Supplemental Information:

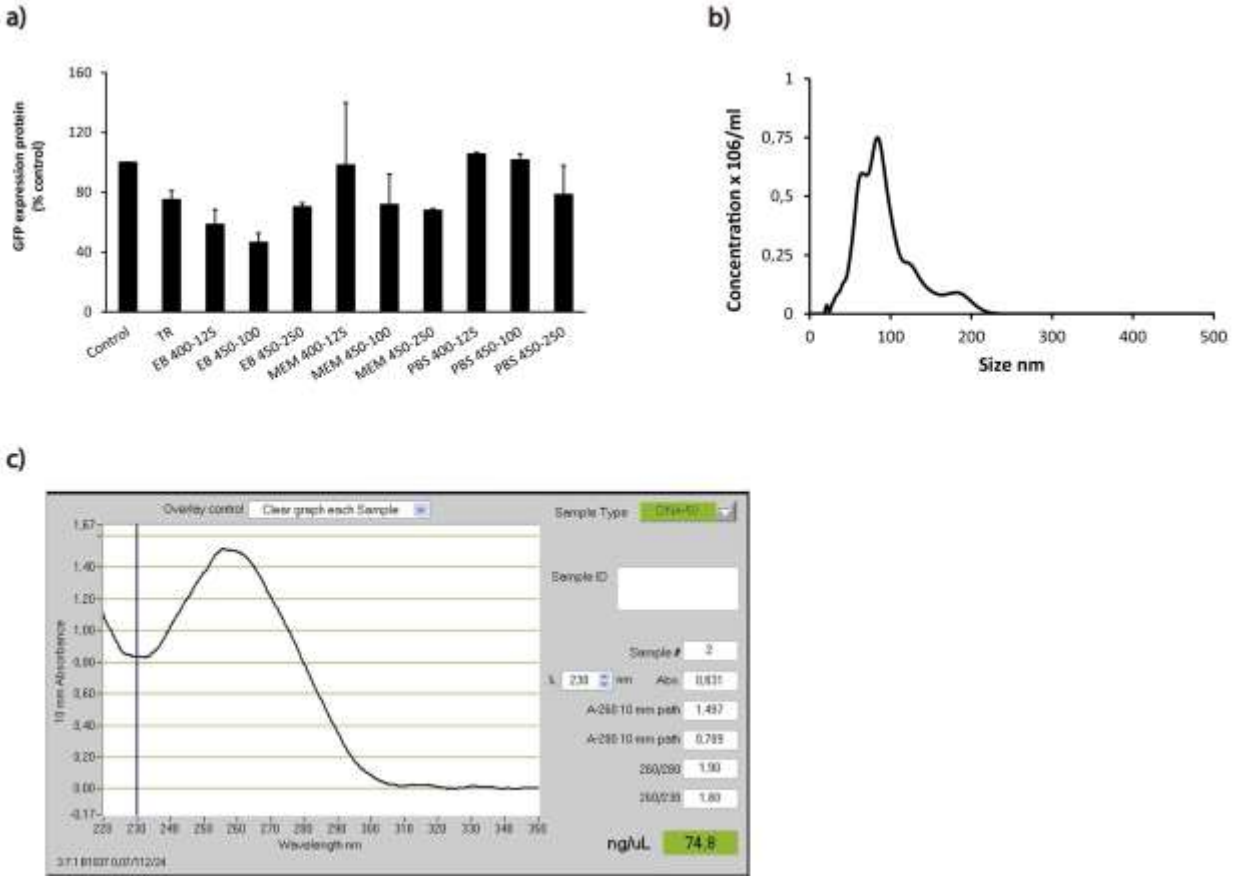

Figure S1. Analysis of RVG-exosomes loaded with anti GFP shRNA-MC. a) The efficacy of anti GFP shRNA-MC loading of RVG-exosomes was assessed using GFP expressing SH-SY5Y cells. Anti GFP shRNA-MC (1 μg) was loaded into RVG-exosomes (3 μg protein) using different electroporation voltages and intensity conditions stated on the graph and various buffers: electroporation buffer (EB) (10), Minimum Essential Media (MEM) or PBS and purified by ultracentrifugation. Cells were incubated with the loaded RVG-exosomes (3 μg) or transfected with anti GFP shRNA-MC (1 μg) using transfection reagent (TR). After 72 hours GFP protein levels were quantified relative to actin levels by Western blot. Ratios were normalized to control cells and expressed as mean ± SEM (n=3). b) The size profile of the RVG-exosomes loaded with anti GFP shRNA-MC (EB, 450V, 100 mA) was analyzed by nanoparticle tracker analysis (NTA) using NanoSight. c) Anti GFP shRNA-MC DNA extracted from electroporated exosomes after DNase digestion was quantified by nanodrop.

