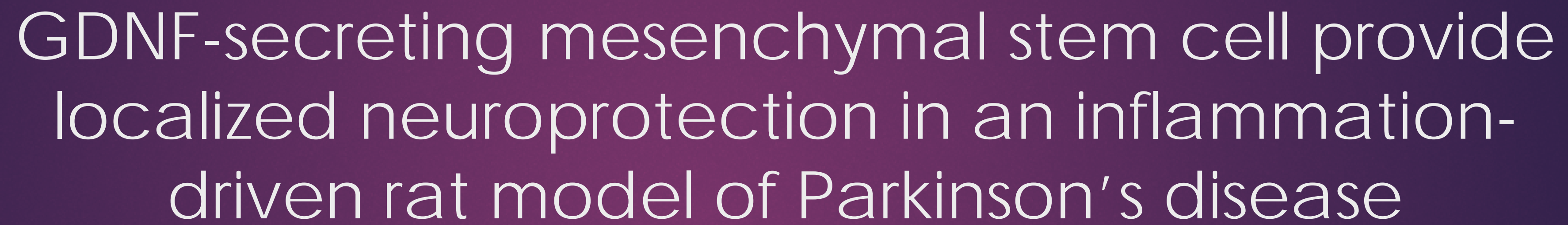

GDNF-secreting mesenchymal stem cell provide localized neuroprotection in an inflammation-driven rat model of Parkinson′s disease

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INTRODUCTION

Parkinson's disease

- progressive neurodegenerative disorder characterized by progressive loss of dopaminergic neurons from the nigrostriatal pathway
- symptoms include resting tremor, bradykinesia, rigidity and postural instability
- current therapies provide only symptomatic relief
- disease modifying therapy remains a vital clinical need

GDNF – glial cell line-derived neurotrophic factor

- **Neurotrophic factor,** in particular **GDNF** (glial cell line-derived neurotrophic factor), have emerged as a promising therapeutic candidate
- Distinct protective effects on survival of midbrain dopaminergic neurons in both *in vitro* and *in vivo* studies
- Neuroprotective and neuroregenerative effects in preclinical trials
- Improvement in motor function, partial restoration of the nigrostriatal pathway and reduction in medication induced dyskinesia (GDNF infused directly in the putamen via catheters, open-label human clinical trial)
- Lack of efficacy in randomized, double-blinded, placebo-controlled trial - ??? negative impact of delivery method : intracerebral infusion ???

GDNF-transduced MSCs

- Alternative approach for delivery of neurotrophin
- Ex vivo gene therapy
- Bone marrow-derived mesenchymal cells (MSCs) are genetically engineered to overexpress GDNF
- Transduction with the retrovirus – GDNF stably integrated into the host genome
- Rationale for the utilization of MSCs : previously exploited for regenerative and reparative effects in neurodegenerative diseases (neutrophic growth factors, chemokines, cytokines, extracellular matrix proteines), easily cultured and expanded, the ability to be easily genetically manipulated

The aim of the study

- To assess the neurotrophic potential of GDNF-transduced MSCs in inflammation-driven LPS model of Parkinson′s disease

- Specifically, to assess the ability of GDNF-MSCs to protect against neuroinflammation, neurodegeneration and behavioral impairment

Experimental design

- 30 male Spraque-Dawley rats, all of which underwent habituation and baseline testing on Stepping and Whisker test
- 3 groups: LPS lesion only, LPS lesion + GFP-MSCs, LPS lesion + GDNF-MSCs (n=10 per group)
- Transplanted rats received unilateral injection of GDNF-MSCs or GFP-MSCs (control) into left striatum
- One day post transplantation all groups received unilateral infusion of LPS into the left substantia nigra
- Behavioral testing were performed the day after cell transplant and continued for 14 days (to assess the functional protection)
- The animals were sacrificied on day 21 post-transplant and day 20 post-LPS
- Post mortem assessment of nigrostriatal neurodegeneration, microgliosis and grafted cell survival was made using quantitative immunohistochemistry and image analysis (to assess the anatomical protection)
- All behavioral testing and quantitative immunohistochemistry were completed blinded to the treatment of the rats

