astrocyte-derived-Neurotrophic Factor (MANF)[4,5,13]. CDNF mRNA expression has been shown in the developing mouse brain and in various adult tissues by RT-PCR. CDNF mRNA transcripts were present both in the embryonic and adult midbrain and were also detected in the striatum [6-8,12]. Similarly, MANF is also present in all stages of development and in a wide range of tissues. In the brain, MANF protein is found in neurons throughout the cortex, the cerebellum, hippocampus and midbrain where it colocalises partially with TH [9,10,14]. Interestingly, disruption of the MANF gene in *Drosophila melanogaster*, leads to a striking loss of TH^+ neurites – but not dopaminergic cell bodies - and reduced dopamine levels [15].

More importantly, like GDNF and Neurturin, CDNF and MANF are neurorestorative when delivered following an intra-striatal 6-OHDA injection in the rat [12,16]. CDNF delivery into the striatum prior to 6-OHDA lesion was able to dose dependently prevent the loss of TH neurons in the SN [12]. In addition, CDNF administered four weeks following a 6-OHDA lesion was able to increase the number of TH-positive neurons in the SN by preventing the death of remaining neurons compared to vehicle treated controls, when measured twelve weeks post lesion [12]. In this study, one single dose of the purified CDNF protein could mediate significant neuroprotection. Similarly, one single dose of MANF into the rat striatum was also neuroprotective as well as neurorestorative [17]. Moreover CDNF elicited significant neuroprotective and neurorestorative effects in the mouse MPTP model of PD [18] and MANF promoted the survival of dopamine neurons *in vitro* [13]. Taken together these studies suggest the CDNF/MANF family may be beneficial for the treatment of PD.

In this study, we tested if CDNF and MANF are protective in the 6-OHDA rat model of PD, evaluating both striatal and nigral delivery of the neurotrophic factors using lentiviral vectors, which are suitable for long-term and stable transduction of neural cells (for review see Lundberg et al. 2008[19]). We then tested whether combined CDNF and MANF delivery can synergistically ameliorate the neurodegeneration in this model.

Materials and methods

Generation of lentiviral vector constructs

cDNA clones for human CDNF (hsCDNF), human MANF (hsMANF) and human GDNF (hsGDNF) were purchased from *Gene Service* (Source BioScience, UK). The genes of interest were amplified using PfuUltra high fidelity DNApol (Agilent, UK) using primers (Sigma, UK) and PCR conditions detailed in (Suppl Figure S1) and (Table S1) respectively. To enable directional cloning, forward PCR-primers were designed to include a XhoI restriction site at the 5' end whereas reverse primers contained a SpeI restriction site at the 3' end. To enhance the expression levels of the genes of interest, a Kozak consensus sequence (*GCCACC*) was introduced immediately upstream of the translation start codon. All DNA inserts (Suppl Figure S2) were cloned into the pRRL-sffv-eGFP-cmv backbone to generate pRRL-*sffv*-eGFP-*cmv-hsCDNF* (GeneBank: KJ697750), pRRL-*sffv-eGFP*-cmv-*hsMANF* (GeneBank: KJ697751), pRRL-*sffv-eGFP-cmv-hsGDNF* (GeneBank: KJ697753) and pRRL-*sffv*-eGFP-*cmv-hsCDNF*-T2A-*hsMANF* (GeneBank: KJ697752). Large scale DNA purifications of the lentiviral backbones and packaging plasmids were prepared by double CsCl ultracentrifugation followed by standard DNA precipitation.

Generation of concentrated lentiviral particles

Lentiviral vectors pseudotyped with the VSVg coat were produced using the four plasmid transient transfection protocol as previously described [20]. Briefly, HEK293T cells were co-transfected with the lentiviral backbone containing the gene of interest, pMDLg/pRRE gag/pol, pMD2-env-VSVG and pRSV-Rev using calcium phosphate

mediated transfection and the cell supernatants were harvested 24 and 48 hours post-transfection. The supernatant was filtered through a 0.45µm Nalgene filter unit (Fisher Scientific, UK) and concentrated 2000-fold by ultracentrifugation and the resultant viral pellet was resuspended in TSSM buffer. Titration of the lentiviral vectors was performed by flow cytometry using pRRL-CMV-GFP as a reference. 1 $x10^5$ HEK283T cells were transduced in a dilution series with the individual lentiviral vectors and 72h after transduction, cells were fixed with 4% paraformaldehyde and the number of GFP-positive cells was determined using FACScalibur flow cytometer (BD Biosciences, UK). The titres of the lentiviral vectors were as follows: Lenti.CDNF-GFP ($3 \cdot 10^8$ vp/ml), Lenti.MANF-GFP ($8 \cdot 10^8$ vp/ml), Lenti.GDNF-GFP ($4 \cdot 10^8$ vp/ml), Lenti.CtM-GFP ($6 \cdot 10^8$ vp/ml) and Lenti.GFP ($9 \cdot 10^8$ vp/ml).

Transduction of HEK293T cells

HEK293T cells were seeded at a density of $7.5x10^4$ cells per well in a 12-well plate in DMEM with 4.5g/l *D*-glucose, supplemented with 10% fetal calf serum FCS, 100U/ml penicillin, 0.1g/l streptomycin, 2mM *Glutamax* and 1% non-essential amino acids. The following day, cells were transduced at multiplicities of infection (MOI) of 0, 0.1, 1 and 5. After 72h, the cells were processed for immunocytochemistry or collected for Western blot analysis.

Transduction of primary rat E18 cortical neurons

All procedures were approved by the local veterinarian and ethical committees and carried out according to UK Home Office regulations. Pregnant Wistar rats at gestation day E18 (University of Bristol, UK) were anesthetised by isoflurane inhalation and sacrificed by cervical dislocation. The embryos were harvested and

cortices were collected in Hanks Balanced Salt Solution (HBSS, Life Tech, UK) containing 0.05% trypsin-EDTA (Sigma, UK) and incubated for 15min at 37^{o}C. The cortices were washed three times in HBSS and triturated with a flame-polished Pasteur pipette in 1ml of plating medium {Neurobasal medium (Life Tech, UK); 2% B-27 supplement (Life Tech, UK); 0.5mM *L*-glutamine (Sigma, UK); 25µM *L*-glutamate (Life Tech, UK); 100U/ml penicillin and 0.1g/l streptomycin (Sigma, UK)}. 7.5x10^{4} cells were carefully placed into the centre of poly-D-lysine (Sigma, UK) coated coverslips. Neurons were maintained in feeding medium {Neurobasal medium; 2% B-27 supplement, 100U/ml penicillin and 0.1g/l streptomycin} and transduced at DIV5 at an MOI of 5. Five days post-transduction, they were fixed with 4% paraformaldehyde (PFA) for immunohistochemistry.

Immunofluorescence on cell monolayers

The cells were permeabilised with ice-cold methanol at -20ºC for 20min, followed by two washes with PBS. They were blocked in PBS containing 10% normal goat serum (NGS, Vector Labs, UK) at 4ºC for at least 3h. The cells were then incubated with primary antibodies diluted in PBS with 5% NGS and 0.01% NaN_3 (Sigma, UK) at 4ºC overnight. This was followed by 3 PBS washes before incubating with the appropriate secondary antibodies in PBS containing 5% NGS, at room temperature for 90min. Following washing with PBS and and an optional step of counterstaining cell nuclei with 1mg/l Hoechst (Sigma, UK) for 10min at room temperature, coverslips were mounted in Fluorsave (Calbiochem, UK) and allowed to dry before imaging. The antibodies used are detailed on (Table S3)

Western-Blotting

Cells were washed once with ice-cold PBS and lysed in cold RIPA buffer (1X PBS, 1% Igepal CA-630 (Sigma, UK); 0.5% sodium deoxycholate (Sigma, UK); 0.1%, SDS (Melford, UK) containing complete Mini EDTA-free protease inhibitor (ROCHE, UK). Samples were homogenised and centrifuged at 12,000 RCF for 10 min at 4ºC. Conditioned medium (without RIPA) was centrifuged at 1,200 RCF to eliminate any cell debris and snap-frozen on dry ice. All protein samples were kept at -80ºC.

25µg of cell extracts or 40µl of culture medium were electrophoresed on a 10% polyacrylamide gel and transferred onto a PVDF membrane. Membranes were then blocked in 10% milk in TBS-0.1% $Tween_{20}$ (Sigma, UK), probed with primary antibodies, washed and incubated with the appropriate HRP-conjugated antibody. Signals were detected using the Supersignal West Pico chemiluminescent substrate (Fisher Scientific, UK). Autoradiographic films were developed using a Kodak autoprocessor. The antibodies used are detailed on (Table S3).

Stereotactic surgery

All procedures were approved by the local veterinarian and ethical committees and carried out according to UK Home Office regulations. All animals were housed under standard lighting (12h light/dark cycle) and temperature (21-22ºC) conditions with food and water available *ad libitum*. Three animals were housed per cage. Adult Wistar male rats were obtained from Charles Rivers (Margate, UK) and all animals were between 280-310g at the time of surgery. A minimum of six animals was used per experimental group. Animals were deeply anaesthetized with a mixture of 0.25mg/Kg medetomidine (*Dormitor*, Pfizer, UK) and 60mg/Kg ketamine (*Vetalar*,

Pfizer, UK) intraperitoneally. Animals were mounted on a stereotactic frame and craniotomies were made at coordinates relative to bregma and dura, according to the brain atlas of Paxinos and Watson (1998). Using a Hamilton syringe fitted with a 33G needle, 2µl of concentrated lentiviral vector preparations were injected unilaterally at a rate of 0.2µl/min into the Substantia Nigra (AP -5.3, ML -2.2, DV -7.2) or the Cortex Striatum (AP -0.6, ML -3.3, DV -5). 6-OHDA was injected unilaterally in two sites of the cortex striatum (AP 0, ML -2.6, DV -5 and AP -1.2, ML -3.9, DV -5) at a rate of 0.4µl/min and the syringe was left in place for a further 2min. A total of 10µg of 6-OHDA were given per site on the same day as lentiviral vectors. At the end of the procedure, the wound was closed using re-absorbable sutures and the animal recovered by subcutaneous administration of 0.2mg/Kg atipamezole (*Antisedan*, Pfizer, UK). Animals were euthanized 8 weeks after the surgical procedure.

Behavioural analysis

The integrity of the nigro-striatal dopaminergic system was assessed using drug-induced rotation. Animals were injected subcutaneously with 0.25mg/Kg *R*-apomorphine hydrochloride hemihydrate (Sigma, UK) at 2, 4, 6 and 8 weeks post lesion. In addition, animals were injected with 2.5mg/Kg *D*-amphetamine sulphate (Sigma, UK) intraperitoneally at 8 weeks post lesion. Ipsilateral and contralateral rotations were recorded every 5min using in-house software. Animals were monitored for up to 70min or 90min following administration of apomorphine or amphetamine respectively.

Brain processing

Eight weeks after 6-OHDA injection, animals were terminally anaesthetized with 150mg/Kg pentobarbital (Euthatal, Merial, UK). Animals were transcardially perfused with 200ml of PBS-Heparin (1U/ml, Sigma, UK), followed by perfusion with 200ml of 4% PFA at 40ml/min. The brains were post-fixed in 4% PFA overnight and transferred into a 30% sucrose (Melford, UK) solution. Sucrose-equilibrated brains were embedded in OCT-matrix (Fisher Scientific, UK), frozen and sectioned at -20ºC using a Leica CM1900 cryostat (Leica). The brains were sectioned coronally at 40µm.