- Evaluation of the in vivo efficacy of shRNA-MC RVG-exosomes in the brain:

150 µg RVG-exosomes were electroporated with either 100 µg or 150 µg anti GFP shRNA-MC and purified as described in the Materials and methods. 150 µg electroporated RVG-exosomes were administered to EGFP mice by intravenous injection. Mice were sacrificed after 30 days and the brain dissected and frozen for mRNA and protein analyses. GFP mRNA levels were quantified relative to actin mRNA using qPCR (Suppl Fig 2a). Levels were significantly decreased in the olfactory bulb (100 µg plasmid: decreased by 46% p=0.032; 150 µg plasmid: decreased by 58% p=0.009), midbrain (100 µg plasmid: decreased by 60% p=0.034; 150 µg plasmid: decreased by 70% p=0.009) and striatum (100 µg plasmid: decreased by 55% p=0.032; 150 µg plasmid: decreased by 65% p=0.009). The decrease was always greater in the mice treated with 150 µg GFP shRNA-MC loaded exosomes (Sup Fig 2a). While GFP mRNA was decreased (35-45%) in the cortical region this did not reach statistical significance. Relative to the controls the GFP protein levels were decreased in all regions studied but was only statistically significant in the olfactory bulb (decreased by 55%, p=0.02) and midbrain (decreased by 35%, p=0.032) at the higher dose of plasmid used (Sup Fig 2b).

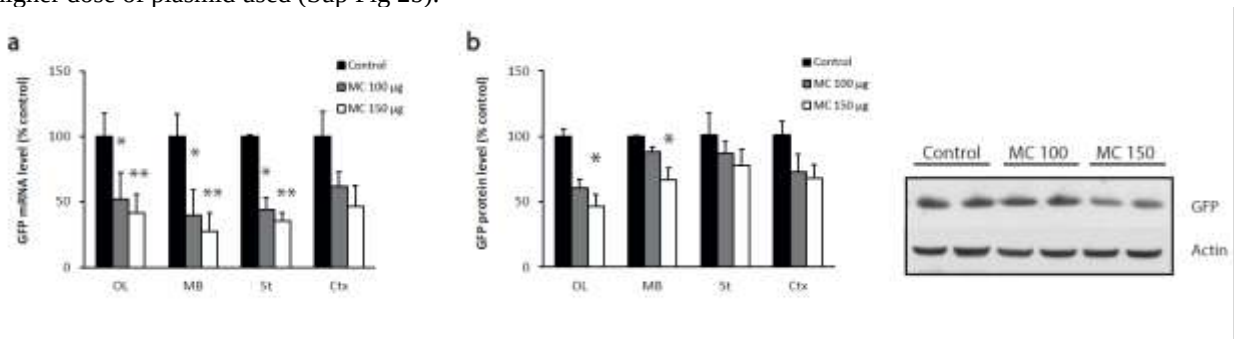

Figure S2. In vivo anti GFP shRNA-MC RVG exosome therapy of EGFP mice. EGFP transgenic mice were injected (iv) with 150 µg RVG-exosomes after electroporation with either 100 µg or 150 µg anti GFP shRNA-MC. Mice were sacrificed after 30 days. Olfactory bulb (OL), midbrain (MB), striatum (St) and cortex (Ctx) samples were analysed for GFP mRNA and protein levels by a) qPCR and b) Western blot respectively and quantified relative to actin. Values are normalised to controls and expressed as mean +/- SEM (n=4) Non-parametric Kruskal-Wallis test, statistical analyses compared with the control group, *$p < 0.05$, **$p<0.01$. Typical blot of the midbrain is shown.