Animals and MSC isolation

- Animals were housed in groups of four per cage, on a 12:12 –h light:dark cycle, at 19-23°C, with the humidity level between 40%-70% and food and water available *ad libitum*
- Bone-marrow-derived MSCs were isolated from the femora and tibiae bone marrow of 8-12-weeks-old Sprague-Dawley GFP transgenic rats and were characterized as MSCs
- GFP-MSCs were virally transduced with a murine leukemia virus with human GDNF transgene expression
- GDNF-MSCs were transduced with a retrovirus to overexpress the GDNF transgene

GFP- green fluorescence protein – used for labelling and tracking cells

GDNF ELISA

- To assess quantitatively the secretion of GDNF by GFP-MSCs and GDNF-MSCs in vitro (human GDNF ELISA, R&D Systems, Minneapolis, MN, USA)
- $5{,}7 \times 10^{3}$ cells cm^{-2} in T-75 flasks, 24h adherence, 24h incubation with the cells
- GDNF concentration in the conditioned medium was measured
- The assay was performer in triplicate

Behavioral testing

- The Stepping test of forelimb akinesia was perfomed

The rat was held with both hindlimbs and one forelimb restrained, the body of the rat was held in a horizontal position with the unrestrained forelimb on a table top (90 cm in 5 s). Number of adjusting steps was counted on both the ipsilateral and contralateral sides of the rat's body.

- The Whisker test of sensorimotor integration was completed

The rat was held with both hindlimbs and one forelimb restrained. Vibrissae-elicted forelimb placing made by unrestrained forelimb were counted when the rat's whiskers were brushed against the side of a table top. These was completed 10 times on both the ipsilateral and contralateral sides of the rat's body.

Stereotaxic surgery

- Rats were sacrificed on day 21 after transplantation by terminal anesthesia (50mg/kg pentobarbital i.p)
- Cell transplantation surgery: the left striatum was infused unilaterally with two injections of sterile transplantation medium containing either 100,000 GFP-MSCs or 100,000 GDNF-MSCs, grafts were implanted at a total of six different sites, total volume was 3 µl at a rate a 1 µl/min
- Unilateral intra-nigral LPS surgery : one day after cell transplantation, 10 µg in 2 µl sterile saline

Both cell transplants and LPS were delivered to the brain using 30-G stainless steel cannulae attached to a Hamilton syringe. The Hamilton syringe was depressed using an automated pump

Immunohistochemistry

- LPS-induced nigrostriatal degeneration and grafted cell-induces neuroprotection was assessed by **tyrosine hydroxylase immunohistochemistry**
- Microgliosis induced by LPS lesion was assessed using quantitative immunohistochemistry for **OX-42**

Image analysis

- Photomicrographs of immunostained sections were taken using a NIKON DXM1200C digital camera mounted on a microscope
- Image analysis was performed using ImageJ software
- Extent of LPS-induced nigrostriatal neurodegeneration was determinate by:
- optical density of tyrosine hydroxylase staining in three coronal photomicrographs through the striatum,
- the number of tyrosine hydroxylase-positive (TH+) cell bodies was counted staining in three coronal photomicrographs through the substantia nigra

Statistical analysis:

- Behavioral data were analyzed using a 2-way ANOVA followed by post-hoc Bonferroni test
- Immunohistchemical data was analyzed by unpaired student's t-test or one-way ANOVA followed by post-hoc Bonferroni test
- $P < 0.05$

RESULTS

Assessment of GDNF secretion in vitro & GDNF release in vivo:

- The secretion of GDNF in vitro from GDNF-MSCs is significantly higher than the secretion of GDNF from GFP-MSCs (GDNF ELISA) Fig 1-A

- GDNF was released from GDNF-MSCs after implantation in striatum (qualitative photomicrographs, GDNF immunostaining) Fig 1- B

Note: lack of GDNF staining by Day 21!!!

