



GDNF-Transfected Macrophages Produce Potent Neuroprotective Effects in Parkinson's Disease Mouse Model

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Abstract

The pathobiology of Parkinson's disease (PD) is associated with the loss of dopaminergic neurons in the substantia nigra pars compacta (*SNpc*) projecting to the striatum. Currently, there are no treatments that can halt or reverse the course of PD; only palliative therapies, such as replacement strategies for missing neurotransmitters, exist. Thus, the successful brain delivery of neurotrophic factors that promote neuronal survival and reverse the disease progression is crucial. We demonstrated earlier systemically administered autologous macrophages can deliver nanoformulated antioxidant, catalase, to the *SNpc* providing potent anti-inflammatory effects in PD mouse models. Here we evaluated genetically-modified macrophages for active targeted brain delivery of glial cell-line derived neurotrophic factor (GDNF). To capitalize on the beneficial properties afforded by alternatively activated macrophages, transfected with GDNF-encoded *pDNA* cells were further differentiated toward regenerative M2 phenotype. A systemic administration of GDNF-expressing macrophages significantly ameliorated neurodegeneration and neuroinflammation in PD mice. Behavioral studies confirmed neuroprotective effects of the macrophage-based drug delivery system. One of the suggested mechanisms of therapeutic effects is the release of exosomes containing the expressed neurotrophic factor followed by the efficient GDNF transfer to target neurons. Such formulations can serve as a new technology based on cell-mediated active delivery of therapeutic proteins that attenuate and reverse progression of PD, and ultimately provide hope for those patients who are already significantly disabled by the disease.

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files.

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Introduction

An extraordinary goal, such as brain delivery of therapeutic proteins, requires exceptional measures. The blood brain barrier (BBB) is a major obstacle to the successful treatment of many devastating diseases of central nervous system (CNS). Among them are neurodegenerative disorders (PD, Alzheimer's disease, and amyotrophic lateral sclerosis [1–3]), infectious diseases (meningitis, encephalitis, prion disease, and HIV-related dementia [4,5]), stroke [6,7], and lysosomal storage diseases [8,9]. PD is the second most common neurodegenerative disorder of people over 65 year age; 70,000 new cases are registered in US every year. Regrettably, no therapies are currently available that can attenuate disease progression. The use of existing drugs provides only symptomatic relief to patients that are hindered by the development of drug resistance and progressive adverse side effects such as motor complications and dyskinesia [10]. In addition, most

current therapies are based on pharmacological replacement of lost striatal dopamine, which only masks or reduces the declining dopaminergic activity in PD patients. Thus, novel therapies are urgently required.

Disease symptoms are characterized by lack of neurotransmitter dopamine due to the loss of dopaminergic neurons located in the nigrostriatal system. A growing body of evidence indicates the usefulness and efficacy of neurotrophic factors for the treatment of PD [11–17]. It has been confirmed that BDNF expression levels are decreased in the *SNpc* of PD patients [18]. Thus, the neurotrophic factors, and in particular GDNF, can promote regeneration of DA neurons and protect these cells from toxic insults [19]. Furthermore, intracranial infusions of GDNF [20,21] and BDNF [22] have been shown to provide protection and restoration of DA neurons in rodent models of PD, and in 1-methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine (MPTP)- or 6-hydroxydopamine (6-OHDA)-lesioned and intact aged primates

[15,17,23–25]. Unfortunately, intracranial infusion is an invasive procedure that carries a high risk of adverse effects. Thus, the development of new drug delivery systems for neurotrophic factors that can be administered systemically is crucial.

The majority of CNS disorders have in common an inflammatory component [26] resulting in the excessive production of reactive oxygen species (ROS) and subsequent neurodegeneration. Immunocytes that include mononuclear phagocytes (dendritic cells, monocytes and macrophages) exhibit an intrinsic homing property of migrating toward the inflammation site *via* the processes known as diapedesis and chemotaxis [27]. Thus, immunocytes are reported to cause BBB breakdown following brain inflammation [28–31] trafficking primarily between adjacent endothelial cells, *i.e.* paracellularly through the junctional complexes [32,33]. Considering that immunocytes readily home to the sites with inflammation, we propose harnessing this natural mechanism, and use macrophages for the active targeted delivery of therapeutic proteins to the brain.

Two approaches are utilized in cell-mediated drug delivery. *First*, host cells are loaded with a drug, usually incorporated into a protective container, and then carry the drug to the disease site. One of the main obstacles of this approach is efficient disintegration of the entrapped foreign particles by monocytes. *Second*, cell-carriers are genetically modified to produce therapeutically active molecules. For example, neurotrophic factors, such as glial cell-line derived neurotrophic factor (GDNF), neurturin (NTN), or vascular endothelial growth factor (VEGF) produced by transfected neural stem cells (NSC) [34–36] and bone marrow-derived macrophages and microglia [37,38] were used in PD mouse models. In particular, bone marrow stem cells were transduced *ex vivo* with lentivirus expressing a GDNF gene driven by a synthetic macrophage-specific promoter and then transplanted into recipient mice. Macrophage-mediated GDNF delivery dramatically ameliorated PD-related inflammation and neuronal degeneration in *SNpc* [37] resulting in axon regeneration and reversed hypoactivity in mice. Furthermore, transfected NSC were used for delivery of neurotrophic factors [39–43] in Alzheimer's disease mouse model.

Our earlier studies suggest cell-carriers offer distinct advantages over standard drug administration regimens by providing disease-specific homing, sustained on-site drug delivery, and improved therapeutic efficacy [44–52]. Based on our previously developed cell-mediated drug delivery system, present work utilized genetically-modified autologous macrophages for active targeted delivery of GDNF to the inflamed brain. The overall scheme of these investigations is depicted in **Figure 1**. Macrophages were transfected *ex vivo* to produce GDNF, and administered intravenously in mice with PD model. This resulted in significant increases in dopaminergic neurons survival and decreases in inflammation in *SNpc*. Of note, genetically-modified macrophages released exosomes with incorporated GDNF that may facilitate GDNF transport into the target cells and preserve it against degradation.

Exosomes are nanosized vesicles secreted by a variety of cells, in particular, cells of the immune system: dendritic cells [53], macrophages [54], B cells [55], and T cells [56]. These extracellular vesicles were initially thought to be a mechanism for removing unneeded proteins. Recent studies revealed, they are actually specialized in long-distance intercellular communications facilitating transfer of proteins [57], functional mRNAs and microRNAs for subsequent protein expression in target cells [58,59]. To shuttle their cargo, exosomes can attach by a range of surface adhesion proteins and specific vector ligands (tetraspanins,

integrins, CD11b and CD18 receptors), and fuse with target cellular membranes delivering their payload [53,60,61]. Indeed, exosomes, comprised of cellular membranes, have an exceptional ability to interact with target cells. Thus, we demonstrated here that exosomes showed an extraordinary ability to abundantly adhere and overflow neuronal cells as was visualized by confocal microscopy. This mechanism may play a significant role in GDNF-mediated protection effects, increasing the blood circulation time, reducing immunogenicity, and facilitation of the protein transfer across the BBB and into target neurons. In addition, the membranotropic properties of GDNF-carrying exosomes may facilitate GDNF binding to GFR α -1 receptors expressed on DA neurons [62]. The present data indicate, intrinsic properties of macrophages can overcome the limitations of current common therapies, alleviate and reverse the symptoms, and may ultimately improve the quality of life of patients with various neurodegenerative disorders.