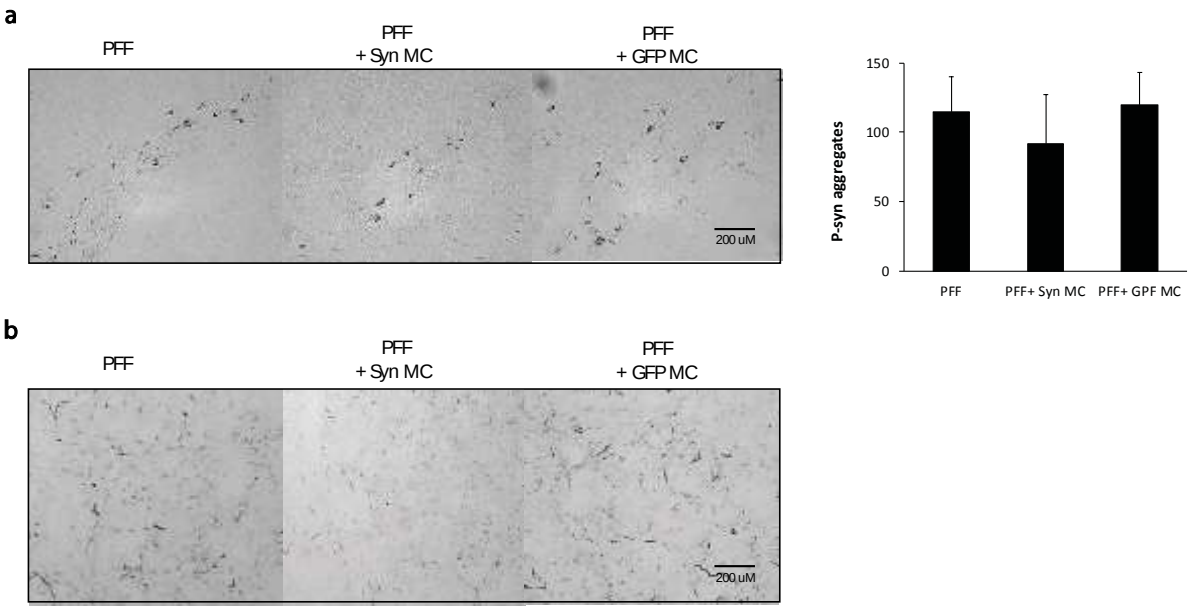

Figure S3. Effect of anti alpha-synuclein shRNA-MC RVG-exosome treatment 30 days after administration of alpha-synuclein PFF in mice. a) Immunohistochemical analysis of midbrain sections stained with antibodies to S129 phospho-alpha-synuclein in alpha-synuclein PFFs treated mice (PFF) and following anti alpha-synuclein shRNA-MC RVG-exosome therapy (PFF + Syn MC) or anti GFP shRNA-MC RVG-exosome therapy (PFF + GFP MC). Quantification of S129 phospho-alpha-synuclein positive aggregates in the midbrain. b) Representative images of ipsilateral striatal sections immunolabeled with S129 phospho-alpha-synuclein after 30 days. Data expressed as mean ± SEM (n=4). Non-parametric Kruskal-Wallis test.