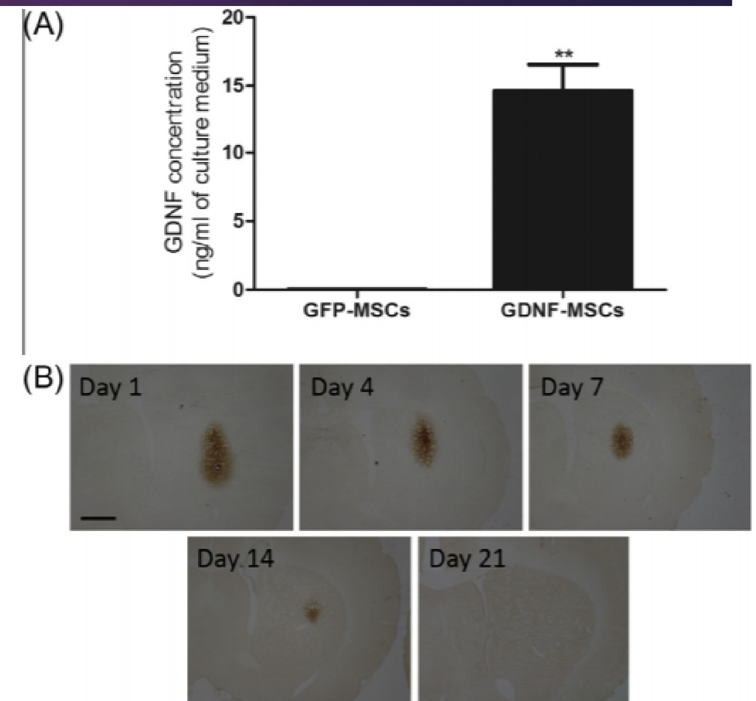

Fig. 1. GDNF release from GFP-MSCs and GDNF-MSCs *in vitro* and *in vivo*. GDNF ELISA (A) revealed that GFP-MSCs are inherently capable of secreting a small amount of GDNF into the culture medium. However, transduction of the cells with a retrovirus to over-express GDNF resulted in the cells secreting significantly more GDNF into the culture medium (**$P < 0.01$ vs. GFP-MSCs, using an unpaired *t*-test). Data are shown as mean ± s.e.m. Qualitative photomicrographs (B) show GDNF immunostaining in the brain at Days 1, 4, 7, 14 and 21 after intra-striatal transplantation of GDNF-MSCs. Note the pronounced GDNF expression at Day 1 (the time-point at which the LPS lesion was given) and the apparent lack of GDNF staining by Day 21 (the time-point of sacrifice for the main study). Scale bar = 500 µm.

Assesment of motor function after LPS lesion

- Intra-nigral LPS administration did not impair ipsilateral motor performance in the Whisker test and the Stepping test Fig 2 A&B
- Intra-nigral LPS administration resulted in a transient contralateral motor impairment in Whisker test and reduced contralateral adjusting steps in the Stepping test Fig 2 C&D
- No functional protection was afforded by the intra-striatal transplantation of either GFP-MSCs or GDNF-MSCs prior to induction of the lesion Fig 2 C&D