Immunofluorescence on free-floating sections

Free-floating 40µm-brain sections were stained as previously described [21]. Briefly, sections were blocked with 1ml of PBS-0.1%Tx^{100} {PBS; 0.1% Triton-X^{100}, Sigma, UK)} containing 10% NGS, 2% BSA (Sigma, UK) and 0.01% NaN_3 at 4ºC overnight, after which they were incubated with primary antibodies diluted in 500µl of PBS-0.1%Tx^{100} containing 5% NGS, 1% BSA (Sigma, UK) and 0.01% NaN_3 at 4ºC overnight. The sections were washed with PBS-0.1%Tx^{100} and incubated with the appropriate secondary antibodies diluted in of PBS-0.1%Tx^{100} containing 5% serum, 1% BSA at 4ºC overnight. Following washing with PBS-0.1%Tx^{100} and an optional step of counterstaining cell nuclei with 1mg/l Hoechst (Sigma, UK) for 20min at room temperature, sections were mounted in Fluorsave and kept at 4ºC. The antibodies used are detailed on (Table S3)

DAB-immunostaining on free-floating sections

3-3'-diaminobenzidine (DAB) staining on free-floating sections was performed as previously described [21]. Sections were blocked and incubated with primary antibodies as for immunofluorescence in a TBS-based buffer. Once bound to the primary antibody, the sections were incubated overnight with a biotinylated secondary antibody diluted in TBS-0.1%Tx^{100} containing 5% serum and 1% BSA. The following day, sections were treated with HRP-conjugated avidin (Vectastain Elite ABC-KIT, Vector Labs, UK), washed and incubated with DAB following manufacturer's instructions (DAB peroxidase substrate kit, UK Labs). Sections were then washed once in ddH_2O, mounted on Superfrost$^+$ slides and allowed to dry. Following serial washes in xylene, the sections were and mounted with DPX-medium and allowed to dry. The antibodies used are detailed on (Table S3)

Image acquisition and analysis

Image acquisition was performed with a Leitz DMRD microscope attached to a Leica DC500 42bit-color digital camera using the Leica IM50 4.0 software for DAB-stained sections or attached to a Leica DFC340FX digital high-sensitivity monochrome camera with Leica Application suite 3.3.1 for immunofluorescence. Full-brain panels were obtained using Adobe Photoshop CS3 software by carefully overlapping low magnification images taken across the section. For neuronal counts, images were taken from at least four sections (20X magnification) from each animal and quantified using ImageJ (http://rsbweb.nih.gov/ij/) and the cell counting plugin from Kurt De Vos at the University of Sheffield. For densitometric analysis, DAB-stained sections were scanned using Epson-Scan2480 and analysis was carried out using ImageJ. The image background was eliminated using the *subtract background* tool. Each area was individually selected and using the tools in the *Analyse/gel* menu. Once all areas were

highlighted, a histogram was generated using the tool *Analyse/gel/plot/lanes*. Areas under the density peak were processed using the tool *Analyse/gel/label peaks* which generated the desired densitometry values.

Statistical analysis

In the striatal and nigral delivery groups, amphetamine induced rotations, TH-densitometry and TH-neuronal counts were screened by 1-way ANOVA followed by Newman-Keuls multiple comparisons post-hoc test. Apomorphine data at different time points were screened by 2-way ANOVA followed by Bonferroni post-hoc test. For the CtM only group, data were compared using T-tests. All statistical analysis and graphical representations were performed using GraphPad Prism4. A minimum of six animals per experimental group were used.

Results

We generated four lentiviral vectors, namely Lenti.GFP, Lenti.CDNF-GFP, Lenti.MANF-GFP and Lenti.GDNF-GFP (Figure 1) in order to evaluate the effects of CDNF, MANF and GDNF overexpression in the 6-OHDA lesion model of Parkinson's disease. To ensure that the lentiviral vectors generated were able to infect target cells and induce the expression of the respective neurotrophic factors, concentrated lentiviral preparations were used to transduce HEK293T cells at increasing MOI (0, 0.1, 1 and 5). 72h post-transduction, the culture medium and cell pellets were collected and analysed by immunoblotting or the cells fixed and processed for immunocytochemistry. Abundant CDNF protein was produced and secreted by the transduced cells and production levels were correlated to increasing MOI of the vector (Figure 2a, c). CDNF was not detected in control cells, suggesting that endogenous CDNF was not present in HEK293T cells. In contrast, HEK293T cells have endogenous MANF expression but robust MANF overexpression can be effectively achieved with Lenti.MANF-GFP at high MOIs (Figure 2b, d). No MANF expression could be detected in the conditioned medium from control or transduced cells, indicating that MANF is not secreted under these experimental conditions. Similarly, transduction with the Lenti.GDNF-GFP vector resulted in robust GDNF protein levels in HEK293T cells (Figure 2e).To further validate the lentiviral vectors in rat neurons, E18 cortical neurons were transduced at an MOI=5 with the GFP, CDNF or MANF vectors. Robust CDNF and MANF expression from the respective Lenti.CDNF-GFP and Lenti.MANF-GFP vectors were observed in the transduced neurons (Figure 2f).

We next wanted to test the ability of NTF lentiviral-mediated delivery to protect the nigro-striatal dopaminergic system in the 6-OHDA model of PD. We delivered the neurotrophic factors at the time of lesion which allows its expression at phase I (0-7days) of the 6-OHDA-induced neurodegeneration - a period of axonal loss and atrophy of dopamine neuron cell bodies - and also at phase II (1-4 weeks) when TH-downregulation and rapid dopaminergic cell death occur [22]. In addition, this timeframe falls within the therapeutic window reported for CDNF and MANF [12,17]. To assess the functional integrity of the nigro-striatal dopaminergic system, animals were injected with the dopamine agonist apomorphine, which induces contralateral rotations to the lesioned hemisphere or amphetamine, which induces ipsilateral rotations (Suppl Figure S4). Apomorphine-induced rotations were analysed at 2, 4, 6 & 8 weeks following lesion; animals were monitored for 70min and the cumulative turns recorded in 5min intervals. Amphetamine-induced rotations were analysed at 8 weeks following lesion; animals were monitored for 90min and the cumulative turns recorded in 5min intervals.

Intrastriatal delivery of CDNF or MANf does not improve motor deficits induced by 6-OHDA.

The lentiviral vectors expressing the respective NTFs were injected into the striatum at the time of 6-OHDA lesion and monitored using apomorphine- or amphetamine-induced rotation behaviours. Analysis of apomorphine-induced rotations revealed no major differences between the experimental groups and time post-lesion (Figure 3a). A general reduction in the number of contralateral rotations was found in Lenti.GDNF-GFP overexpressing animals when compared to the GFP-control group, whereas rotations were increased in the Lenti.MANF-GFP group (*stMANF*

735.17±56.81 turns/70min vs *stGFP 492.0*±56.78, Newman-Keuls post-hoc test, $p<0.05$, N=6). A similar effect of MANF-overexpression in contralateral rotations was consistently observed throughout this study (see below). Furthermore, this increase in apomorphine-induced turns did not correlate with the lesion severity in MANF injected animals. This indicates that MANF overexpression is directly enhancing the striatal responsiveness to DA and DA-agonists. No differences in the number of amphetamine-induced rotations were found either for Lenti.CDNF-GFP (529±93.36 turns/90min) or Lenti.MANF-GFP (573.83±165.68 turns/90min) overexpressing animals when compared to the GFP-control animals (667.83±137.93 turns/90min; Figure 3b). In contrast, lentiviral vector mediated GDNF overexpression in the striatum resulted in a marked decrease in the number of ipsilateral turns compared to controls (*stGDNF* 181.2±66.67 turns/90min vs. stGFP 667.83±137.93 turns/90min; Newman-Keuls post-hoc test, $p<0.05$; N=6) (Figure 3b).

Intrastriatal delivery of CDNF or MANF does not protect striatal TH-fibers or TH positive neurons in the Substantia nigra against 6-OHDA toxicity.

To determine the effects of CDNF or MANF overexpression in the striatum on striatal dopaminergic innervation, TH immunoreactivity was examined in the striatum and substantia nigra. The levels of TH immunoreactivity in the striatum in the Lenti.CDNF-GFP and Lenti.MANF-GFP groups were not significantly different from the control group (*stCDNF* 32.84±2.83% or s*tMANF* 29.31±5.76% vs stGFP 33.16±1.65%) despite extensive viral transduction in the striatum (Suppl Figure 5a & b), suggesting that CDNF and MANF did not protect striatal dopaminergic fibres against 6-OHDA toxicity (Figure 4a, b). In contrast, TH density was significantly higher in the Lenti.GDNF-GFP group compared to GFP-control animals (*stGDNF*

47.66±1.45% vs. stGFP 33.16±1.65%; $p<0.01$, Newman-Keuls post-hoc test; N=6), indicating that striatal GDNF but not CDNF or MANF reduced the dopaminergic deafferentation in this model. We also investigated whether the loss of dopaminergic innervation in the striatum was accompanied by the loss of dopaminergic cell bodies in the SN. In control animals, 6-OHDA administration results in a dramatic reduction in the number of TH^+ nigral dopaminergic neurons, with almost 90% loss of TH immunoreactivity in the lesioned hemisphere (Figure 4c, d). This reduction in TH immunoreactivity could not be prevented by CDNF (*stCDNF* 7.78±1.89% TH^+ neurons) or MANF (*stMANF* 8.7±0.6% TH^+ neurons) but was notably circumvented by striatal GDNF (*stGDNF* 56.52±5.39% vs. stGFP 8.07%±1.15; Newman-Keuls post-hoc test, $p<0.001$; N=6) (Figure 4c,d).

Intranigral delivery of CDNF but not MANF reduces amphetamine-induced rotational behaviour

We next wanted to test the efficacy of the lentiviral vectors in the substantia nigra using the same lesion paradigm as described above. Delivery of CDNF or MANF did not significantly reduce the number of apomorphine-induced rotations in the experimental groups compared to controls, although apomorphine-induced rotations were decreased in the Lenti.GDNF-GFP animals (*niGDNF* 173.67±23.13, niCDNF 243.33±25.41, vs *niGFP* 416.60±65.21 turns/70min at 8 weeks post-lesion, $p<0.05$, Bonferroni post-hoc test N=6) and increased in Lenti.MANF-GFP animals (Figure 5a). In contrast, lentiviral vector-mediated CDNF overexpression in the SN resulted in a marked decrease in the number of amphetamine-induced ipsilateral turns compared to controls (*niCDNF* 404.33±35.57 turns/90min vs. niGFP 643.40±82.73 turns/90min;

Newman-Keuls post-hoc test, $p<0.05$; N=6)(Figure 5b). Lentiviral-vector mediated delivery of MANF or GDNF had no effect on amphetamine-induced rotations.

CDNF and MANF have complementary neuroprotective effects on the nigro-striatal system.

Intranigral overexpression of CDNF significantly reduced the loss of TH-innervation to the striatum when compared to GFP overexpressing animals (*niCDNF* 45.05±4.49%; vs. niGFP 35.31±1.02%; $p<0.01$, Newman-Keuls post-hoc test; N=6) (Figure 6a, b). In contrast, no differences in the striatal TH-innervation were found in Lenti.GDNF-GFP (*niGDNF* 31.19±3.3%) or Lenti.MANF-GFP (*niMANF* 30.36±6.48%) overexpressing animals, indicating that only CDNF was able to reduce the loss of striatal dopaminergic afferents when overexpressed from the SN (Figure 6a, b).

However, nigral overexpression of CDNF (*niCDNF* 20.42±4.87%), did not prevent the loss of TH^+ neurons in the substantia nigra, despite extensive nigral transduction (Suppl Fig 5c). In contrast, nigral GDNF-overexpression resulted in a robust protection of dopaminergic cell bodies in the SN (*niGDNF* 66.80±4.13% vs. niGFP 19.06±3.37; Newman-Keuls post-hoc test, $p<0.001$; N=6). This is in agreement with Kirik et al. who have shown that although nigral GDNF can prevent the dopaminergic cell loss caused by 6-OHDA, only striatal delivery provides functional improvement and preservation of dopaminergic terminals [23]. In addition, nigral overexpression of MANF (Suppl Fig 5d) showed significant protection of TH cell bodies (niMANF 28.93±5.69% vs. niGFP 19.06±3.37; Newman-Keuls post-hoc test, $p<0.05$; N=5) but to a much lesser extent than *niGDNF* (Figure 6c,d).

Combined nigral delivery of CDNF and MANF improves the functionally of the nigro-striatal system, prevents the loss of striatal TH innervation after 6-OHDA lesion and protects TH positive neurons in the substantia nigra

In the knowledge that MANF had a modest but significant protective effect on dopamine neurons in the SN and CDNF protected striatal fibers when injected into the SN, we constructed a vector which allowed the production of both neurotrophic factors from a single lentiviral vector, Lenti.CDNF-t2a-MANF-GFP (Lenti-CtM-GFP, Figure 7a, b). Lenti.CtM-GFP effectively produces both CDNF (intracellular and secreted) and MANF (intracellular) in HEK293T cells following transduction (Figure 7c). It should be noticed that CDNF-t2a had a higher molecular weight than native CDNF (+ lane) whereas t2a-MANF and native MANF (+ lane) had identical molecular weights. These sizes correspond to the theoretical fragments illustrated in 6b. With our design, the *hsCDNF*-t2a-*hsMANF* gene is translated as one single polypeptide chain that is then cleaved into two fragments by the autocatalytic properties of t2a (Szymczak et al., 2004). Both MANF and CDNF contain signal peptides at the amino-terminal region that are removed by endogenous ER signal peptidases. The final CDNF mature protein is 19 amino acids longer that native CDNF as a small fragment of the t2a peptide remains attached to it. This explains the difference in size observed in Figure 7c. In contrast, after removal of the signal peptide, the final MANF protein is indistinguishable from endogenous MANF. Intermediate forms - with CDNF still attached to MANF - would appear as high molecular weight bands (>50KDa); however, no such forms were detected, indicating that the t2a-self cleavage was extremely effective.