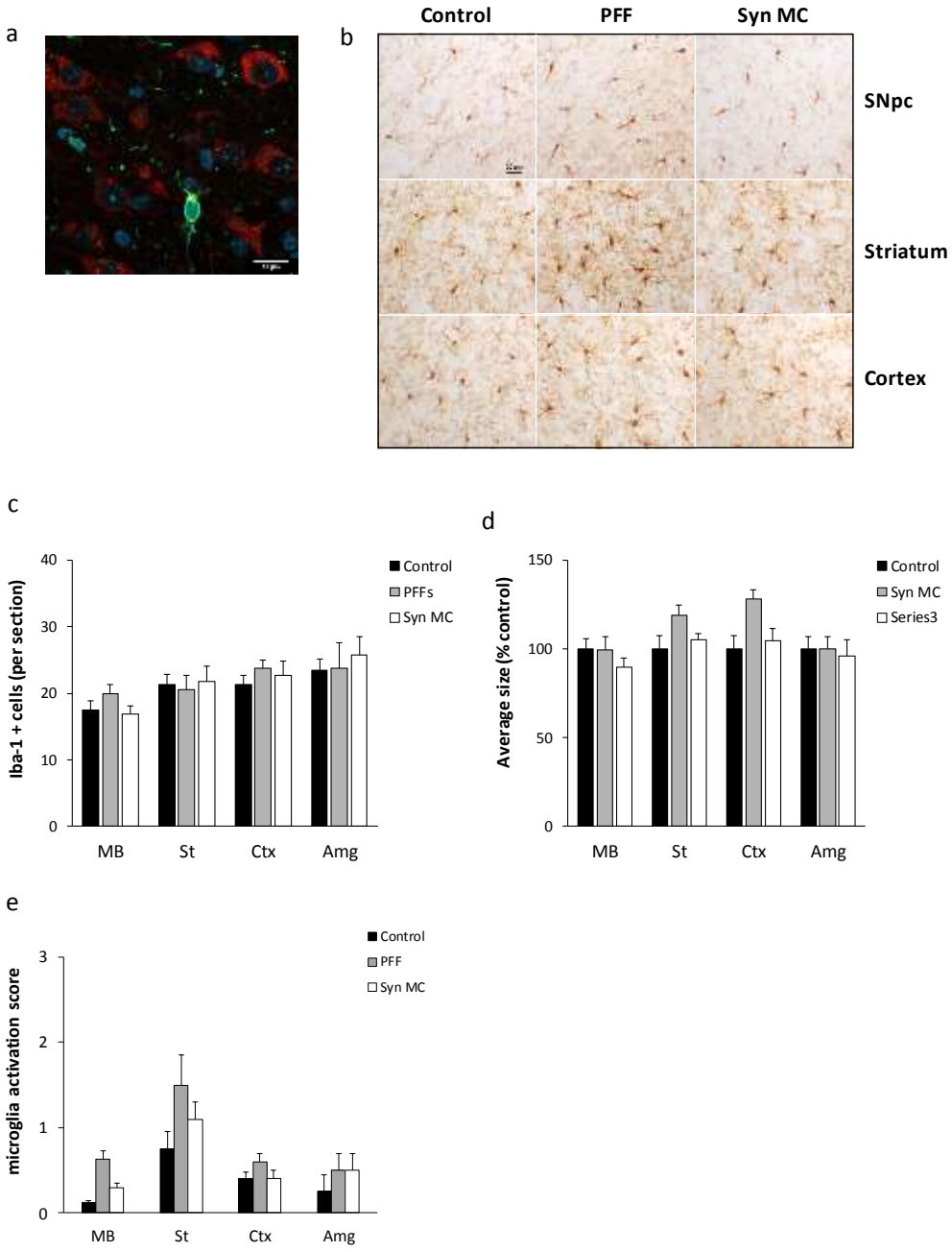

Figure S4. Analysis of microglial activation in Syn PFF treated mice and after anti syn shRNA-MC RVG exosome therapy a) Representative image of ipsilateral midbrain section immunolabeled with microglia marker iba1 (green) and TH (red) b) Representative images of ipsilateral substantia nigra, striatal and cortical sections immunolabeled with microglia marker iba1 after 90 days. Quantification of the number (per section) (c) and average size (d) of Iba1-positive cells in the ipsilateral mid brain (MB), striatum (St), frontal cortex (Ctx) and amygdala (Amg) (e) Qualitative rating of microgliosis revealed by Iba1 immunohistochemistry using Colburn scale (assessed 4-6 sections per region) . Data are expressed as mean ± SEM (n=5). Non-parametric Kruskal-Wallis test, statistical analyses compared with untreated control group.