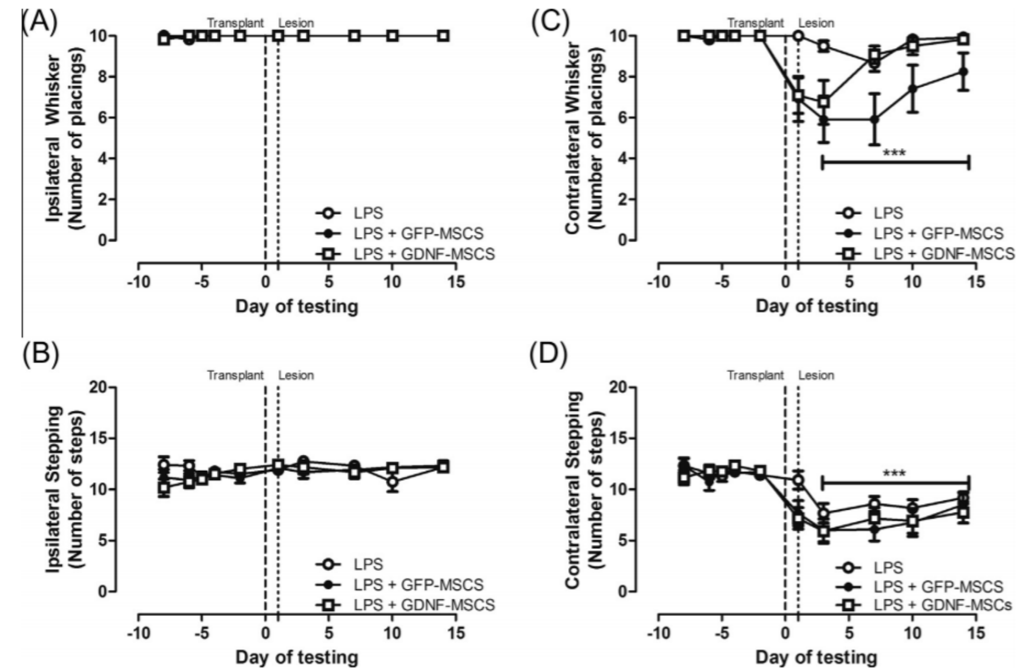

Fig. 2. Impact of GDNF-MSCs on ipsilateral motor function after unilateral intra-nigral LPS administration. Unilateral injection of LPS into the substantia nigra did not cause any ipsilateral dysfunction in the (A) Whisker or (B) Stepping tests of spontaneous motor function. Unilateral injection of LPS into the substantia nigra induced contralateral deficits in the (C) Whisker and (D) Stepping tests (***$P < 0.001$ vs. Baseline by 2 way ANOVA with post hoc Bonferroni). This was not affected by intra-striatal transplantation of MSCs (confirmed by 2-way ANOVA restricted to the post-graft period only). The dashed line at Day 0 indicates the day of cell transplantation surgery, the dotted line at Day 1 indicates the day of lesion surgery. Data are shown as mean ± s.e.m.

Assessment of nigrostriatal microgliosis

- Intra-nigral LPS administration induced localized microgliosis in the substantia nigra at the site of injection (photomicrographs of OX-42 immunohistochemica; staining) FIG 3- A

- Intra-stratial transplantation of neither GFP-MSCs nor GDNF-MSCs had any effect on the lesion-induced nigral microgliosis (spatial separation between the site of lesion and the site of cell transplant) FIG 3

- Intra-striatal transplantation of MSCs did induce microgliosis surrounding the graft site in the striatum