Materials and Methods

Plasmids

The gWI high expression vectors encoding the reporter gene green fluorescent protein (GFP) (gWIGFP) under control of an optimized human cytomegalovirus (CMV) promoter followed by intron A from the CMV immediate-early (IE) gene were used throughout the study (Gene Therapy Systems, San Diego, CA). Human GDNF cDNA (NM_199234) was provided by OriGene (Rockville, MD). All plasmids are expanded in DH5 α E.coli and isolated using Qiagen endotoxin-free plasmid Giga-prep kits (Qiagen, Valencia, CA) according to the supplier's protocol.

Reagents

GenePORTER 3000 transfection agent was purchased from AMS Biotechnology, England). Lipopolysaccharides (LPS), 6-hydroxydopamine (6-OHDA), and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO, USA). A lipophilic fluorescent dye, 1,1'-dioctadecyl-3,3',3'-tetramethylindo-carbocyanine perchlorate (DIL), was purchased from Invitrogen (Carlsbad, CA). Interferon gamma (INT- γ), and murine macrophage colony-stimulating factor (MCSF) were purchased from Peprotech Inc (Rocky Hill, NJ).

Cells

Raw 264.7, a mouse macrophage cell line, was purchased from ATCC (cat # TIB-71), and cultured in Dulbecco's Modified Eagle's Media (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 2.5% horse serum and 15% FBS. Neuronal PC12 rat adrenal pheochromocytoma cell line was obtained from ATCC, and cultured in Dulbecco's modified Eagle medium (Hyclone, Utah, USA) supplemented with 10% FBS, and 1% (v/v) of both penicillin and streptomycin. The cells were grown in an incubator with optimal culture conditions of 37°C and 5% CO $_2$, and the medium was routinely replaced every 2–3 days.

Animals

BALB/C female mice (Charles River Laboratories, USA) eight weeks of age were treated in accordance to the Principles of Animal Care outlined by National Institutes of Health and approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

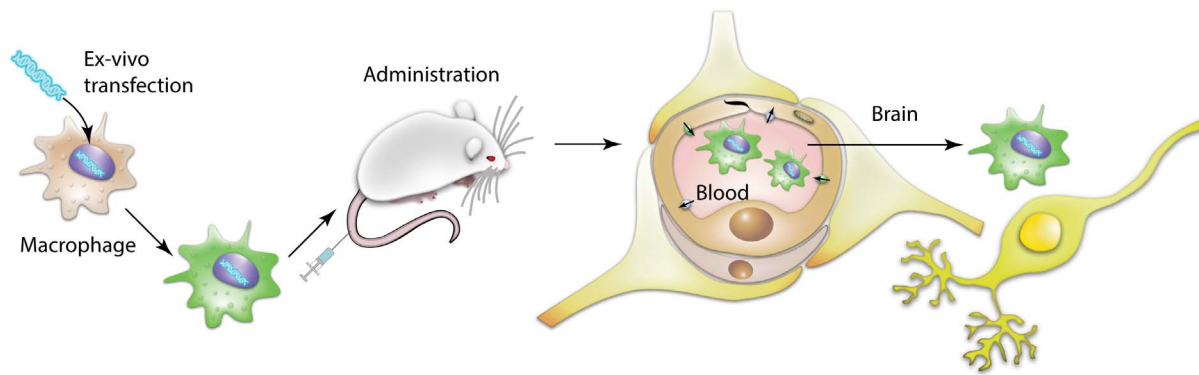


Figure 1. Schematic representation of macrophage-mediated drug delivery approach. Autologous macrophages were transfected with GDNF-encoding *pDNA* *ex vitro* and systemically administered into mice with brain inflammation. Driven by chemotaxis, genetically-modified cell-carriers home the inflamed brain tissues, and deliver the expressed neurotrophic factor to the dopaminergic neurons protecting them from toxic insults. The release of overexpressed GDNF in exosomes protected it against proteases degradation, facilitated the GDNF transfer into target neurons and as a result, improved therapeutic efficacy of this drug formulation. doi:10.1371/journal.pone.0106867.g001

Transfection of Macrophages with GDNF-encoding *pDNA* and Differentiation toward M2 Regenerative Subtype

Macrophages were incubated with a mixture of 13.6 μg GFP, or GDNF *pDNA* and GenePORTER 3000 in serum free media for four hours. Following incubation, an equal volume of full media containing 20% FBS was added bringing final serum concentration to 10%. To exclude the possibility that cell death explains the release of GDNF and GFP, percentage of live macrophages on the fourth day after transfection was accounted by Fluorescence Activated Cell Sorting (FACS). Transfected cells were collected, washed, stained with Alexa 488 LIVE/DEAD dye according to manufacturer's protocol, and the amount of accumulated LIVE/DEAD dye was assessed. The macrophage transfection efficiency using GenePORTER 3000 reagent was around 40% as determined by FACS for GFP-encoding *pDNA* [48].

To promote specific cell differentiation, Raw 264.7 macrophages were cultured in the presence of: (i) Interleukin 4 (IL-4) (to promote M2 anti-inflammatory subtype); or (ii) Interferon gamma (IFN- γ) and LPS (to obtain M1 pro-inflammatory subtype). For M2 subset differentiation, macrophages were supplemented with IL 4 (20 ng/ml) for 48 hours. For M1 subset differentiation, the cells were cultured in the mixture of IFN- γ (20 ng/ml) and LPS (100 ng/ml) for 48 hours. Following the incubation, media was replaced with a mixture of antibodies to mannose receptor (M2 type marker, anti-CD 206, BD Bioscience, 1 $\mu\text{g}/\text{ml}$), and antibodies to CD86 (M1 type marker, anti-CD 86, BD Bioscience, 2 $\mu\text{g}/\text{ml}$). The cells were incubated with the antibodies for 1 hour, washed, fixed, and examined by confocal microscopy as described below (CD 86, $\lambda = 405$ nm, and CD 206, $\lambda = 647$ nm). An overexpression of specific markers related to M1 or M2 subset of macrophages upon cell differentiation was confirmed by RT-PCR.