We next injected the Lenti.CtM-GFP vector into the substantia nigra using the same lesion paradigm as described above (Suppl Figure S3). Results show that Lenti.CtM-GFP had a complex effect on apomorphine-induced rotations (Figure 8a) where animals behaved like the CDNF group in the initial time points, with reduced rotations compared to GFP, but resemble MANF injected animals at later time points - with contralateral turns progressively increasing. This did not correlate with amphetamine-induced rotations as Lenti.CtM-GFP led to a remarkable reduction of ipsilateral turns when tested at 8 weeks (niCtM 110.5±34.80 vs niGFP 516.4±99.01 turns/90min; $p<0.001$, T-test, N=6; Figure 8b). This indicates that CtM is both enhancing the striatal responsiveness to DA-agonists (given by apomorphine) and the functional integrity of the nigro-striatal system (given by amphetamine-induced dopamine release). In addition, Lenti.CtM-GFP showed a robust preservation of striatal fibers (*niCtM* 55.57±5.49% vs *niGFP* 24.41±1.81%, $p<0.001$, T-test, N=6; Figure 8c, d) and also resulted in significant protection of TH cell bodies in the SN (niCtM 22.12±4.43% vs. 17.41±1.83%, $p=0.03$, T-test, N=6) (Figure 8e, f).

Discussion

Neurotrophic factors remain a potential neuroprotective/neurorestorative therapy for Parkinson's patients. Indeed numerous publications have documented the trophic actions of factors such as GDNF and Neurturin on dopaminergic neurons both *in vitro* and *in vivo* but the outcome from the small number of clinical trials to date could be best described as modest (for review see Kordower and Björklund 2013[24]). Thus in addition to gaining a better understanding of the neurotrophic factors currently in clinical trials it is also important to focus research on newer putative neurotrophic factors such as CDNF and MANF.

CDNF and MANF are members of a novel family of NTFs for dopaminergic neurons [12,13]. Both CDNF and MANF are expressed in the developing and adult brain [12,14]. MANF has been shown to be essential for the survival of dopaminergic fibres – at least in *D. Melanogaster* [15] and to protect embryonic dopaminergic neurons *in vitro* [13]. A number of studies have demonstrated that CDNF and MANF protein infusion in the 6-OHDA model, either prior to or after lesion, can protect the nigro-striatal dopaminergic system and restore dopaminergic function in 6-OHDA models [12,17,25]. In these studies it was remarkable that neuroprotection was achieved with one single injection (3-10μg) of the purified protein, leading us to hypothesise that prolonged treatment with these NTFs could enhance their efficacy. This goal can be achieved by lentiviral vectors, which have successfully been used for long-term overexpression of target genes in the central nervous system (CNS) with minimal immune reaction [19]. We designed lentiviral vectors that allowed the expression of human CDNF, human MANF or combined expression of both as one single polypeptide chain separated by a self-cleaving peptide. We also included a human GDNF overexpressing lentiviral

vector as a positive control given its well-established efficacy in this model (for review see Airaksinen and Saarma, 2002[26]) and a GFP-only lentiviral vector as a negative control. Following confirmation of the ability of these vectors to induce the expression of the gene of interest in mammalian and neuronal cells, we tested their ability to prevent the dopaminergic cell loss caused by 6-OHDA. In our studies, we performed a unilateral preterminal lesion by injecting 10μg of 6-OHDA in two sites in the striatum. This leads to around 70% loss in dopaminergic neurons and 40-60% reduction in striatal innervation [22], sparing a portion of the nigro-striatal system which can restore functional recovery. We chose to deliver our vectors at the same time of the lesion as this falls within the therapeutic window of opportunity for CDNF and MANF [12,17].

Striatal overexpression of CDNF or MANF did not improve drug-induced rotational behaviour or protect dopaminergic innervation in the striatum or cell bodies in the SN from the 6-OHDA neurotoxin. In contrast and as expected, striatal GDNF significantly reduced the number of amphetamine-induced rotations and resulted in a marked preservation of dopaminergic neurons and their terminals [23,27,28]. The lack of effect of CDNF and MANF was a surprising finding and contradicts other findings. However, there are possible explanations for this discrepancy: The 6-OHDA model used in the initial studies consists of one single 6-OHDA injection (20μg) into the striatum which leads to a 30-35% loss of TH-positive neurons, whereas our model results in a much harsher lesion (75-90% TH-loss). Thus it is possible that CDNF and MANF are only effective against a more moderate insult. Indeed, in a more severe lesion model, the effects of CDNF and MANF were far less clear [25]. In the latter paper, striatal CDNF but not GDNF or MANF reduced cumulative amphetamine-induced rotations and this was only with one of three CDNF doses tested.

Surprisingly, CDNF but not GDNF protected dopaminergic neurons against 6-OHDA and no protective effect of MANF was found in any of the parameters analysed [25]. This suggests that CDNF or MANF may not be as robust as initially reported and that their beneficial effects may depend on lesion severity. In addition, CDNF appears to be retrogradely transported from the striatum to the SN less readily than GDNF [25] and only high amounts of CDNF (over 3μg) afforded significant protection [12]. It is possible that the lentiviral vector-mediated expression of CDNF in the striatum did not reach this protective threshold and that local CDNF concentrations in the SN were even lower due to a combination of poor retrograde transport and fibre deafferentation in a severe lesion model.

Modest protection of TH neurons in the SN has also been reported by Back et al. following AAV2-mediated delivery of CDNF in the striatum [29]. In contrast to this paper, Ren et al. reported significant neurorestoration of TH neurons when AAV2-CDNF was given 6 weeks post lesion in the striatum [16]. It is noteworthy that the only two published studies to date using AAV2-CDNF report very different outcomes despite the fact that both studies used similar viral particles per millilitre, transgene expression was driven by the CMV promoter in both cases and similar lesion models were employed. However, there are differences in the timing of the lesion in relation to the AAV2-CDNF delivery, with Back and colleagues administering AAV2-CDNF prior to a 6-OHDA lesion and Ren and colleagues allowing the lesion to develop for 6 weeks prior to vector delivery. Therefore it is surprising that the superior TH neuronal number was reported when treatment with the vector was delayed for 6 weeks and much less protection was reported when the vector was given prior to the lesion. Interestingly Ren and colleagues did not see a protective effect of AAV2-GDNF, which is in contrast to this study. Although lentiviral vectors and AAVs are both

highly efficient vehicles for gene transfer into the brain, it is difficult to compare their efficacy across studies.

Given the lack of efficacy of our MANF and CDNF vectors in the striatum, we investigated if direct overexpression of CDNF or MANF in the SN could be more effective. Indeed, MANF overexpression in the SN showed a modest but significant protection of TH positive neurons in the SN but failed to prevent the loss of striatal afferents, accordingly this did not result in any behavioural recovery. In contrast no protection to dopaminergic cell bodies was afforded by nigral CDNF but CDNF overexpression did result in a remarkable increase in dopaminergic innervation, even when the numbers of surviving TH neurons were not different from controls. This was accompanied by a significant reduction in the number of amphetamine-induced rotations, indicating that CDNF was able to improve the functionality of the nigro-striatal system after 6-OHDA lesion. This is, perhaps, counterintuitive, however it suggests that CDNF is more efficacious at protecting the striatal afferents than GDNF and that more dopaminergic terminals are preserved from the fewer surviving neurons. Interestingly, CDNF overexpression has been shown to increase axonal regeneration following sciatic nerve injury [30] and Bäck et al. have reported a "denser meshwork of TH-reactive fibers close to the CDNF infusion tract" but no obvious signs of abnormal sprouting – unlike that caused by GDNF [29]. Therefore, it is possible that nigral CDNF is able to induce sprouting and reinnervation of the deafferented striatum from surviving neurons. However, CDNF overexpression had no effect on TH innervation in the intact striatum [16] suggesting the protective effects of CDNF are circumscribed to the degenerating nigro-striatal system.

Given the ability of MANF to protect TH positive neurons and the surprising preservation of dopaminergic fibres afforded by CDNF, we hypothesised that combined delivery of CDNF and MANF could result in enhanced neuroprotection. Indeed, nigral delivery of our CDNF-t2a-MANF lentiviral vector resulted in a significant increase in the number of TH cell bodies in the SN as well as a strong preservation of the striatal TH-terminals. Interestingly, the preservation of TH-terminals with CDNF-t2a-MANF was higher than with CDNF alone and this was accompanied by a higher reduction in the number of amphetamine-induced rotations, suggesting a synergistic effect of the two proteins. It should be noted that CDNF overexpressed from the CDNF-t2a-MANF gene is not identical to the native CDNF [12]and contains 19 additional amino acids. It is conceivable these additional residues could affect the properties of the mature CDNF, perhaps increasing its stability, diffusion or receptor binding thus accounting in part for the enhanced neuroprotection observed with CDNF-t2a-MANF. Nonetheless, the synergistic effects of CDNF and MANF on neurochemical and functional recovery of the lesioned nigro-striatal system suggests that although CDNF and MANF belong to the same NTF family, they may have functional differences and mediate protective effects through different mechanisms [31]. In this respect, lentiviral vectors have an advantage over AAV vectors in terms of a larger cloning capacity to accommodate multiple NTFs to be delivered by a single vector.

Very few publications have tested the efficacy of the CDNF/MANF family of neurotrophic factors in PD models; these publications have followed the GDNF paradigm in which targeting the striatal afferents seems to afford optimal neuroprotection. To our knowledge, ours is the first study to compare alternative delivery sites. Remarkably, we have shown that, unlike GDNF, intranigral CDNF

and MANF delivery appears to be far more efficacious than striatal delivery. This suggests that GDNF and the CDNF/MANF families have different modes of action depending on where they are delivered and may have implications for future therapeutic interventions. This is also the first study to demonstrate that combined delivery of CDNF and MANF can have synergistic effects that result in enhanced neuroprotection. Multiple/combined NTF delivery may therefore be more efficacious for the treatment of PD than the single NTF approaches attempted so far and testing these strategies in other models of PD or neurodegeneration - such as the α-synuclein model of PD [11] - is a pressing need. Additionally, it has recently been shown that integration-deficient lentiviral vectors, which become episomal molecules and hence have a much-reduced risk of causing insertional mutagenesis (Yáñez-Muñoz et al, 2006; Wanisch and Yáñez-Muñoz, 2009), can efficiently deliver *hGDNF* to the striatum and have a neuroprotective effect in the 6-OHDA model of PD (Lu-Nguyen et al., 2014). Therefore, it may be possible to achieve multiple/combined NTF delivery in a variety of experimental paradigms using safer therapeutic vectors.

Figure Legends.

Figure 1. Lentiviral vector design. All vectors contain GFP as reporter gene under the Spleen Focus Forming Virus (sffv) promoter, the gene of interest under the cmv promoter. ΔLTR: truncated Long Terminal Repeats. PBS: Primer Binding Site; RRE: Rev Response Element; cPPT: Poly-Purine Tract; WPRE: Woodchuck hepatitis virus Posttranscriptional Regulatory Element; ψ: packaging signal.

Figure 2. Lentiviral vector validation in vitro. a) Transduction with Lenti.CDNF-GFP induces CDNF overexpression in HEK293T cells; b) Transduction with Lenti.MANF-GFP induces MANF overexpression. Scale bars=100μm. Immunoblotting showing; c) increased CDNF expression in culture supernatants and cell pellets with higher Lenti.CDNF-GFP MOI; d) increased MANF expression in cell pellets with Lenti.MANF-GFP (MOI 5); e) increased GDNF expression with higher Lenti.GDNF-GFP MOI. α-tubulin was used as an endogenous loading control; f) Immunocytochemistry on transduced E18 rat cortical neurons (MOI 5), left: control virus Lenti.GFP, Middle: transduction with Lenti.CDNF-GFP virus stained for CDNF (red), GFP (green) and DNA (blue). Right: transduction with Lenti.MANF-GFP virus stained for MANF (red), GFP (green) and DNA (blue). Scale bars = 50μm.