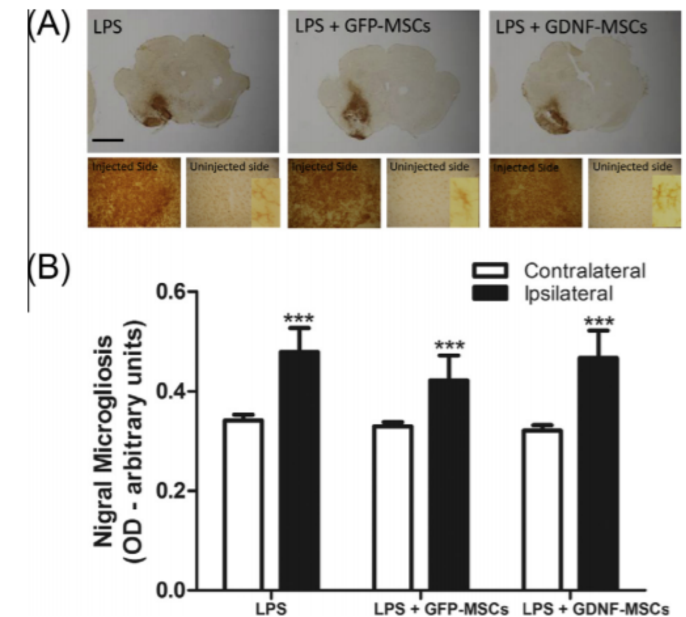

Fig. 3. Impact of GDNF-MSCs on nigrostriatal microgliosis after unilateral intra-nigral LPS administration. (A) Photomicrographs of OX-42 immunohistochemical staining confirmed that injection of LPS into the substantia nigra resulted in pronounced microgliosis at this site. High magnification photographs of uninjected and injected sides are also included. Morphology of resting microglia is shown inset in the uninjected high magnification images. (B) Quantitative analysis confirmed that intra-nigral administration of the inflammagen induced a significant microgliotic reaction at the site of administration (when compared to the intact side), and that this was not affected by intra-striatal transplantation of MSCs. Data are shown as mean ± s.e.m. $^{***}P < 0.001$ vs. Contralateral by 2 way ANOVA with post hoc Bonferroni. Scale bar = 1.5 mm.

Assessment of cell survival:

- Survival of transplanted MSCs was assessed at 21 days post-transplantation (photomicrographs) Fig 4 –A
- Surviving cells were observed in only 40% of the transplanted animals
- The results confirm the progressive death of the cells
- No difference in grafted cell survival between the two transplanted cell types (quantitative analysis) Fig 4-B

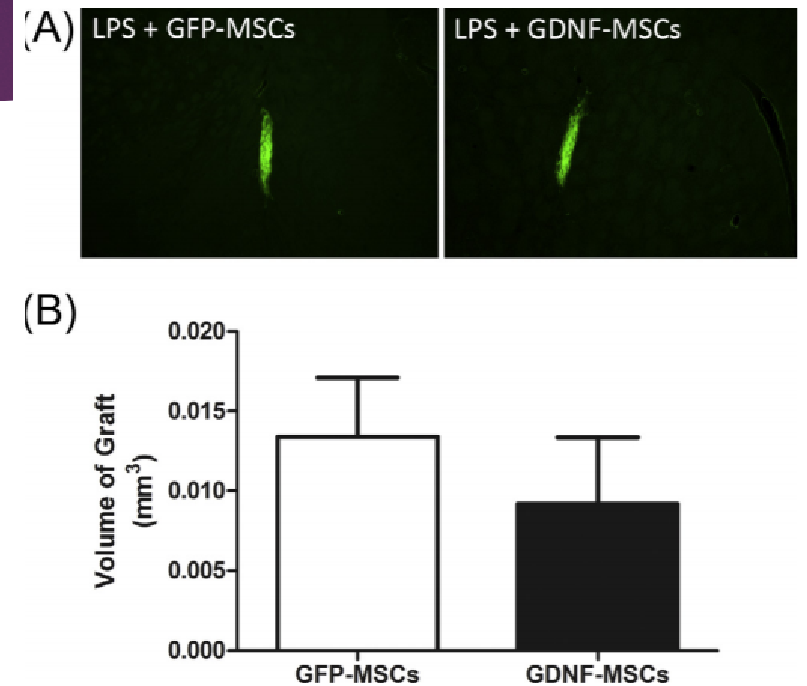

Fig. 4. Survival of transplanted GFP-MSCs and GDNF-MSCs in the LPS-lesioned striatum. (A) Representative photomicrographs showing that transplanted GFP-MSCs and GDNF-MSCs were identifiable *in vivo* due to their strong GFP expression at 21 days post-transplantation. (B) Quantitative analysis of the volume of the graft showed that there was no significant difference in cell survival between either cell type. Data are shown as mean ± s.e.m.