Isolation of Exosomes

Conditioned media from genetically-modified Raw 264.7 macrophages grown on T-75 flasks (20×10^6 cells/flask) was collected, and exosomes were isolated using ultra centrifugation [53]. In brief, the culture supernatants were cleared of cell debris and large vesicles by sequential centrifugation at $300 \times g$ for 10 min, $1000 \times g$ for 20 min, and $10,000 \times g$ for 30 min, followed by filtration using 0.2- μm syringe filters. Then, the cleared sample was spun at $100,000 \times g$ for 1 hour to pellet the exosomes, and supernatant was collected. The collected exosomes (10^{11} – 10^{12} exosomes/flask) were washed twice with PBS. To avoid contam-

ination of the FBS-derived exosomes, FBS was spun at $100,000 \times g$ for 2 hours to remove exosomes before use. The recovery of exosomes was estimated by measuring the protein concentration using the Bradford assay and by Nanoparticle Tracking Analysis (NTA). The obtained exosomal fraction was re-suspended in PBS (500 μl , 1 mg/mL total protein), and characterized for size and polydispersity by Dynamic Light Scattering (DLS) and Atomic Force Microscopy (AFM). The obtained exosomal fraction was evaluated for protein content.

Western Blot Analysis

Western blots were utilized to evaluate the presence of GDNF in transfected macrophages as well as in exosomes secreted by GDNF- or empty vector-transfected macrophages. Genetically-modified macrophages and exosomes isolated from the conditioned media were treated with lysis buffer (1% Triton X-100) and protease inhibitors (Sigma) and protein concentration was determined by BCA assay. Samples were mixed with 2 \times SDS sample buffer, boiled for 5 min and then separated on a precast 4–20% SDS-PAGE gel (BioRad, USA). Proteins were transferred to nitrocellulose membranes and GDNF was visualized with sheep polyclonal antibodies to GDNF (Millipore, AB5252, 1:2000 dilution) and secondary antibodies donkey anti-sheep IgG-HRP (Jackson ImmuneResearch; 1:5000 dilution). To correct for loading differences in cellular lysates and exosomal fractions, the levels of proteins were normalized to constitutively expressed β -actin in cells with goat polyclonal antibodies to β -actin (Abcam, ab8229; 1:500 dilution); and TSG101 in exosomes with goat polyclonal antibodies to TSG101 (Santa Cruz, SC6037; 1:200 dilution). Furthermore, for the characterization of different subtypes of polarized macrophages and released from them exosomes, membranes with corresponding transferred protein bands from macrophages or exosomes were blotted with rabbit polyclonal antibodies to CD63 (Santa Cruz, SC15363; 1:200 dilution), iNOS (Santa Cruz, SC650; 1:200 dilution), Arg1 (Santa Cruz, SC18351; 1:200 dilution) and CD206 (Santa Cruz, SC6037; 1:200 dilution) overnight at 4 $^{\circ}\text{C}$, and incubated with appropriate HRP-conjugated secondary antibodies: goat anti-rabbit IgG-HRP (Santa Cruz, SC2004; 1:2500 dilution), or donkey anti-goat IgG-HRP (Santa Cruz, SC2020; 1:5000 dilution). Membranes were washed and the expression levels were visualized with chemiluminescent substrate (Thermo Scientific) and a FluorChem E imaging

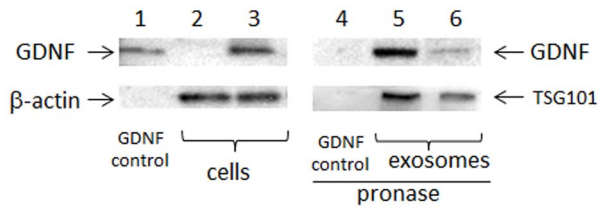


Figure 2. Expression of GDNF by genetically-modified macrophages. Raw 264.7 macrophages were pre-transfected with GDNF-encoding pDNA and GenePorter 3000 reagent for 4 hours. Then, exosomes were collected from concomitant macrophages media for 24 hours, and GDNF levels in cellular lysates (lines 2–3) and in exosomes (lines 5–6) were examined by western blot. Commercially available GDNF (line 1) served as a positive control. Significant amount of GDNF was detected in the cells (line 3) and exosomes released from GDNF-transfected macrophages (line 5), but not in macrophages transfected with empty vector (line 2). Expressed GDNF was protected in exosomes against degradation by pronase (line 5), while control GDNF was degraded at these conditions (line 4). Destruction of exosomes by sonication eliminated their protective effect (line 6). β -actin and TSG101 served as controls for cell lysates and exosomes, respectively. doi:10.1371/journal.pone.0106867.g002

system (Protein Simple). Specific protein bands were quantitated by densitometry (Bio-Rad Laboratories, Hercules, CA).

RT-PCR analysis

Total RNA of macrophages as well as exosomal RNA was extracted using RNeasy mini kit (Qiagen, CA, USA) according to manufacturer's instructions. Residual genomic DNA of macrophages and exosomes was removed by incubating with Rnase-free DNase set (Qiagen). RNA was analyzed and quantified using nanodrop 2000c (Thermo Scientific, USA). As quality controls of RNA samples purity from contaminating DNA and chaotropic salts was obtained by absorbance Ratio A260/A280 and A260/A230, respectively. RNA (1 μ g) isolated from resting and polarized macrophages cells and their respective exosomes was reverse transcribed with Superscript III First-Strand synthesis system for RT-PCR (Invitrogen, CA, USA) according to manufacturer's protocol. To quantify mRNA levels, quantitative reverse transcription PCR was performed using an ABI StepOne Plus Detection System (Applied Biosystems, MA, USA). TaqMan PCR Universal Master Mix and Expression Assays were from Applied Biosystems. Assay IDs: iNOS Mm00440502_m1, CD206

Mm00485148_m1 and CD63 Mm01966817_g1. CD63 was used as exosomal marker and CD11b as macrophage cells marker.

Confocal Microscopy Studies

To visualize exosomal accumulation in target neurons, Raw 264.6 macrophages were transfected with GDNF-encoding DNA, and cultured in exosome-free media for two days. Exosomes were isolated from macrophage conditioned media by ultracentrifugation, and stained with the lipophilic fluorescent dye, DIL (red). PC12 neurons were cultured with DIL-labeled exosomes or PLGA nanoparticles (as a control) for three days, washed with PBS, and stained with phalloidin for actin microfilaments (green). The accumulation levels were examined by confocal microscopy.

To track systemically injected macrophages in the inflamed brain, macrophages were transfected with GFP-encoding DNA as described above. For 6-OHDA intoxications, mice were stereotactically injected with 6-OHDA solution (10 μ g 6-OHDA in 0.9% NaCl with 0.02% ascorbic acid), flow rate of 0.1 μ L/min into the striatum (AP: +0.5; L: -2.0 and DV: -3.0 mm) [52]. Animals with brain inflammation induced were injected with GFP-expressing Raw 264.7 macrophages on day twenty one after intoxication with 6-OHDA *via* the intrajugular vein (*i.v.*). Two days later, animals were sacrificed and perfused as described [52], brains were removed, washed, post-fixed in 10% phosphate-buffered paraformaldehyde, and evaluated by confocal microscopy.