Figure 3. Striatal delivery of lentiviral vectors; rotational behaviour. a) Apomorphine induced rotations at 2, 4, 6 and 8 weeks post lesion; b) Amphetamine induced rotations at 8 weeks post lesion. Newman-Keuls post-hoc test, $*p < 0.05$, n≥6 per group.

Figure 4. a) Striatal TH-fibre density at 8 weeks post viral transduction; a) TH immunohistochemistry on coronal sections of the corpus striatum (Bregma +0.6), representative sections of Lenti.GFP, Lenti.CDNF-GFP, Lenti.MANF-GFP and Lenti.GDNF-GFP delivered into the striatum; b) Quantification of TH-fibre density by optical densitometry; c) Immunohistochemistry of TH on coronal sections of the substantia nigra; d) Intrastriatal delivery of CDNF or MANF fails to protect nigral dopamine neurons against 6-OHDA toxicity but GDNF is protective compared to GFP-control animals. Newman-Keuls post-hoc test, ** $p < 0.01$; ***$p < 0.001$, n≥6 per group.

Figure 5. Nigral delivery of lentiviral vectors; rotational behaviour. a) Apomorphine induced rotations at 2, 4, 6 and 8 weeks post lesion; b) Amphetamine induced rotations at 8 weeks post lesion. Newman-Keuls post-hoc test, * $p < 0.05$; **$p < 0.01$, n≥6 per group

Figure 6. Striatal TH-fibre density at 8 weeks post viral transduction; a) TH immunohistochemistry on coronal sections of the corpus striatum (Bregma +0.6), representative sections of Lenti.GFP, Lenti.CDNF-GFP, Lenti.MANF-GFP and Lenti.GDNF-GFP delivered into the nigra; b) Quantification of TH-fibre density by optical densitometry; c) Immunohistochemistry of TH on coronal sections of the substantia nigra; d) Intranigral delivery of CDNF fails to protect nigral dopamine neurons against 6-OHDA toxicity but both MANF and GDNF are protective compared to GFP-control animals. Newman-Keuls post-hoc test * $p < 0.05$; **$p < 0.01$; ***$p < 0.001$, n≥6 per group.

Figure 7. Processing of CDNF-t2a-MANF. a) translation of the CDNF-t2a-MANF gene as one single polypeptide; b) removal of signal peptides (SP) and t2a-induced self-cleaving yields four peptidic fragments, including CDNF+19 aa_s and native MANF; c) Increased intracellular CDNF and MANF as well as secreted CDNF with higher Lenti.CDNF-t2a-MANF-GFP MOI (+ = positive control).

Figure 8. Nigral delivery of Lenti.CDNF-t2a-MANF-GFP (Lenti.CtM) vector. Intranigral Lenti.CtM-GFP reduces the number of ipsilateral rotations, increases the TH-striatal innervation and increases the number of surviving neurons at weeks post lesion. a) Apomorphine induced rotations at 2, 4, 6 and 8 weeks post lesion; b) Amphetamine induced rotations at 8 weeks post lesion; c) Striatal TH-fibre density at 8 weeks post viral transduction; TH immunohistochemistry on coronal sections of the corpus striatum (Bregma +0.6), representative sections of Lenti.GFP and Lenti.CtM-GFP delivered into the nigra; d) Quantification of TH-fibre density by optical densitometry; e) Immunohistochemistry of TH on coronal sections of the substantia nigra; f) Intranigral delivery of CtM protects nigral dopamine neurons against 6-OHDA toxicity compared to GFP-control animals. *T-test;* $*p < 0.05$; $p < 0.01$**; ***$p < 0.001$, n≥6 per group.

Supplementary figure legends

Supplementary Figure 1 - Primer sequences. Forward primers contain 17-22bp complementary to the beginning of the target gene and the consensus kozak sequence with a XhoI restriction site immediately upstream of the starting codon (green). Reverse primers contain 17-22bp complementary to the end of the target gene and a SpeI restriction site immediately after the stop codon (red). For illustrative purposes, the sequence of reverse primers in the table is the reverse-complement of the actual PCR primers. The CDNF-t2a-MANF insert was generated by overlap-extension PCR involving three steps. PCR-I: (primers I & II) a XhoI restriction site and the sequence of the t2a self-cleaving peptide are introduced by PCR flanking the hsCDNF gene. PCR-II: (primers III & IV) a SpeI restriction site and the sequence of the t2a self-cleaving peptide are introduced by PCR flanking the hsMANF gene. Extension PCR: (primers I & IV) XhoI-hsCDNF-t2a and t2a-hsMANF-SpeI are used as a template for a final PCR yielding the full-length DNA construct. PCR settings are detailed on Table S2

Suppl Figure 2 - Insert sequences. Annotated sequences of the inserts cloned into the pRRL-sffv-eGFP-cmv backbone.

Suppl Figure 3 - Experimental design. Nigro-striatal lesions were performed by unilateral injection of 10μg 6-OHDA in two sites of the cortex striatum. On the same day, neurotrophic factors (NTF) were delivered into the Cortex striatum or the Substantia nigra. All stereotactic coordinates are given relative to Bregma and Dura (AP: antero-posterior, ML: medio-lateral, DV: dorso-ventral) The integrity of the nigro-striatal dopaminergic system was assessed using amphetamine and

apomorphine induced rotations. Animals were euthanized 8 weeks after the surgical procedure.

Suppl Figure 4 - Schematic of drug-induced rotations in the hemilesioned rat. Left: Amphetamine induces dopamine release from surviving dopaminergic neurons. DA release is higher in the intact hemisphere and this imbalance results in ipsilateral rotations. Right: lesioned hemisphere is hyper-responsive to dopamine. Apomorphine, a dopamine receptor agonist causes over-activation in the lesioned hemisphere and leads to contralateral rotations. Right hemisphere: lesion side

Suppl Figure 5- Lentiviral vector transduction spread. Immuno-fluorescence on 40μm coronal sections at 8 weeks following unilateral preterminal lesion with 6-OHDA. Lentiviral transduction in the cortex striatum following striatal delivery of a) Lenti.CDNF-GFP or b) Lenti.MANF-GFP. Lentiviral transduction in the substantia nigra following intranigral delivery of c) Lenti.CDNF-GFP or b) Lenti.MANF-GFP. Sections were stained for CDNF or MANF (Red), GFP (Green) and DNA (Blue). Scale bars=100μm

Acknowlegments

This study was funded by Parkinson's UK (grant numbers K-1104, G-0915) and by the Elizabeth Blackwell Institute, University of Bristol (MAC). BCH was supported by an MRC Industrial CASE Studentship. RJYM received financial support from the 7th EU Framework Programme (Neugene; Grant Agreement No. 222925). The project was conceived and directed by LFW and MAC. All surgical procedures, behavioural analysis, lentiviral vector production and validation were performed by OCL and BCH. Immunohistochemical analysis and stereological counts were performed by OCL, BCH and FR. HT carried out the VM cultures and neurite outgrowth assays. RJYM and JBU contributed with novel reagents and their lentiviral vector expertise. The manuscript was written by OCL, LFW and MAC. All authors contributed to the edition and revision of the final article.