Assessment of LPS-induced nigrostriatal neurodegeneration:

- Intra-nigral injection of LPS result in significant nigrostriatal neurodegenereation at the level of both the cell bodies and the terminals (tyrosine hydroxylase immunohistochemical staining) Fig 5
  - Loss of approximately 20% of dopaminergic cell bodies in substantia nigra and 20% of dopaminergic terminals in the striatum

- Neither the GFP-MSCs nor the GDNF-MSCs were capable of protecting the nigrostriatal pathway as a whole

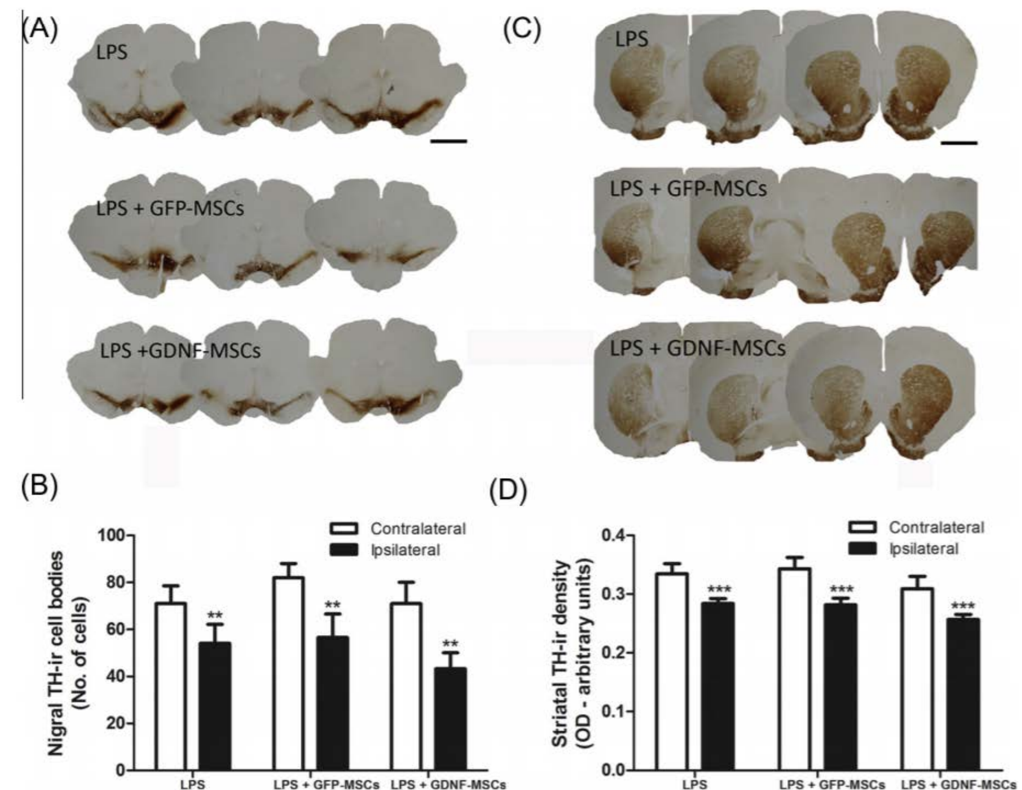

Fig. 5. Impact of GDNF-MSCs on nigrostriatal integrity after unilateral intra-nigral LPS administration of MSCs. (A) Tyrosine hydroxylase immunohistochemical staining revealed the extent of nigrostriatal neurodegeneration after injection of LPS into the substantia nigra (the lesion is on the left side). Photomicrographs show the immunostaining of the cell bodies in the substantia nigra and the terminals of the striatum. (B) Quantitative analysis revealed that neither GFP-MSCs nor GDNF-MSCs were able to protect the dopaminergic cell bodies from degeneration with the number of cell bodies significantly reduced compared to the contralateral intact (right) side. (C) Additionally, neither cell type was capable of protecting the striatal terminals from degeneration with the density of striatal fibers reduced when compared to the contralateral intact (right) side. Data are shown as mean ± s.e.m. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. Contralateral 2 way ANOVA with post hoc Bonferroni). TH-ir: Tyrosine hydroxylase immunoreactivity. Scale bar = 1.5 mm.