Immunohistochemical and Stereological Analyses

6-OHDA-intoxicated mice were *i.v.* injected with PBS, or GDNF-transfected macrophages, or macrophages transfected with empty vector 48 hours after intoxication. Twenty four days later, animals were sacrificed, perfused; brains were removed, washed, post-fixed, and immunohistochemical analysis was performed in 30 μ m thick consecutive coronal brain sections [46]. For detection of microglia activation, tissue sections were incubated with primary monoclonal rat anti mouse anti-CD11b antibodies (AbD Serotec, Raleigh, NC) 1:500 dilution), and secondary biotinylated goat anti-rat antibodies (Vector Laboratories, Burlingame, CA, 1:200 dilution). For the assessment of neuroprotection effect, tyrosine hydroxylase (TH) staining was used to quantitate numbers of dopaminergic neurons [63]. The total number of TH-positive SN neurons and CD11b-positive microglia cells were counted by using the optical fractionator module in StereoInvestigator software (MicroBrightField, Inc., Williston, VT) [46].

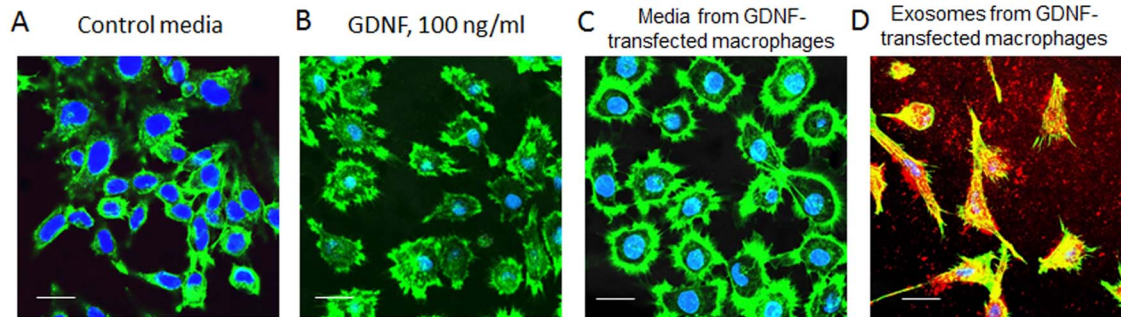


Figure 3. Effect of exosomes released from GDNF-transfected macrophages on the axonal growth in PC12 neurons. PC12 neurons were cultured for 3 days in: (A) control media without GDNF; (B) in the presence of 100 ng/ml GDNF; or supplemented with (C) conditioned media collected from GDNF-transfected macrophages; or (D) exosomes isolated from conditioned media released from GDNF-transfected macrophages. Exosomes were fluorescently labeled with lipophilic dye, DIL (red) before the addition to the neurons (D). Following incubation, the cells were washed with PBS, and stained with phalloidin for actin microfilaments (green). Confocal images revealed the pronounced development of axons upon treatment with media (C) and especially exosomes (D) released from GDNF-transfected macrophages. The bar: 20 μ m. doi:10.1371/journal.pone.0106867.g003

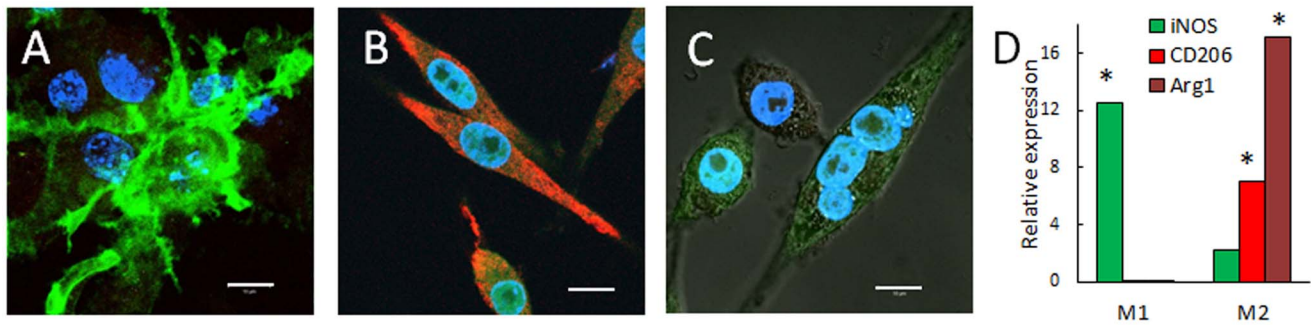


Figure 4. Differentiation of macrophages toward "alternatively" activated M2 subtype. Raw 264.7 macrophages were cultured in the presence of: (A) Interferon gamma (IFN- γ) and lipopolysaccharides (LPS) for M1 pro-inflammatory subtype; or (B) Interleukin 4 (IL 4) for M2 anti-inflammatory subtype for two days as described in **Materials and Methods** section. Then, the cells were stained with a mixture of antibodies to CD 86 (green) and mannose receptor CD206 (red) for M1 and M2 phenotype, respectively, and examined by confocal microscopy. Macrophages differentiated in the presence of INF- γ /LPS showed high expression of CD86, but low if any mannose receptor levels indicating classically activated M1 subtype (A). In contrast, cells differentiated in the presence of IL-4 showed high expression of mannose receptor, and low expression of CD 86 that is attributed to M2 macrophages (B). Non-differentiated Mo macrophages served as a control (C). Bar: 10 μ m. RT-PCR studies confirmed statistically significantly elevated levels of inducible Nitric Oxide Synthases (iNOS) mRNA in M1 cells, and high levels of CD206 and Arginase 1 (Arg1) mRNA in M2 macrophages (D), compared to Mo macrophages ($p < 0.05$). doi:10.1371/journal.pone.0106867.g004

Behavioral Tests

For the traditional constant speed rotarod test, mice were trained and tested as previously described with slight modifications [64]. 6-OHDA-intoxicated mice were *i.v.* injected with PBS, or GDNF-transfected macrophages 48 hours after intoxication and the latency to fall from the rotarod was determined at three speeds (4, 5, and 7 rpm). Healthy mice *i.c.* injected with PBS were used as a control [65]. For apomorphine test, the animals were injected with apomorphine (0.05 mg / kg, *s.c.*) and rotations were scored every 10 min for 90 min [66].

Statistical Analysis

For the all experiments, data are presented as the mean \pm SEM. Tests for significant differences between the groups in *in vitro* experiments investigating transfection of macrophages, as well as in *in vivo* evaluations of therapeutic effects of different drug

formulations were performed using a one-way ANOVA with multiple comparisons (Fisher's pairwise comparisons) using GraphPad Prism 5.0 (GraphPad software, San Diego, CA, USA). A standard T-test was performed when only two groups (for example, for the evaluation of expression levels of specific proteins by western blot) were compared. A minimum p value of 0.05 was chosen as the significance level for all tests.