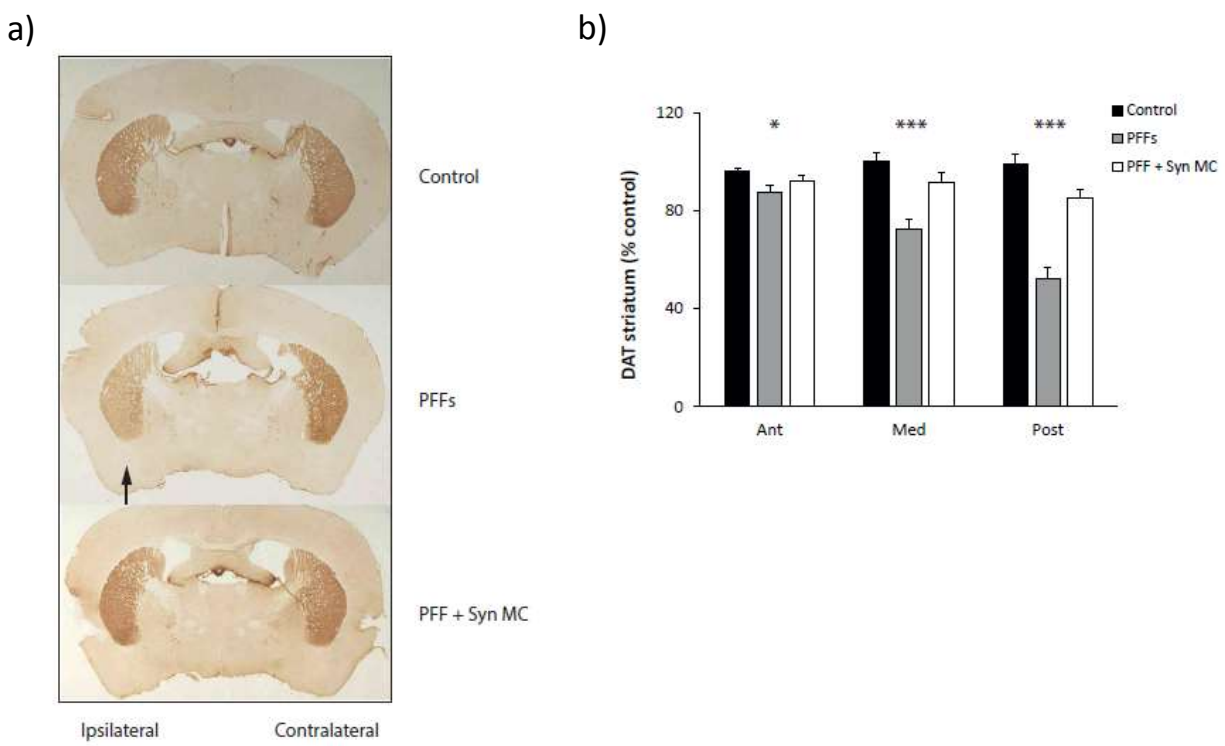

Figure S5. DAT immunostaining in the striatum in Syn PFF treated mice and after anti alpha-synuclein shRNA-MC RVG-exosome treatment. a) DAT immunostraining in a coronal section of the forebrain in control mice, 90 days after alpha-synuclein PFF treatment alone (PFFs) and following injection of 150 µg anti alpha-synuclein shRNA-MC RVG-exosome (PFF + Syn MC) treatment. b) Optical density measurements of striatal DAT in the ipsilateral side normalized to the contralateral striatum, images were quantified by Image J. Data normalized to control values and expressed as mean ± SEM (n=8). Non-parametric Kruskal-Wallis test, statistical analyses compared with untreated control group, *$p < 0.05$, ***$p<0.001$.