Assessment of local neuroprotection in the striatum:

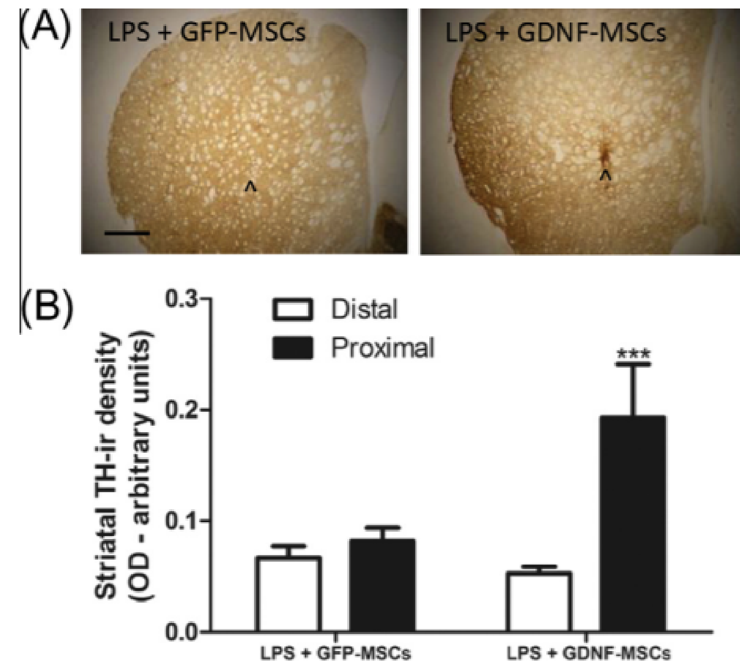

Fig. 6. GDNF-MSCs induced local neuroprotection of dopaminergic terminals in the striatum. (A) Tyrosine hydroxylase-positive staining in the striatum was assessed directly in the vicinity of the transplant site. In animals that received GDNF-MSCs, discrete regions of dense immunoreactive fibers were present in the vicinity of the transplant. This effect was not observed in the animals that received GFP-MSCs. (B) Quantitative analysis of the density of immune-staining in the denervated striatum (distal) compared with the density of immune-staining within 0.5 mm of the graft (proximal) confirmed that this effect was exclusive to the animals transplanted with GDNF-MSCs and not GFP-MSCS. Data are shown as mean ± s.e.m. ***$P < 0.001$ vs. distal by 2 way ANOVA with post hoc Bonferroni. TH-ir: Tyrosine hydroxylase immunoreactivity. Scale bar = 500 µm. The arrowhead indicates the location of the cell graft.

- The tyrosine hydroxylase staining in the striatum focused on vicinity of the transplanted cells (within 0,5 mm of the cell transplant) showed dense pocket of TH-positive staining despite the apparent loss of immunoreactivity in the striarum as a whole Fig 6-A
- The effect was observed only in GDNF-MSCs transplanted group and not in GFP-MSCs group
- Transplanted GDNF-MSCs provided local neuroprotection of dopaminergic terminals in the striatum

Discussion:

- The study was performed at an inflammation-model of Parkinson disease – it is not clear whether neuroinflammation is a cause or consequence of dopamine degeneration
- Separation between the lesion (substantia nigra) and the site of cell implant (striatum)
- no anti-inflammatory and/or immunomodulatory effects on nigral microgliosis
- more consistent improvements in terms of dopamine neuron survival, dopamine fiber outgrowth and behavioral motor impairment following intrastriatal delivery of GDNF was shown in previous studies (retrograde transport of the neutrophic factor)
- Discret local neuroprotection of dopaminergic terminals was observed in the striatum (the effect observed only for GDNF-MSCs group indicated the protective/regenerative effects of GDNF rather than MSCs)
- Poor survival of the cell after transplantation could be the reason for modest neuroprotective results – late time-point of sacrifice
- Increasing cell survival could reveal the true potential of that therapy approach
- Invasiveness of the transplantation must be carefully considered

THANK YOU FOR YOUR ATTENTION!!!