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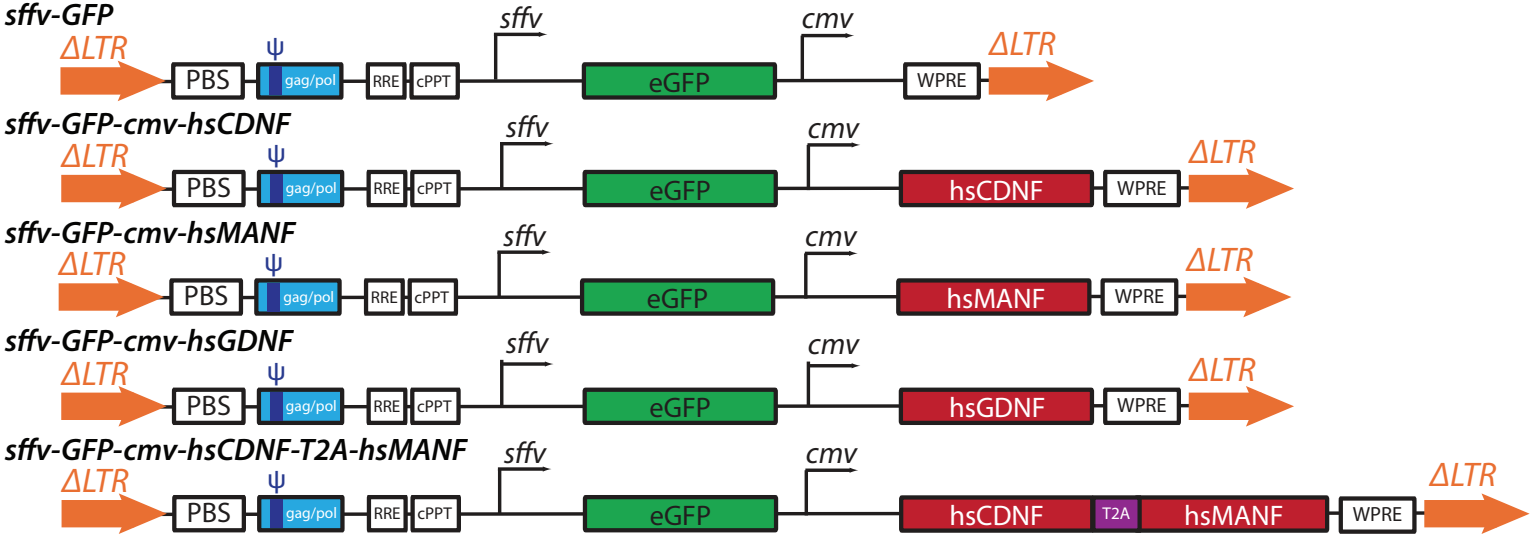
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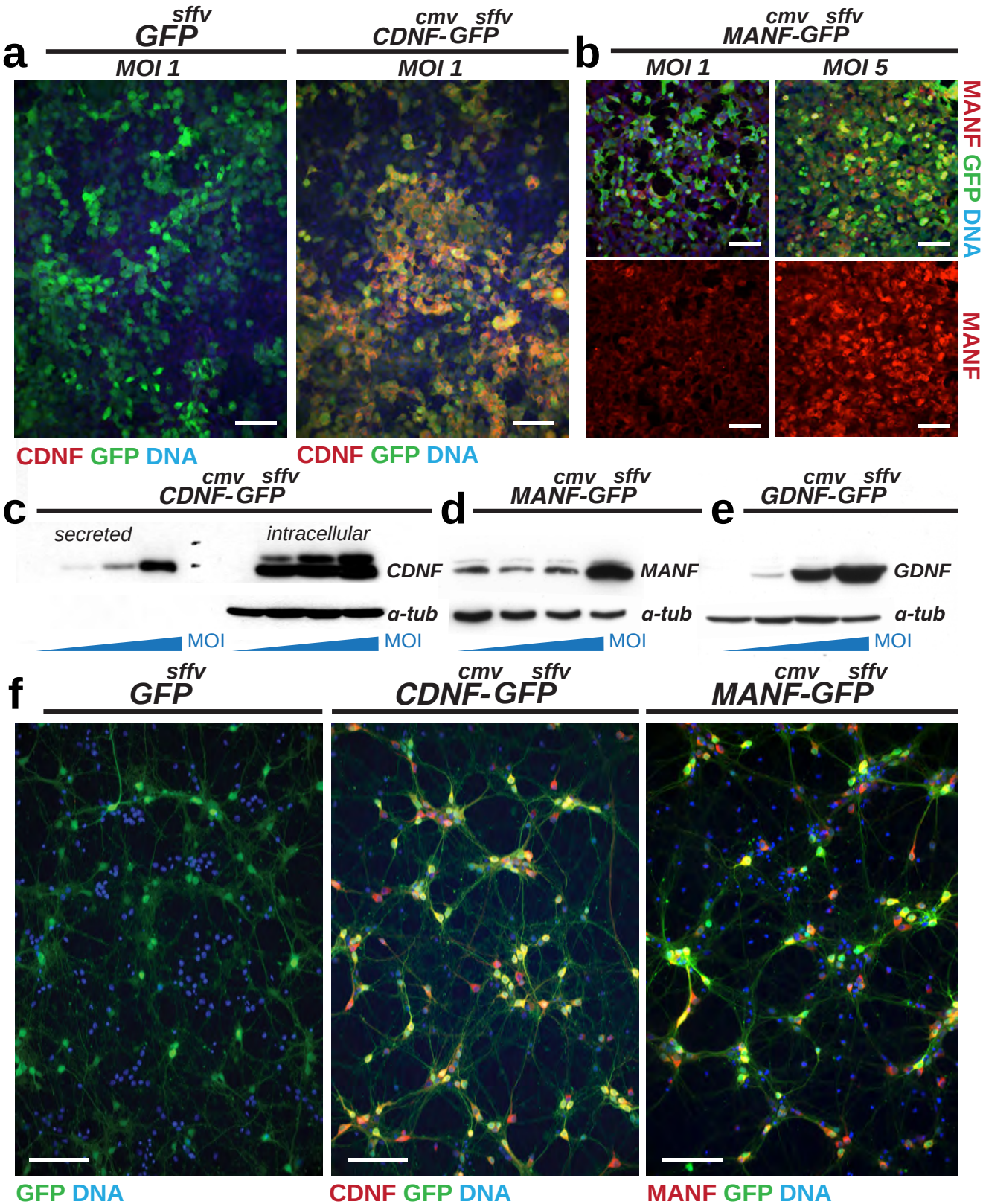
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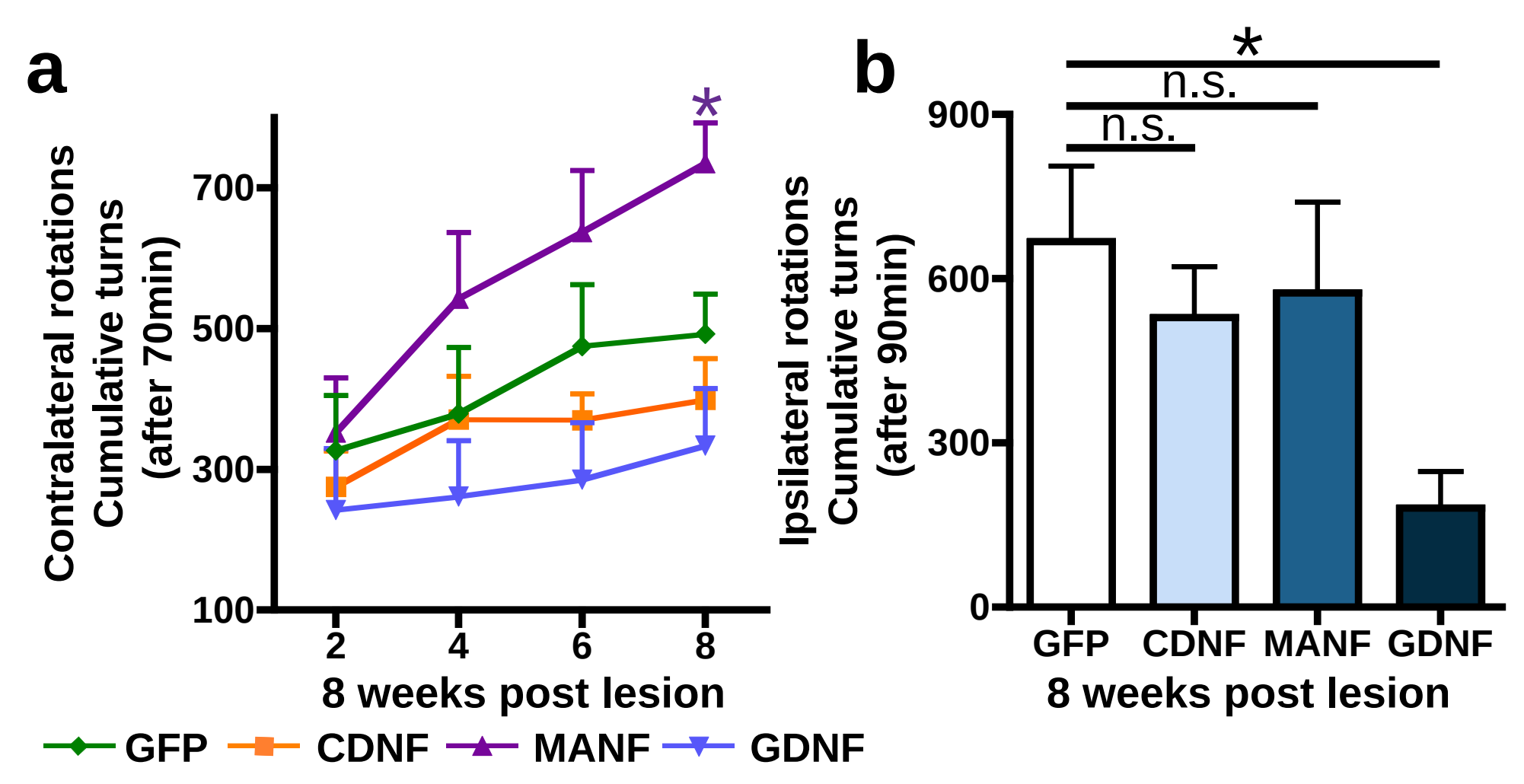
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Cumulative turns
(after 70min)
700
500
300
100
2
4
6
8
8 weeks post lesion
*
GFP
CDNF
MANF
GDNF
b
Ipsilateral rotations
Cumulative turns
(after 90min)
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600
300
0
n.s.
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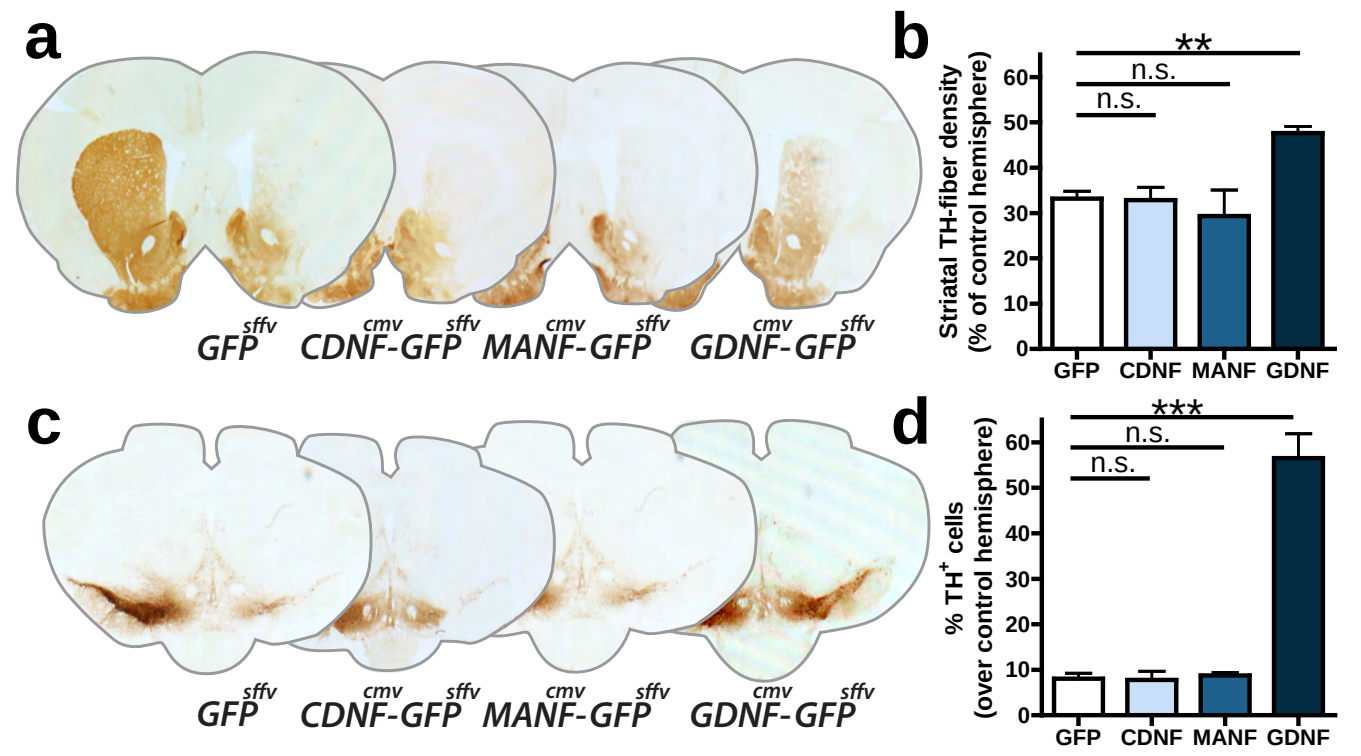
8 weeks post lesion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GFP sffv
CDNF-GFP cmv sffv
MANF-GFP cmv sffv
GDNF-GFP cmv sffv
b
Striatal TH-fiber density
(% of control hemisphere)
**
n.s.
n.s.
60
50
40
30
20
10
0
GFP
CDNF
MANF
GDNF
c
GFP sffv
CDNF-GFP cmv sffv
MANF-GFP cmv sffv
GDNF-GFP cmv sffv
d
% TH+ cells
(over control hemisphere)
***
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n.s.