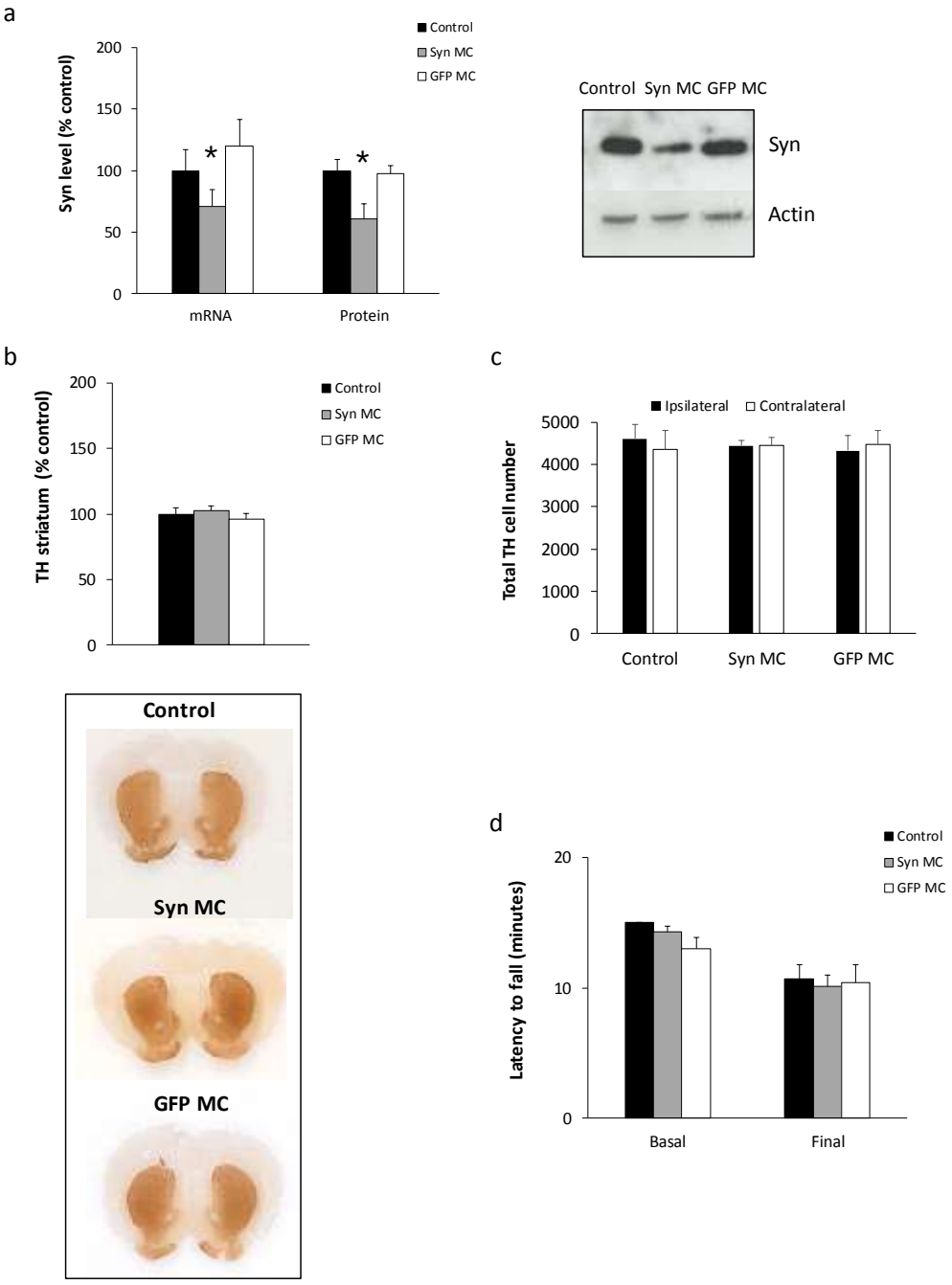

Figure S6. Effect of anti alpha-synuclein shRNA-MC RVG-exosome treatment in control mice. Normal C57BL6/C3H F1 mice sham injected intrastriatally were treated with 2 injections (iv, at 2 and 45 days) of 150 µg RVG-exosomes electroporated with 150 µg anti alpha-synuclein shRNA-MC (Syn MC) or 150 µg anti GFP shRNA-MC (GFP MC). Analyses at 90 days of (a) alpha-synuclein mRNA expression and protein levels normalized to actin in ipsilateral midbrain. Values expressed as mean ± SEM (n=10), data compared with untreated controls (Control). Typical Western blot of the midbrain is shown.(b) Optical density measurements of striatal TH, images were quantified by Image J. Data normalized to control values and expressed as mean ± SEM (n=8). (c) Dopaminergic neurons were stained with anti TH antibodies in coronal sections of the midbrain. Unbiased stereology was employed to quantify the number of nigral dopaminergic neurons on each side of the brain. (d) Wire hanging tests

were evaluated prior to the sham injection (basal) and after 90 days (Final). Non-parametric Kruskal-Wallis test, *$p < 0.05$.

Table S1. Sequences used for the siRNA, shRNA MC constructs and various qPCR analyses

NAME	SEQUENCE
Syn siRNA	GACAAAUGUUGGAGGAGCAdTdT
Syn shRNA MC	TTAATTAATACTAAGCTTAAAAAGACAAATGTTGGAGGAGCACTCTTGATGCTCCTCCAACATTTGTCGGGGATCTCTATCACTGATAGGGAA CTTATAAGATTCCCAAATCCAAAGACATTTCACGTTTATGGTGATTTCCCAGAACACATAGCGACATGCAAATATTGCAGGGCGCCACTCCCCT GTCCCTCACAGCCATCTTCCTGCCAGGGCGCACGCGCGCTGGGTGTTCCCGCCTAGTGACACTGGGCCCGCGATTCCTTGGAGCGGGTTGAT GACGTCAGCGTTCGAATTATCTGCAGTTAATTAA
GFP shRNA MC	GAACGCTGACGTCATCAACCCGCTCCAAGGAATCGCGGGCCCAGTGTCACTAGGCGGGAACACCCAGCGCGCGTGCGCCCTGGCAGGAAGA TGGCTGTGAGGGACAGGGGAGTGGCGCCCTGCAATATTTGCATGTCGCTATGTGTTCTGGGAAATCACCATAAACGTGAAATGTCTTTGGAT TTGGGAATCTTATAAGTTCTGTATGAGACCACAGATCCCCAAGCTGACCCTGAAGTTCATTCAAGAGATGAACTTCA GGGTCAGCTTTTTTT
α-Syn F	GCCAAGGAGGGAGTTGTGGCTGC
α-Syn R	CTGTTGCCACACCATGCACCACTCC
GFP F	CGCTACATCGCTCTTTCTTCA
GFP R	GACAGGCACATGCACTTCAAGA
Human GAPDH F	GAAGGTGAAGGTCGGAGT
Human GAPDH R	GAAGATGGTGATGGGATTTC
Mouse actin F	HK-SY-mo-600 against murine actin (PrimerDesign)
Mouse actin R	HK-SY-mo-600 against murine actin (PrimerDesign)

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Table S2. Inflammatory cytokine levels in transgenic S129D alpha-synuclein mice 45 days after RVG-exosomal treatment. Blood samples were collected 45 days after RVG-exosomal therapy and were analysed for 4 different cytokines. Values are mean +/- SEM, ND non detectable. Values were all within expected ranges for the RVG exosomal therapies and did not induce significant changes in cytokine levels, consistent with an immunologically inert profile of the treatment.

	Experimental group		
Cytokine	**Control (n=10)**	**+ GFP MC (n=5)**	**+ Syn MC (n=10)**
TNFα (pg/mL)	ND	4,16 ± 0.4	0,53 ± 0,1
IFNγ (pg/mL)	ND	ND	ND
IL-6 (pg/mL)	0,41 ± 0,1	3,65 ± 0,1	5,54 ± 0,2
IP-10 (pg/mL)	49,95 ± 4,7	37 ± 3,8	22,29 ± 4.1

Table S3. Inflammatory cytokine levels in PFFs alpha-synuclein treated mice analyzed 90 days after the first treatment. Blood samples were collected 90 days after the first RVG-exosomal therapy and were analysed for 6 different cytokines. Values are mean +/- SEM, ND non detectable. There were no significant changes in mean level of any cytokine analyzed suggesting the immunologically inert profile of the treatment and that the RVG-exosome treatment does not activate the peripheral immune response.