Results

Exosomes secreted from genetically-modified macrophages contain GDNF

The optimal conditions that provide for high levels and duration of therapeutic proteins expression in macrophages identified previously [48] were used to transfect Raw 264.7 macrophages. Next, exosomes were collected from conditioned macrophages

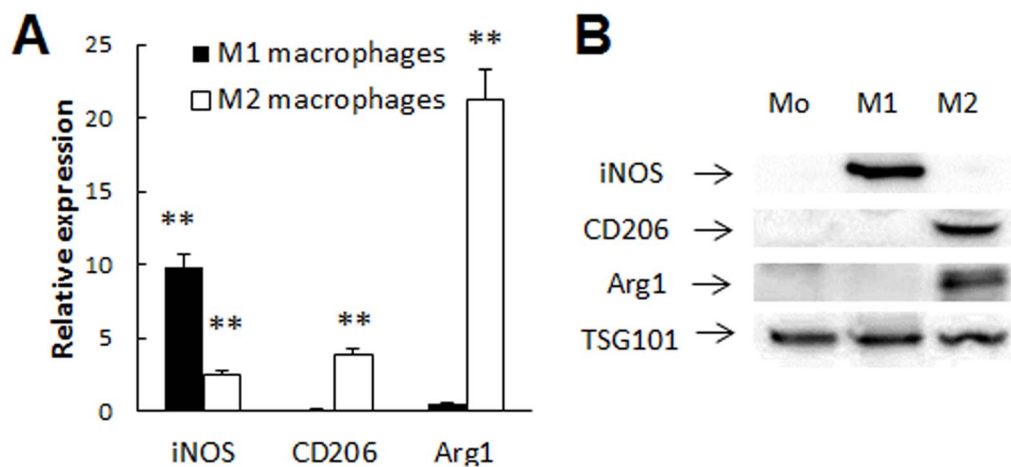


Figure 5. Characterization of exosomes released from differentiated subtypes of macrophages. Exosomes were isolated from conditional media of differentiated macrophages and examined for the presence of specific markers by RP-PCR (A) and western blot (B). Expression of Arg1 and CD206 mRNA and protein (markers for M2 subtype) was detected in exosomes originated from M2 macrophages, but not in those secreted from M1 macrophages. In contrast, expression of iNOS mRNA and protein was detected in exosomes released from M1 macrophages, but not in those secreted by M2 macrophages. TSG101 was used as house-keeping protein for exosomes. Values are means \pm SEM ($N = 4$), and $p < 0.05$ compared with the expression levels in Mo macrophages. doi:10.1371/journal.pone.0106867.g005

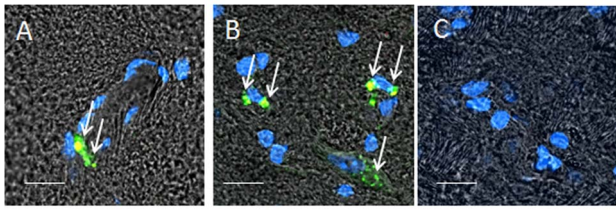


Figure 6. Recruitment of GFP-expressing M2 macrophages to SNpc in 6-OHDA-intoxicated mice. Macrophages were transfected with GFP-encoding *pDNA* as described in Materials and Methods section and stained with primary antibodies to CD206, a marker for M2 macrophages, and secondary fluorescently-labeled anti-Mouse-IgG-atto 647N (red). BALB/c mice were *i.c.* intoxicated with 6-OHDA into SNpc. Twenty one day later, the animals were *i.v.* injected with GFP-expressing RAW 264.7 macrophages (green, 5×10^6 cells/ mouse in 100 μ l). Twenty four hours later mice were sacrificed, and perfused with PBS and 4% PFA. Brains were frozen, sectioned with a cryostat (10 μ m thick), and examined by confocal microscopy (60 \times magnification) (A, B). Healthy mice without brain inflammation (with PBS *i.c.* injections) were used as a control group (C). Slides were stained with for expression of mannose receptor (CD206 antibodies). Co-localization of GFP-expressing macrophages and CD206 antibodies to mannose receptor manifested in yellow staining (arrows) confirmed presence of significant amounts of the M2 genetically-modified cells in the intoxicated brain endothelial microvessels (A), and parenchyma (B). No fluorescence in the healthy brain was found (C) indicating that systemically administered Raw 264.7 macrophages did not cross the BBB in the absence of brain inflammation. The bar: 20 μ m.
doi:10.1371/journal.pone.0106867.g006

media for 24 hours, and the expression of the encoded protein in the in cellular lysates and exosomes were evaluated by western blot (Fig. 2). Significant amounts of GDNF was detected in the cells (line 3) and exosomes released from GDNF-transfected macrophages (line 5), but not in macrophages transfected with empty vector (line 2). Noteworthy, the expressed GDNF was protected in exosomes against degradation by pronase (line 5); while control GDNF was degraded at these conditions (line 4). Destruction of exosomes by sonication eliminated their protective effect against proteases degradation (line 6). The average size for exosomes released from GDNF-transfected macrophages (96.0 ± 9.1 nm) was slightly greater than those released from non-transfected macrophages (90.5 ± 3.4 nm).

Effects of GDNF-transfected Macrophages on axonal growth *in vitro*

A profound therapeutic effect of GDNF released from pre-loaded M2 macrophages *in vitro* was demonstrated in PC12 neurons that are known to express GDNF receptor (Fig. 3). Neurons were cultured for 72 hours in: (A) control media without GDNF; (B) media supplemented with high concentration of commercially available GDNF (100 ng/ml); (C) conditioned media collected from GDNF-transfected macrophages, or (D) exosomes isolated from GDNF-transfected macrophages. Following the incubation, the cells were stained with phalloidin for actin microfilaments (green) and DAPI for cell nucleus (blue). In parallel, exosomes were stained with a lipophilic fluorescent dye, DID (red). Confocal images revealed the significant axonal growth in neurons cultured with conditioned media from GDNF-preloaded macrophages (Fig. 3 C) suggesting GDNF was released from the cell-carriers in a functional state. Noteworthy, treatment of PC12 neurons with exosomes released from pre-transfected macrophages resulted in the drastic increase of neuronal maturation with a pronounced outgrowth of axons and dendrites (Fig. 3 D). The effects were greater than those caused by a high

dose of commercially-available GDNF (Fig. 3 B). It is likely, similar to catalase-transfected macrophages [48], exosomes with incorporated overexpressed neurotropic factor promote GDNF transfer, and secure its efficient accumulation and favorable intracellular localization in target neurons.