60
50
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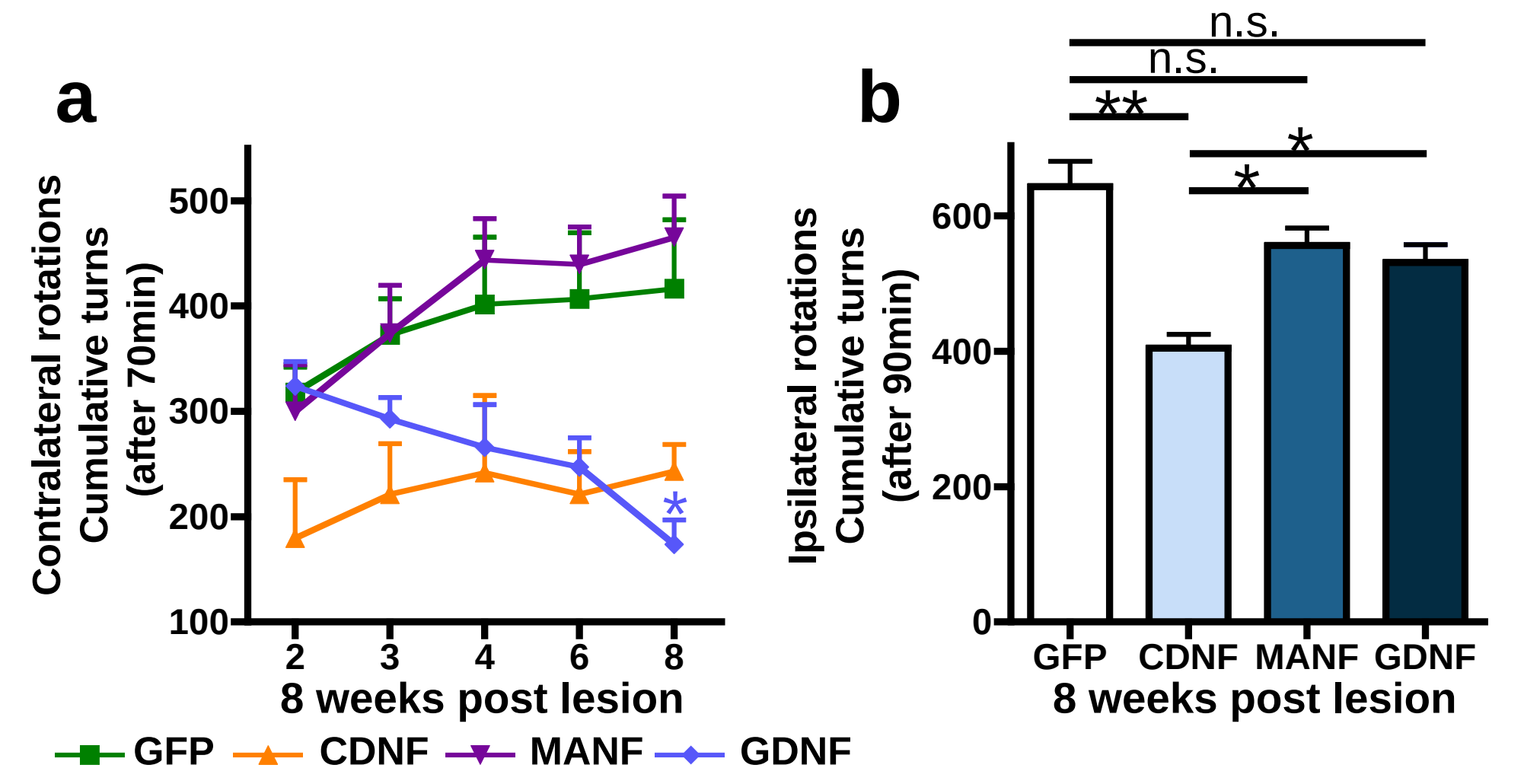
CDNF
MANF
GDNF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Contralateral rotations
Cumulative turns
(after 70min)
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8 weeks post lesion
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GFP
CDNF
MANF
GDNF
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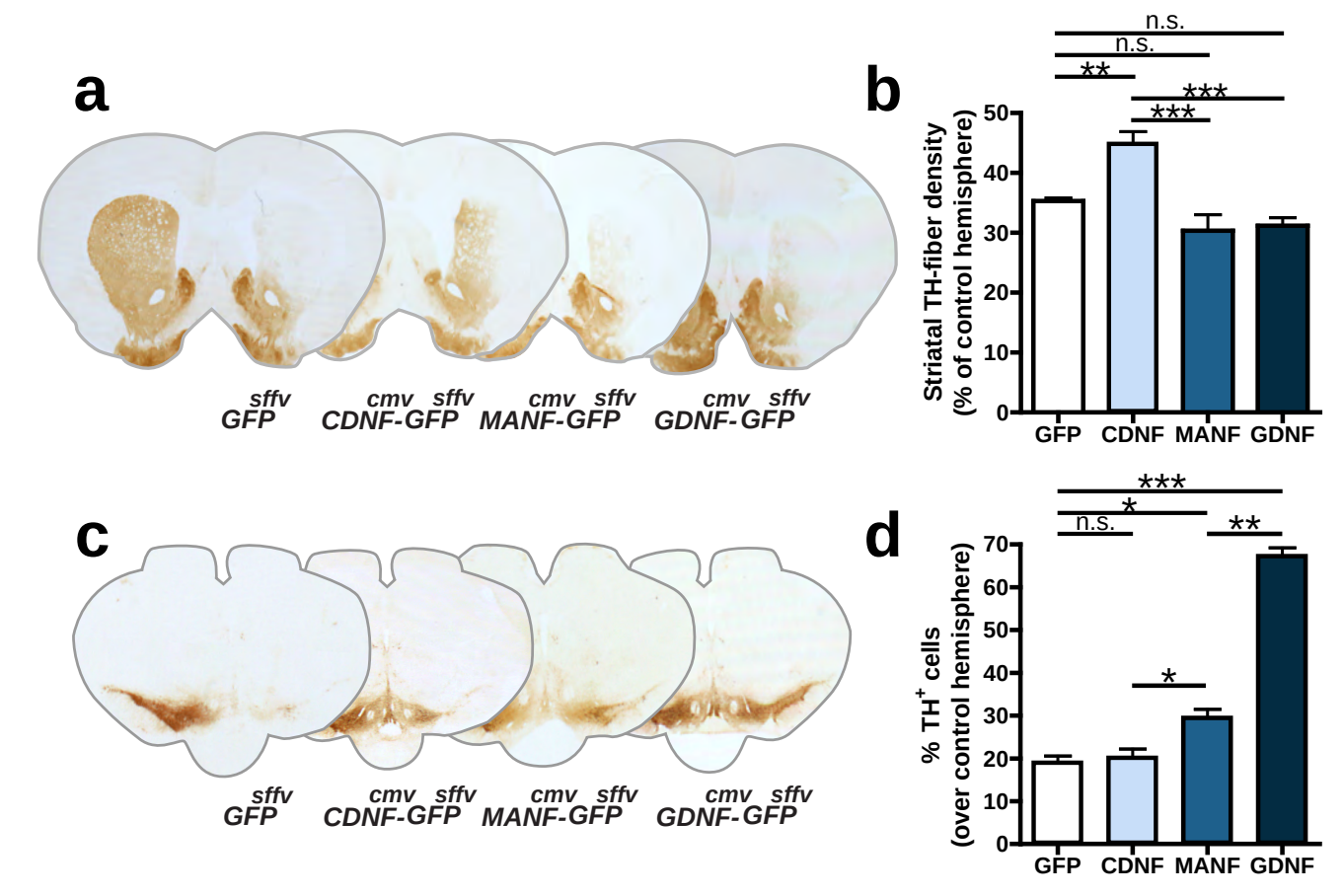
Ipsilateral rotations
Cumulative turns
(after 90min)
600
400
200
0
GFP
CDNF
MANF
GDNF
8 weeks post lesion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sffv GFP
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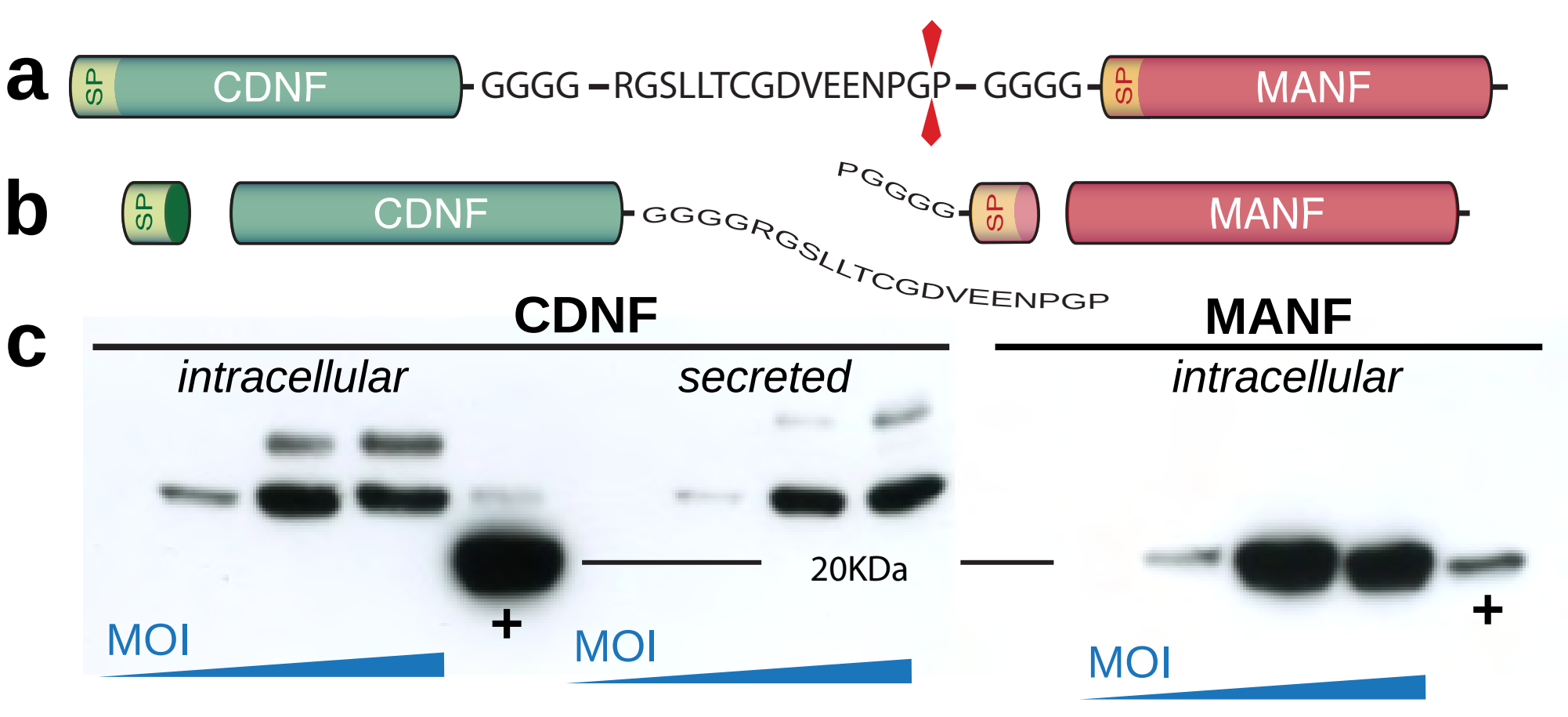
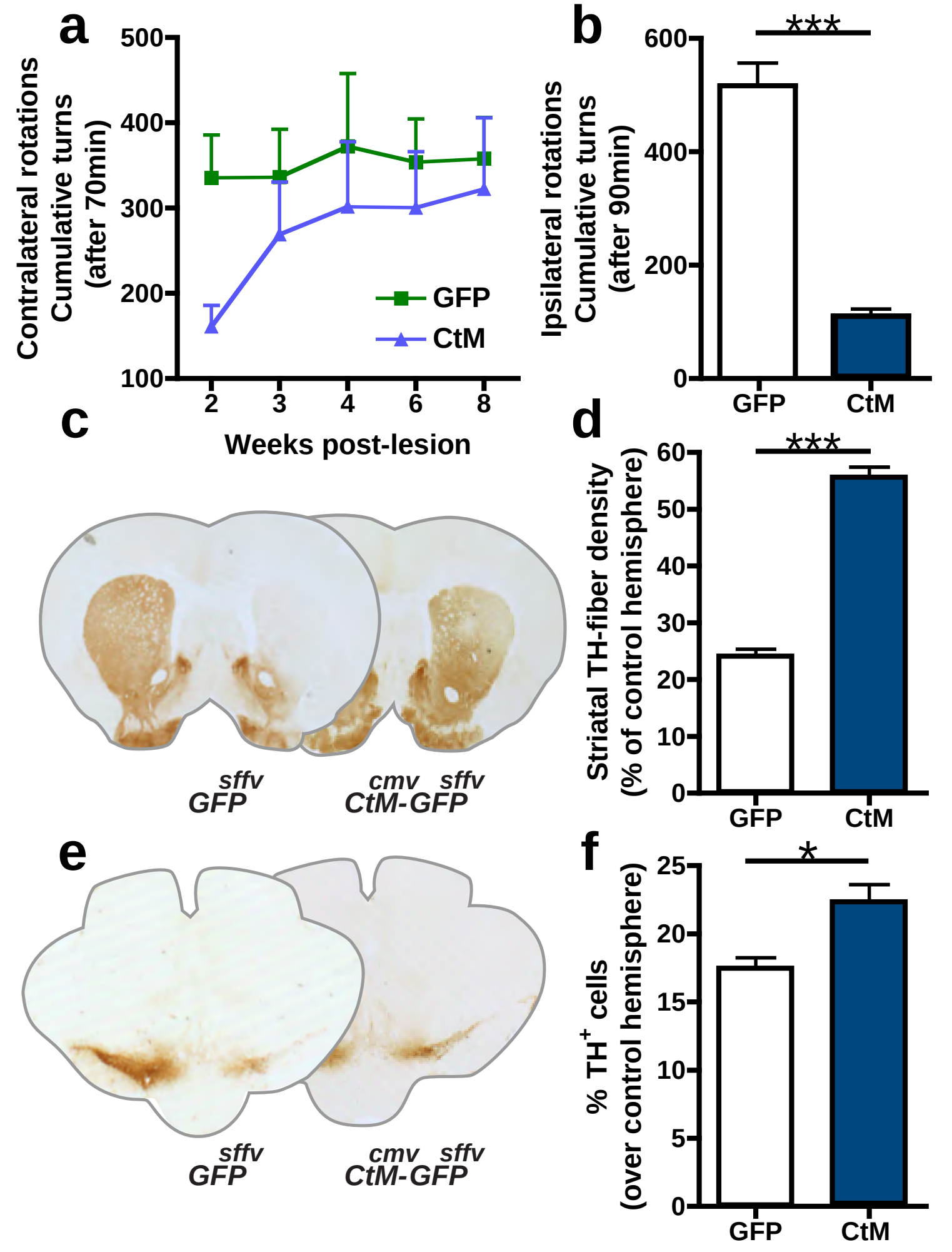
cmv sffv GDNF-GFP
b
n.s.
n.s.
**
***
***
Striatal TH-fiber density (% of control hemisphere)
50
40
30
20
10
0
GFP
CDNF
MANF
GDNF
c
sffv GFP
cmv sffv CDNF-GFP
cmv sffv MANF-GFP
cmv sffv GDNF-GFP
d
***
*
n.s.
**
*
% TH+ cells (over control hemisphere)
70
60
50
40
30
20
10
0
GFP
CDNF
MANF
GDNF