	Experimental group		
		PFF treated Mice	
Cytokine	**Control (n=10)**	**Control (n=10)**	**+ Syn MC (n=10)**
TNFα (pg/mL)	3,6 ± 0,2	10,1 ± 2,3	7,7 ± 3,1
IFNγ (pg/mL)	2,5 ± 0,6	5,7 ± 2,1	2,1 ± 0,8
IL-4 (pg/mL)	ND	ND	ND
IL-5 (pg/mL)	4,0 ± 1	5,3 ± 1,2	2,4 ± 0,5
IL-6 (pg/mL)	25,0 ± 7,1	12,3 ± 3,4	5,4 ± 0,9
IL-12p70 (pg/mL)	ND	ND	ND

Table S4. Changes in specific gene expression following Syn PFF and Syn MC treatments. Transcriptomic analysis of contralateral cortex from control (G1), alpha-synuclein PFFs treated mice (PFF, G2) after 90 days following anti alpha-synuclein shRNA-MC RVG-exosome treatment (PFF + Syn MC, G3). Genes differentially expressed at significant levels are reported. The up-regulated genes are highlighted in green colour, whereas the down-regulated genes are highlighted in red. Genes are named according to the Gene Name established by the BioMart database (Ensembl) and listed in order from lower FDR values to the highest ones, FDR limit 0,05. (n=5). Data demonstrated no significant changes in gene expression associated with MC therapy (comparison G2 and G3).

PFFs (G2) vs Ctrl (G1) groups	**Syn MC (G3) vs Ctrl (G1) groups**	**Syn MC (G3) vs PFFs (G2) groups**
Stra6, Cspg4, Prdm11, Fam214a, Pkdrej, Tenm4, Jrk, Mical2, Adgrd1, Gucy2e, Zc3h6	*Tenm4, Xpo4, Adnp*	*Non-detected up-/down-regulated genes*
Tnfrsf25, Mirg, Ccdc117, Clk1, Prpf38b, Akap8I, Cfp, Cys1, Arglu1, Dnajb1, Arl4d, Jmjd6, Zan, mt-Rnr1, Clk4, RasI11a, Dnase1I2, Gm15832, Rpgr, mt-Rnr2	*Mirg, Tnfrsf25, Akap8I, Cfp, Fam193b, Gm28438, Dok3, mt-Rnr1, Rgs11, Shkbp1, mt-Rnr2, Slc2a4rg-ps*	

Table S5. Antibodies used in the study.

Antibody	Source	Host	IHC (Dilution)	WB (Dilution)
Alpha-synuclein	Abcam	Mouse		1/2000
Phospho S129 alpha-synuclein	Abcam	Rabbit	1/5000	
Dopamine transporter (DAT)	Millipore	Rat	1/2000	
Tyrosine hydroxylase (TH)	Chemicon	Mouse	1/2000	
Iba-1	Waco	Rabbit	1/500	
Green Fluorescent protein (GFP)	Millipore	Chicken		1/2000
Actin	Abcam	Mouse		1/30000

Table S6. Description of the qualitative scoring system for microglia activation state.

Score	Activation state	Criteria
0	Resting	Ramified cells with fine processes. Cells evenly distributed.
1	Mild	Ramified microglial, evenly spaced but with a slight increase in the number or density of cells
2	Moderate	Microglia display shortened and clumply processes, densely concentrated with slight overlap between individual microglia
3	Intense	Microglia displaying hypertrophy of cell bodies and retraction of processes, with apparent amoeboid morphology, overlap between individual microglia.

Supplemental methods

- Behavioural assessments

To evaluate the motor function, mice were tested using the wire hang and negative geotaxis tests before treatment and during the study at 30 day intervals and prior to being sacrificed. All tests were conducted between 09:00-13:00 in the lights-on cycle. Mice were habituated to the testing room for 1 hour before tests and the apparatus was cleaned with 70% ethanol in between animals to minimize odour cues.

1. Wire Hang Test: To assess neuromuscular strength and motor coordination, the wire hang test was performed. Each mouse was placed on a wire lid of a conventional housing cage. The lid was lightly agitated to encourage the animal to grip the bars and then was turned upside down. The latency of mice to fall off the wire grid was measured and averaged over two trials (15 min apart). Trials were stopped if the mouse remained on the lid for over 15 min.