Differentiation of Macrophages and Using an Alternatively Activated Neuroregenerative M2 Subtype

To enhance the therapeutic effects of GDNF and avoid the pro-inflammatory neurotoxic effects of classically-activated M1 macrophages [67], Raw 264.7 macrophages were differentiated to M2 regenerative subtype. For this purpose, macrophages were cultured in the presence of Interleukin 4 (IL 4) for M2 subtype; or Interferon gamma (IFN- γ) and lipopolysaccharides (LPS), as a negative control for M1 pro-inflammatory subtype. The obtained subsets of macrophages were characterized by confocal microscopy (Fig. 4 A–C) and RT-PCR (Fig. 4 D). Mannose receptor (CD206), and Arginase 1 (Arg1) were chosen as principal markers form identifying M2 macrophages subtype. Levels of inducible Nitric Oxide Synthases (iNOS, CD86) were examined as marker of M1 macrophages subtype. Noteworthy, Arg1 and iNOS [68] were demonstrated to have anti-inflammatory and pro-inflammatory properties, respectively.

Macrophages differentiated in the presence of INF- γ /LPS demonstrated considerable expression of CD 86, and low, if any, levels of mannose receptor (Fig. 4 A) that is indicative for classically activated pro-inflammatory M1 subtype. In contrast, Raw 264.7 macrophages differentiated in the presence of IL-4 showed high expression levels of mannose receptor, and low, if any, expression of CD 86 (Fig. 4B) that indicative for “alternatively activated” anti-inflammatory macrophages. Non-activated Mo macrophages showed low, if any expression of both CD86 and CD206 receptors (Fig. 4 C). Noteworthy, the polarization to different macrophages subtypes altered the cells morphology. RT-PCR studies confirmed elevated levels of iNOS mRNA in M1 cells, and high levels of CD206 and Arginase 1 (Arg1) mRNA in M2 macrophages (Fig. 4 D).

Next, we hypothesized that exosomal content, at least in part, should reflect the content of parental macrophages. Thus, exosomes released from M2 macrophages may exhibit neuroprotective and regenerative properties by themselves. A presence of Arg1 mRNA and Arg1 protein that indicative for M2 macrophages subtype was evaluated by RT-PCR (Fig. 5 A) and western blot analyses (Fig. 5 B). The obtained data confirmed exosomes secreted by M2 regenerative macrophages, but not M1 pro-inflammatory macrophages showed high levels of Arg1 and CD206 mRNA and Arg1 protein. In contrast, expression of iNOS mRNA and protein (marker for M1 macrophages) was detected in exosomes released from M1 macrophages, but not in those secreted by M2 macrophages. Noteworthy, although the overall trend of the expression of specific markers in different subtypes macrophages and exosomes is analogous, these levels might not be completely the same, as exosomes should reflect, but not copy the content of parental macrophages they were released from. This approach was utilized further to differentiate the cell-carriers towards the M2 phenotype prior to the infusion to capitalize on the beneficial properties afforded by alternatively activated M2 macrophages in the context of PD, and minimize the potential of the cells converting to the pro-inflammatory M1 subtype.

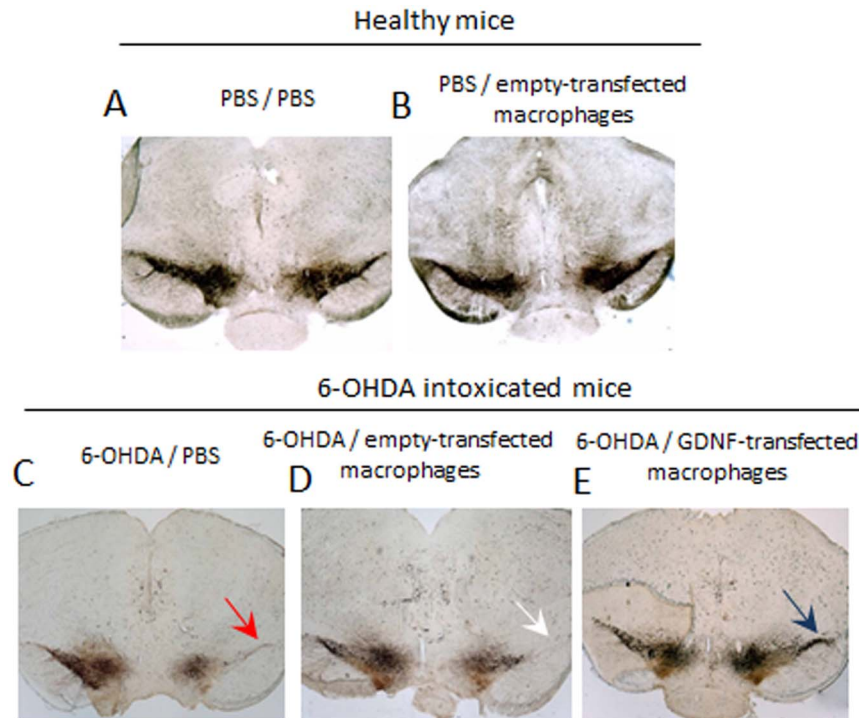


Figure 7. Neuroprotective effects of GDNF-transfected macrophages in PD mouse model. BALB/c mice were *i.c.* injected with 6-OHDA. Forty eight hours later, animals were *i.v.* injected with GDNF-transfected or empty-transfected macrophages, or PBS, and 21 days later they were sacrificed, and mid-brain slides were stained for expression of TH, a marker for dopaminergic neurons. Whereas 6-OHDA treatment caused significant neuronal loss in *SNpc* (red arrow), administration of GDNF-transfected macrophages dramatically increased neuronal survival (blue arrow). Administration of empty-vector transfected macrophages did not affect the number of dopaminergic neurons in healthy mice, and shows mild effect on increased neuronal survival in PD mice (white arrow).
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Systemically-administered M2 Macrophages Home Inflamed Brain Tissues and Sustain their Phenotype in Brain Tissues

Raw 264.6 macrophages were transfected with GFP-encoding *pDNA*, and then differentiated to M2 regenerative subtype as described above. BALB/c mice were *i.c.* intoxicated with 6-OHDA into *SNpc*. Twenty one days later (at the pick of inflammation), mice were systemically injected with GFP-expressing macrophages (5×10^6 cells/ mouse in 100 μ l). Twenty four hours later, the mice were sacrificed, and perfused with PBS and 4% PFA. Healthy mice without brain inflammation were used as a control group. Brain slides were stained with primary antibodies to CD 206, a marker for mannose receptor attributed to M2 subtype of macrophages. Confocal images of brains sections indicate systemically infused macrophages home the inflamed brain and localize around brain endothelial microvessels (Fig. 6 A), and parenchyma (Fig. 6 B). Co-localization of GFP-expressing macrophages (green) and CD206 staining (red) manifested in yellow confirmed, the macrophages sustain their M2 phenotype in the intoxicated brain. No macrophages were found in healthy brain (Fig. 6 C) indicating the cells do not cross the BBB in the absence of inflammation.