a
SP
CDNF
GGGG
RGSLLTCGDVEENPGP
GGGG
SP
MANF
b
SP
CDNF
GGGGRGSLLTCGDVEENPGP
PGGGG
SP
MANF
c
CDNF
MANF
intracellular
secreted
intracellular
20KDa
+
+
MOI
MOI
MOI


a
Contralateral rotations
Cumulative turns
(after 70min)
500
400
300
200
100
2
3
4
6
8
Weeks post-lesion
GFP
CtM
b
Ipsilateral rotations
Cumulative turns
(after 90min)
***
600
400
200
0
GFP
CtM
c
sffv
GFP
cmv sffv
CtM-GFP
d
Striatal TH-fiber density
(% of control hemisphere)
***
60
50
40
30
20
10
0
GFP
CtM
e
sffv
GFP
cmv sffv
CtM-GFP
f
% TH+ cells
(over control hemisphere)
*
25
20
15
10
5
0
GFP
CtM

S1-Primer sequences

Cloning primers I (5'⟶3')

hsCDNF forward	GA CTCGAG (XhoI) GCCACC (kozak) **ATG**TGGTGCGCGAGCCCA
hsCDNF reverse	CCCCAAAACAGAGCTC**TGA** ACTAGT (SpeI) GCC
hsGDNF forward	GA CTCGAG (XhoI) GCCACC (kozak) **ATG**AAGTTATGGGATGTC
hsGDNF reverse	GCTAAAAGGTGTGGATGTATC**TGA** ACTAGT (SpeI) GC
hsMANF forward	GAATT CTCGAG (XhoI) GCCACC (kozak) **ATG**TGGGCCACGCAGGGGC
hsMANF reverse	GTGCACGGACCGATTTG**TAG** ACTAGT (SpeI) GCGCCGCG

Cloning primers II (5'⟶3')

Primer I - XhoI-hsCDNF: GA CTCGAG (XhoI) GCCACC (kozak) **ATG**TGGTGCGCGAGCCCA

Primer II - hsCDNF-$(Gly)_4$-t2a

GCAGCGACACACCCCAAAACAGAGCTC GGAGGAGGAGGA (Glynk) GAGGGCCGCGGCAGCCTGCTGACCTGCGGCGACGTGGAGG (t2a self-cleaving peptide)

TGA

Primer III - t2a-$(Gly)_4$-hsMANF

GCCTGCTGACCTGCGGCGACGTGGAGGAGAACCCCGGCCCC (t2a self-cleaving peptide) GGAGGAGGAGGA (Glynk) **ATG**TGGGCCACGCAGGGGCTGGCGGTG

Primer IV - hsMANF-SpeI: GTGCACGGACCGATTTG**TAG** ACTAGT (SpeI) GCGCCGCG

S2-Insert sequences

XhoI-hsCDNF-SpeI

XhoI Kozak Start

CTCGAG GCCACC ATGTGGTGCGCGAGCCCAGTTGCTGTGGTGGCCTTTTGCGCCGGGCTTTTGGTCTCTCACCCGG
TGCTGACGCAGGGCCAGGAGGCCGGGGGGCGGCCAGGGGCCGACTGTGAAGTATGTAAAGAATTCTTGAACCGAT
TCTACAAGTCACTGATAGACAGAGGAGTTAACTTTTCGCTGGACACTATAGAGAAAGAATTGATCAGTTTTTGCTTGG
ACACCAAAGGAAAAGAAAACCGCCTGTGCTATTATCTAGGAGCCACAAAAGACGCAGCCACAAAGATCCTAAGTGA
AGTCACTCGCCCAATGAGTGTGCATATGCCTGCAATGAAGATTTGTGAGAAGCTGAAGAAGTTGGATAGCCAGATCTG
TGAGCTGAAATATGAAAAAACACTGGACTTGGCATCAGTTGACCTGCGGAAGATGAGAGTGGCAGAGCTGAAGCAG
ATCCTGCATAGCTGGGGGGAGGAGTGCAGGGCCTGTGCAGAAAAAACTGACTATGTGAATCTCATTCAAGAGCTGG
CCCCCAAGTATGCAGCGACACACCCCAAAACAGAGCTCTGA ACTAGT
Stop SpeI

XhoI-hsGDNF-SpeI

XhoI Kozak Start

CTCGAG GCCACC ATGAAGTTATGGGATGTCGTGGCTGTCTGCCTGGTGCTGCTCCACACCGCGTCCGCCTTCCCGCT
GCCCGCCGGTAAGAGGCCTCCCGAGGCGCCCGCCGAAGACCGCTCCCTCGGCCGCCGCCGCGCGCCCTTCGCGCTG
AGCAGTGACTCAAATATGCCAGAGGATTATCCTGATCAGTTCGATGATGTCATGGATTTTATTCAAGCCACCATTAAAA
GACTGAAAAGGTCACCAGATAAACAAATGGCAGTGCTTCCTAGAAGAGAGCGGAATCGGCAGGCTGCAGCTGCCA
ACCCAGAGAATTCCAGAGGAAAAGGTCGGAGAGGCCAGAGGGGCAAAAACCGGGGTTGTGTCTTAACTGCAATAC
ATTTAAATGTCACTGACTTGGGTCTGGGCTATGAAACCAAGGAGGAACTGATTTTTAGGTACTGCAGCGGCTCTTGC
GATGCAGCTGAGACAACGTACGACAAAATATTGAAAAACTTATCCAGAAATAGAAGGCTGGTGAGTGACAAAGTAG
GGCAGGCATGTTGCAGACCCATCGCCTTTGATGATGACCTGTCGTTTTTAGATGATAACCTGGTTTACCATATTCTAAG
AAAGCATTCCGCTAAAAGGTGTGGATGTATCTGA ACTAGT
Stop SpeI

XhoI-hsMANF-SpeI

XhoI Kozak Start

CTCGAG GCCACC ATGTGGGCCACGCAGGGGCTGGCGGTGGCGCTGGCTCTGAGCGTGCTGCCGGGCAGCCGGGC
GCTGCGGCCGGGCGACTGCGAAGTTTGTATTTCTTATCTGGGAAGATTTTACCAGGACCTCAAAGACAGAGATGTCA
CATTCTCACCAGCCACTATTGAAAACGAACTTATAAAGTTCTGCCGGGAAGCAAGAGGCAAAGAGAATCGGTTGTGC
TACTATATCGGGGCCACAGATGATGCAGCCACCAAAATCATCAATGAGGTATCAAAGCCTCTGGCCCACCACATCCCT
GTGGAGAAGATCTGTGAGAAGCTTAAGAAGAAGGACAGCCAGATATGTGAGCTTAAGTATGACAAGCAGATCGACC
TGAGCACAGTGGACCTGAAGAAGCTCCGAGTTAAAGAGCTGAAGAAGATTCTGGATGACTGGGGGGAGACATGCA
AAGGCTGTGCAGAAAAGTCTGACTACATCCGGAAGATAAATGAACTGATGCCTAAATATGCCCCCAAGGCAGCCAGT
GCACGGACCGATTTGTAG ACTAGT
Stop SpeI

XhoI-hsCDNF-Glynk-*T2A*-Glynk-hsMANF-SpeI

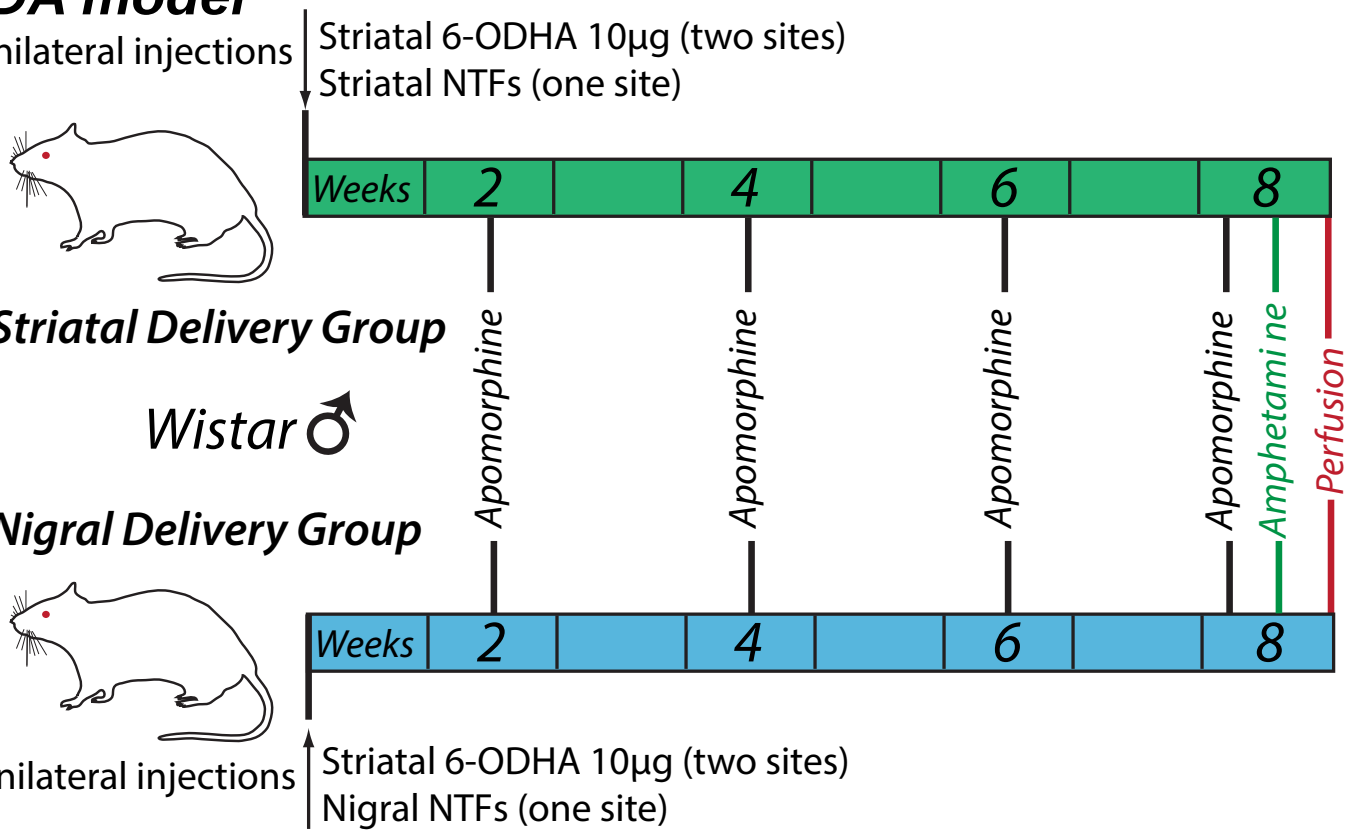
XhoI Kozak Start

CTCGAG GCCACC ATGTGGTGCGCGAGCCCAGTTGCTGTGGTGGCCTTTTGCGCCGGGCTTTTGGTCTCTCACCCGG
TGCTGACGCAGGGCCAGGAGGCCGGGGGGCGGCCAGGGGCCGACTGTGAAGTATGTAAAGAATTCTTGAACCGAT
TCTACAAGTCACTGATAGACAGAGGAGTTAACTTTTCGCTGGACACTATAGAGAAAGAATTGATCAGTTTTTGCTTGG
ACACCAAAGGAAAAGAAAACCGCCTGTGCTATTATCTAGGAGCCACAAAAGACGCAGCCACAAAGATCCTAAGTGA
AGTCACTCGCCCAATGAGTGTGCATATGCCTGCAATGAAGATTTGTGAGAAGCTGAAGAAGTTGGATAGCCAGATCT
GTGAGCTGAAATATGAAAAAACACTGGACTTGGCATCAGTTGACCTGCGGAAGATGAGAGTGGCAGAGCTGAAGCA
GATCCTGCATAGCTGGGGGGAGGAGTGCAGGGCCTGTGCAGAAAAAACTGACTATGTGAATCTCATTCAAGAGCTG
GCCCCCAAGTATGCAGCGACACACCCCAAAACAGAGCTC *GGAGGAGGAGGA* ***GAGGGCCGCGGCAGCCTGCTGACC***
TGCGGCGACGTGGAGGAGAACCCCGGCCCC *GGAGGAGGAGGA* ATGTGGGCCACGCAGGGGCTGGCGGTGGCGCT
GGCTCTGAGCGTGCTGCCGGGCAGCCGGGCGCTGCGGCCGGGCGACTGCGAAGTTTGTATTTCTTATCTGGGAAGA
TTTTACCAGGACCTCAAAGACAGAGATGTCACATTCTCACCAGCCACTATTGAAAACGAACTTATAAAGTTCTGCCGG
GAAGCAAGAGGCAAAGAGAATCGGTTGTGCTACTATATCGGGGCCACAGATGATGCAGCCACCAAAATCATCAATGA
GGTATCAAAGCCTCTGGCCCACCACATCCCTGTGGAGAAGATCTGTGAGAAGCTTAAGAAGAAGGACAGCCAGATAT
GTGAGCTTAAGTATGACAAGCAGATCGACCTGAGCACAGTGGACCTGAAGAAGCTCCGAGTTAAAGAGCTGAAGAA
GATTCTGGATGACTGGGGGGAGACATGCAAAGGCTGTGCAGAAAAGTCTGACTACATCCGGAAGATAAATGAACTG
ATGCCTAAATATGCCCCCAAGGCAGCCAGTGCACGGACCGATTTGTAG ACTAGT
Stop SpeI

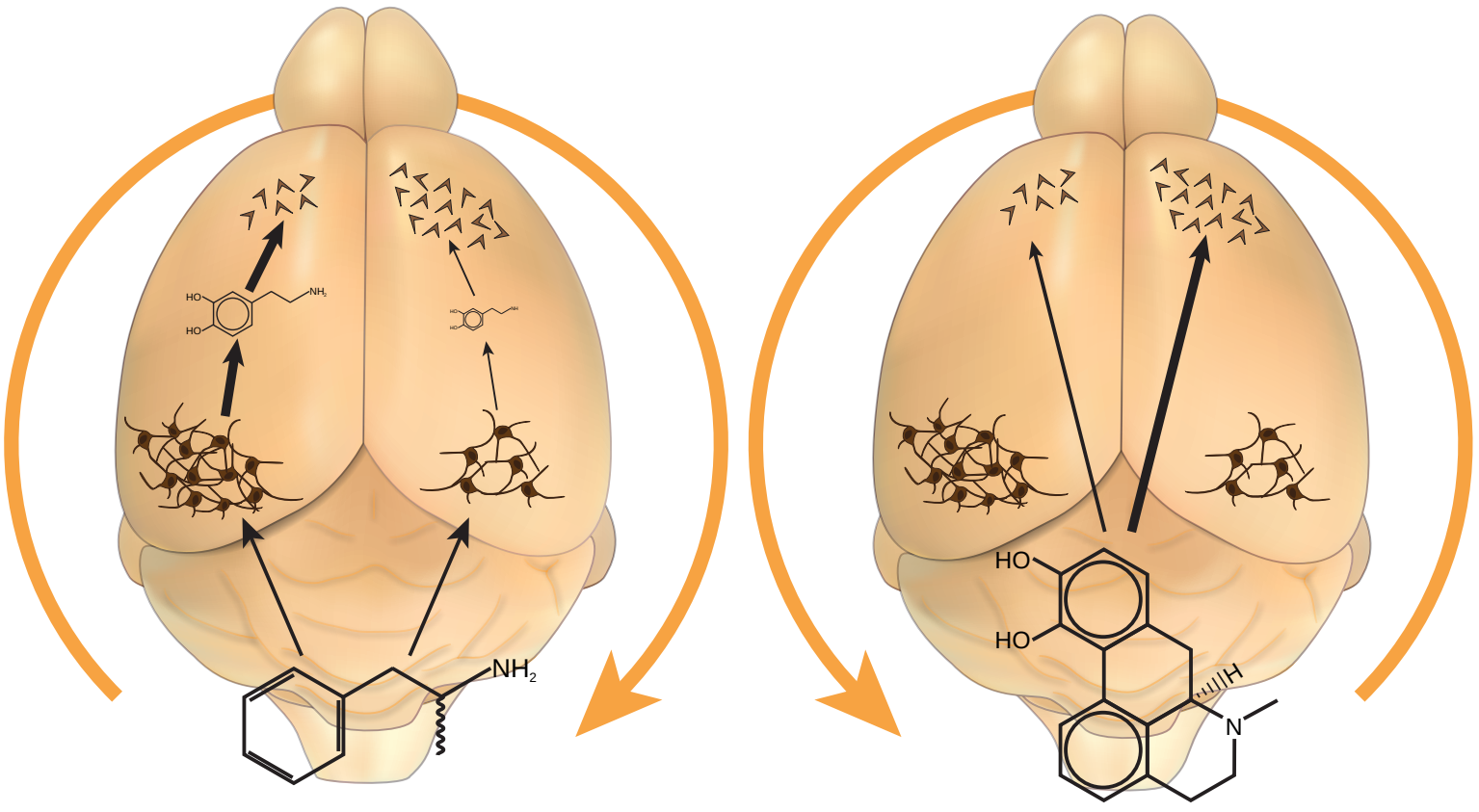
S3
Experimental design

Stereotactic coordinates (mm)	AP	ML	DV
6-OHDA 2X Unilateral injection (10µg)	0	-2.6	-5
	-1.2	-3.9	-5
NTF Striatal Delivery	-0.6	-3.3	-5
NTF Nigral Delivery	-5.3	-2.2	-7.2
AAV-α-Synuclein Nigral Delivery	-5.3	-2.2	-7.2

6-OHDA model



S4 Ipsilateral vs. Contralateral rotations



D/L-Amphetamine
(Ipsilateral rotations)

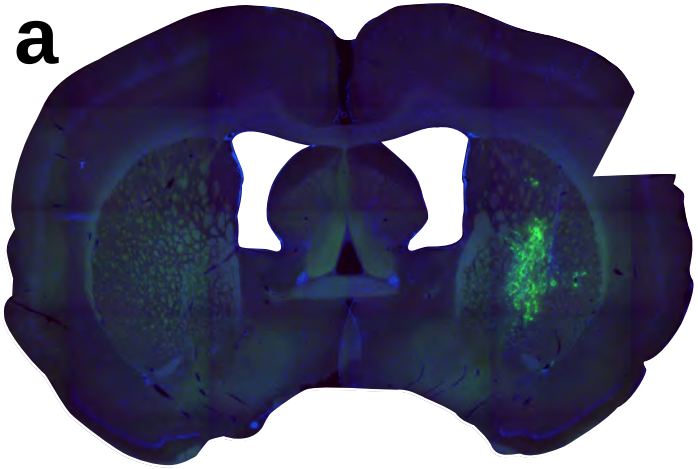
Apomorphine
(Contralateral rotations)

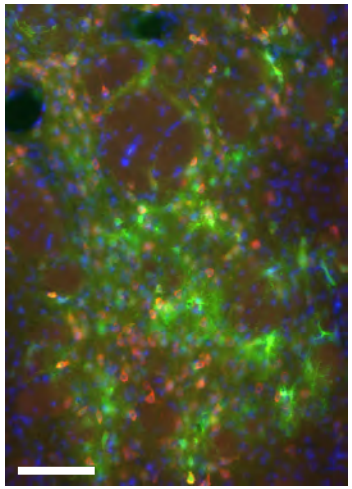
S5 Viral Spread in lesioned hemisphere

STRIATAL DELIVERY

cmv sffv
CDNF- GFP

a

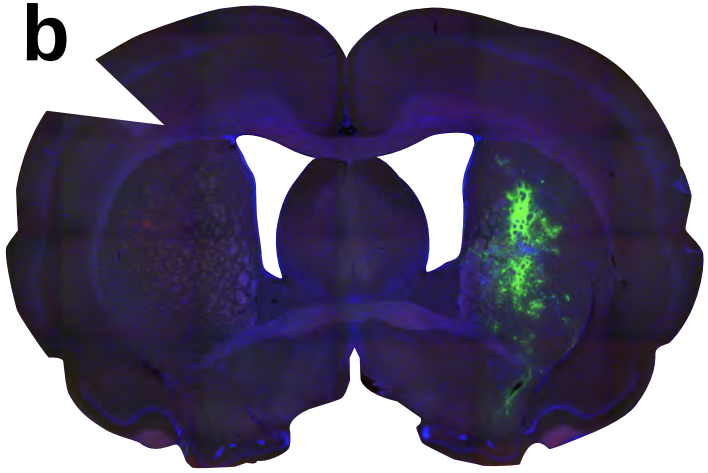


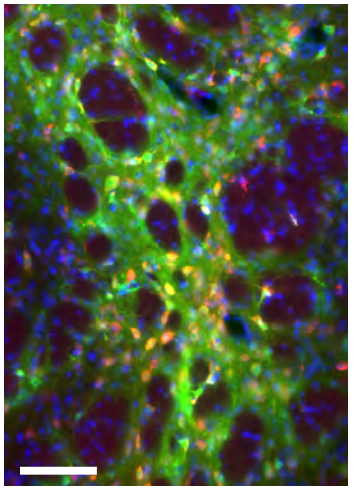


CDNF GFP DNA

cmv sffv
MANF-GFP

b



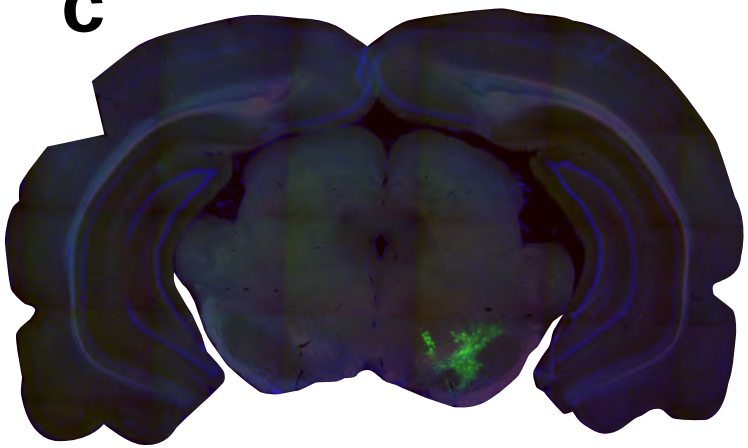


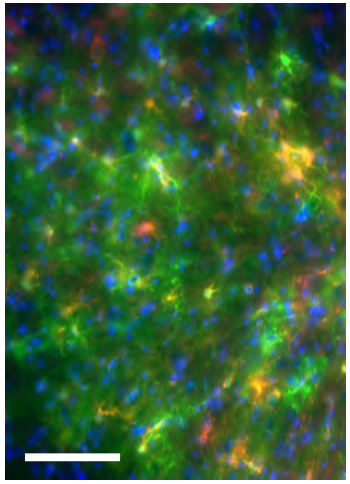
MANF GFP DNA

NIGRAL DELIVERY

cmv sffv
CDNF- GFP

c

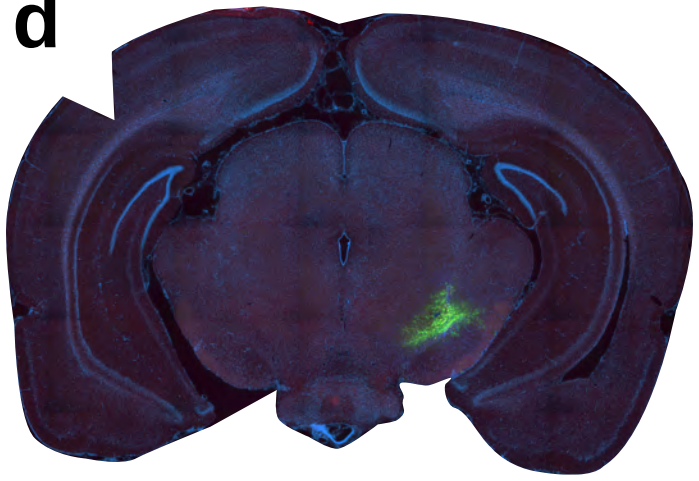


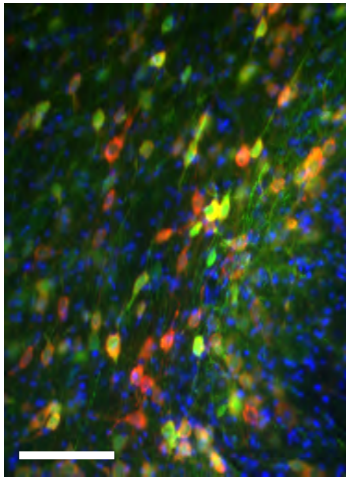


CDNF GFP DNA

cmv sffv
MANF-GFP

d





MANF GFP DNA