Macrophage-mediated Neuroprotection in PD mice

A potent neuroprotective effect of GDNF-transfected macrophages was demonstrated in the 6-OHDA-intoxicated mice (Fig. 7). GDNF-overexpressing Raw 264.7 macrophages were systemically administered to BALB/c mice with brain inflammation (1×10^6 cells/100 μ l) 48 hours after *i.c.* intoxication with 6-

OHDA (PD mouse model). Non-intoxicated mice were used as healthy controls. 21 day later, mice were sacrificed, perfused, and the brain slides were stained for tyrosine hydroxylase (TH)-expressing DA neurons. *I.c.* intoxications of 6-OHDA caused substantial neurodegeneration in the *SNpc* (Fig. 7 C, Table 1) compared to healthy mice (Fig. 7 A, Table 1). Systemic administration of GDNF-transfected macrophages protected DA neurons against 6-OHDA intoxications (Fig. 7 E, Table 1). The numbers of TH+ neurons in *SNpc* of 6-OHDA animals treated with GDNF-transfected macrophages were significantly ($p < 0.05$) greater than those 6-OHDA intoxicated, and then PBS-injected animals (Table 1). Noteworthy, empty-transfected macrophages slightly improved neuronal survival in PD animals, probably due to their regenerative M2 subtype, although this effect was not statistically significant (Fig. 7D, Table 1). Indeed, empty-transfected macrophages did not affect neuronal survival in healthy mice (Fig. 7 B, Table 1). This signifies that GDNF-transfected macrophages can efficiently protect DA neurons against 6-OHDA-induced intoxication. The therapeutic effect of GDNF-transfected macrophages was also manifested in significant decreases in inflammation and levels of activated microglia in *SNpc* (Figure 8, Table 1).

Finally, behavioral tests demonstrated statistically significant improvements in motor functions upon treatment with GDNF-transfected macrophages (Fig. 9). Specifically, the loss of dopaminergic input due to the lesion of the left nigro-striatal pathway resulted in number of full-body contralateral rotations induced by a dopaminergic agent, apomorphine (Fig. 9 A). In contrast, systemic administration of genetically-modified macrophages to 6-OHDA intoxicated mice drastically ($p < 0.005$) reduced number of

Table 1. Effect of GDNF-transfected macrophages on inflammation and neurodegeneration in mice with PD model^a.

Treatment	CD11b+ (cells per mm ²)		Total N of neurons ^b × 10 ³	
	PBS	6-OHDA	PBS	6-OHDA
PBS	10.1 ± 1.2	90.0 ± 11 (**) ^c	5.9 ± 1.4	2.15 ± 0.3 (**)
GDNF-transfected macrophages	n/a	63.5 ± 5.2 (*)	n/a	3.3 ± 0.6 (*, #)
Empty-transfected macrophages	9.8 ± 1.0	87.0 ± 11.1	5.7 ± 1.5	2.5 ± 0.7

^aBALB/c mice were *i.c.* injected with 6-OHDA. Forty eight hours later, the animals were *i.v.* injected with various macrophage-based formulations or PBS. A control group was *i.c.* injected with PBS, and then 48 hours later *i.v.* injected with PBS.

^bTotal number of neurons was calculated in ipsilateral hemisphere.

^cStatistical significance is shown by asterisk: $p < 0.05$ (*), and $p < 0.005$ (**) compared to mice with *i.c.* PBS injections followed by *i.v.* PBS injections (healthy controls); or $p < 0.05$ (#), compared to mice with *i.c.* 6-OHDA injections followed by *i.v.* PBS injections (PD controls). Errors are mean ± SEM, $N = 7$.

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these rotations on the seventh week following the intoxication in apomorphine test to the levels of the non-intoxicated animals. Furthermore, the motor functions were preserved by systemic administration of GDNF-transfected macrophages in 6-OHDA intoxicated animals at the levels similar to those of control non-intoxicated mice, as demonstrated in rotarod test (Fig. 9 B).

Discussion

A long-term objective of these investigations is to develop an active targeted cell-mediated delivery of therapeutic proteins to the brain. We demonstrated here that genetically-modified macrophages can produce therapeutically-active neurotrophic factor, GDNF, and release it in extracellular vesicles, exosomes. The

most important finding of this work is that GDNF-transfected macrophages provide significant neuronal protection in a PD mouse model. Our *in vivo* experiments demonstrated that systemically administered GDNF-expressing macrophages migrate across the BBB towards the inflammation site in large numbers, and provide efficient sustained delivery of their drug payload at the disease site that is consistent with previously reported findings [45–49]. To enforce outcomes of the new formulation, a specific subset of “alternatively activated” (M2) macrophages with regenerative functions was used. Thus, macrophages, polarized to M2 regenerative subtype, represent biologically-active carriers that can promote neuronal regeneration enhancing the therapeutic efficacy of drug formulations.

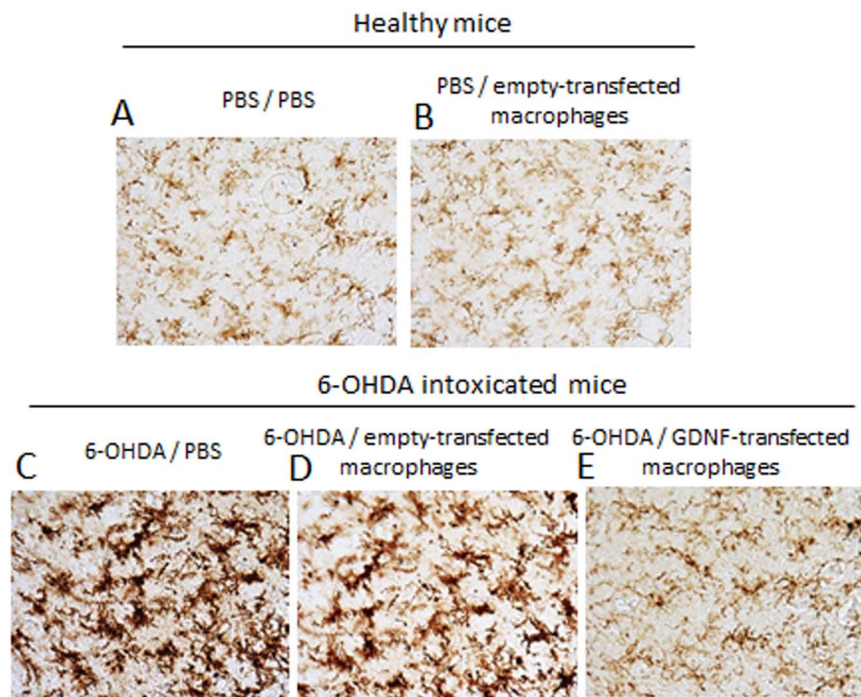


Figure 8. GDNF-transfected macrophages reduce neuro-inflammation in PD mice. BALB/c mice were *i.c.* injected with 6-OHDA. Forty eight hours later, animals were *i.v.* injected with GDNF-transfected or empty-transfected macrophages, or PBS, and 21 days later they were sacrificed, and mid-brain slides were stained for expression of CD11b, a marker for activated microglia. A 6-OHDA-mediated intoxication up-regulated expression of CD11b by microglia within the *SNpc* as exhibited a more amoeboid morphology in 6-OHDA-treated mice compared to ramified microglia in PBS-treated mice. In contrast, treatment of 6-OHDA-intoxicated mice with GDNF-transfected macrophages resulted in the decreased levels of CD11b compared with 6-OHDA-intoxicated control animals. Administration of empty-vector transfected macrophages did not affect the number of dopaminergic neurons in PD or healthy mice.

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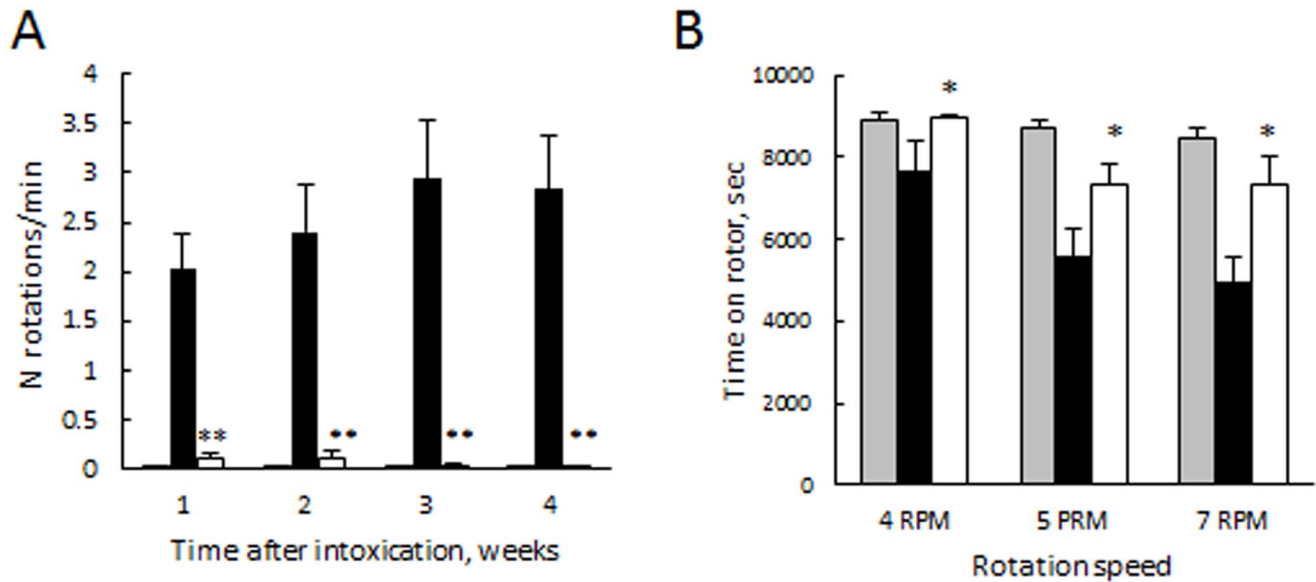


Figure 9. GDNF-transfected macrophages significantly improved motor functions in PD mouse model. 6-OHDA-intoxicated BALB/c mice were *i.v.* injected with GDNF-transfected macrophages (white bars), or PBS (black bars) 48 hours after the intoxication. A control group (grey bars) was *i.c.* injected with PBS, and then 48 hours later *i.v.* injected with PBS. Apomorphine (A) and rotarod (B) tests demonstrated statistically significant improvements in motor functions upon treatment with GDNF-carrying macrophages. Values are means \pm SEM ($N=12$), and $p<0.05$ compared with 6-OHDA-intoxicated mice. doi:10.1371/journal.pone.0106867.g009

The mechanism of the macrophage-mediated drug delivery is not fully understood. Thus, several independent processes may serve to improve GDNF-expressing macrophage therapeutics. In addition to the targeted tissue-specific delivery of therapeutics in macrophages [46,52], drug-carrying macrophages were shown to increase time circulation in the blood, and therefore permit sustained release of the therapeutic protein allowing the drug to enter the brain, independent of carrier cells (“depot effect”) [46,52]. Furthermore, we reported earlier that significant catalase transport across the BBB can be attributed to the efficient interaction of exosomes with brain microvessel endothelial cells followed by facilitated protein transfer to the brain [47]. Similar mechanism may be employed in the GDNF transfer from carrier macrophages across the BBB. We demonstrated that exosomes secreted from preloaded with nanoformulated catalase macrophages attach to the plasma membranes, and discharge their cargo into target cells [47]. As a result, the drug-incorporated nanoparticles were transferred from macrophages to adjacent cells, and diffused broadly throughout the recipient cells avoiding degradation in lysosomes. This mechanism enabled the drug to reach different intracellular compartments such as mitochondria, cytoplasm, and endoplasmic reticulum, and produce a powerful therapeutic effects [47]. Indeed, the mechanism of GDNF action differs from catalase neuroprotective effects, as specific binding to GFR α -1 receptors is required in order to accomplish GDNF-mediated neuroprotection. Nevertheless, we hypothesized that the efficient interactions of exosomal carriers with plasma membranes of DA neurons may facilitate GDNF binding to their receptors, and result in the profound therapeutic effect. Unfortunately, at this stage of understanding, we cannot discriminate, which mechanism is the most important for the therapeutic effects of cell-based formulations. A comprehensive examination and detailed understanding of these mechanisms would be of great value in designing strategies for cell-mediated drug delivery.

Several trials human and nonhuman (primate) were initiated to evaluate efficiency of GDNF for PD treatment as well as its off-side effects. Thus, GDNF showed therapeutic promise when injected intracranially through a surgically implanted shunt [69–71]. Although there were significant improvements followed GDNF infusions, some primate subjects revealed damage in the cerebellum found in brain autopsies. This was attributed to the rapid withdrawal from large amounts of GDNF and damaging tissues by the catheter, rather than to the direct GDNF toxic effects [71]. To this end, we believe that cell-mediated GDNF delivery can eliminate these issues, providing continuous targeted GDNF delivery specifically to the inflamed brain areas. Using genetically-modified macrophages as a cell-mediated drug delivery system will target the therapeutic proteins to the brain, prolong drug half-life, and diminish drug immunogenicity. In addition, properly differentiated immune cells accumulating in traumatic, degenerative, ischemic, infectious, and autoimmune lesions of the nervous system might provide a neuroprotective effect, which may further boost the therapeutic effect of cell-mediated drug delivery systems. It is anticipated that these studies will lead to the developing a new technology based on active targeted cell-mediated delivery of therapeutic polypeptides that produce neuroprotection and neuroregeneration in patients with PD.

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Author Contributions

Conceived and designed the experiments: MH AVK EVB. Performed the experiments: YZ MH RG JB ZH. Analyzed the data: EVB. Contributed to the writing of the manuscript: EVB.

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