2. Negative Geotaxis: Motor coordination was assessed on an inclined plane. Each mouse was placed with its head facing downward on a wire grid that was set at a 45º angle to the plane. The behaviour of the animal was observed during 30 seconds and scored as follows: 0=turns and climbs, 1=turns and freezes, 2=moves, but fails to turn, 3=does not move. The latency to turn 180º to an upright position and initiate climbing was recorder for all animals that received a score of 0.

- Serum collection

Blood samples were collected when the animals were sacrificed. Mice were deeply anesthetized with an overdose of inhaled isofluorane and bled by cardiac puncture. Blood samples were collected in EDTA-free vials and stored at 4ºC for 30 min, followed by centrifugation at 1200 x g for 10 min to isolate serum.

- Minicircles

Minicircles consisting almost only of the gene of interest (GOI) derived from parental plasmids (PP) with that GOI, e.g. an antibiotic resistance marker and an origin of replication within E.coli (ori), as well as two special signal sequences right and left of the GOI. An intra-molecular recombination process (Darquet et al., 1997; Bigger et al., 2001: Chen et al., 2003; Jechlinger et al., 2004) separated the parental plasmid (PP) into a miniplasmid (MP) and a minicircle (MC) (Scheleef., 2013). The GOI (plus one recombination signal sequence element) is present within the MC, that is circular and finally results in only the GOI and the remaining sequence element deriving from the recombination event in a supercoiled circular molecule.

The production of MC DNA is carried out in 2 major steps: the cultivation in a bioreactor and the purification by specific chromatographic steps. The cultivations were carried out at 37°C in a MBR bioreactor (MBR BIO REACTOR, Switzerland) with 5 L, pH adjusted to 7.0 with 2 M sodium hydroxide solution and 2 M phosphoric acid. The air flow rate was fixed at 5 L/min. The oxygen concentration of 60% was controlled by varying the stirrer speed. LB-medium was used without addition of any antibiotics. The bioreactor was inoculated with 50 mL of an E. coli K12 culture transformed with the parental plasmid PP and grown in LB-medium for approximately 15 h. The recombinase expression was induced at an OD600 » 4 by adding L-arabinose. After 1 h of further growth cells were harvested by centrifugation, frozen and purified by the PlasmidFactory contract manufacturing service (Bielefeld, Germany).

After initial optimization runs in a 5 L scale further production runs were carried out in larger scales e.g. 20 L or above. In these cases the pre-culture was scaled-up in a linear way.

After successful recombination, the MC was separated from the MP. This was done by a series of chromatography steps, including an affinity chromatography step separating MP and MC. The approach to selectively bind a sequence motif (identification sequence) with the purpose of separating this from a mixture of different DNAs (Gossen et al., 1993). The recombination product (MC and MP) was further purified by affinity chromatography as previoisly described (Mayrhofer et al., 2008). . The sequence specific DNA binding was optimized with different ionic strength and pH values and resulted in a highly purified supercoiled monomeric MC product.

- cDNA Library Preparation and Ultrasequencing

Integrity and concentration of RNA was determined using the Experion automated electrophoresis system (Bio-Rad). RNA with an integrity value >8,9 underwent further analysis.

Sequencing libraries for mRNA analysis were prepared from 1 μg of total RNA following the RiboZero Human/Mouse/Rat and TruSeq RNA Library (LS) Preparation Kit v2 (Illumina Inc.) guidelines. All libraries were run in a HiSeq 2000 PE100 lane in Rapid mode, pooled in equimolar amounts to a 10 nM final concentration. Library concentration was measured via Qubit 3.0 (Thermo Fisher Scientific) and Experion (Bio-Rad) before high throughput sequencing.

- mRNA bioinformatic analysis

The quality of RNAseq results was assessed using FastQC. The raw reads were trimmed, filtered with a Phred quality score of at least 25 and all adapters removed with Trim Galore2 software. Clean reads were aligned versus the Mus Musculus reference genome (release GRCm38_80, http://ftp.ensembl.org/pub/release-80/gtf/mus_musculus/) using Tophat2 with default parameters. Resulting alignment files were quality assessed with Qualimap2 and sorted and indexed with Samtools software. Quantitative differential expression analysis between conditions was performed both by DESeq2 and edgeR implementations, to compare paired groups. Both methodological analyses, implemented as R Bioconductor packages, perform read-count normalization following a negative binomial distribution model. In order to automate this process and facilitate all group combination analysis, the SARTools pipeline was used. All resultant data was obtained as an .html file and .csv tables, including density count distribution analysis, pairwise scatter plots, cluster dendrograms, Principal Component Analysis (PCoA) plots, size factor estimations, dispersion plots and MA- and volcano plots. Resulting tables, including Ensembl Gene ID, raw counts, normalized counts, Fold-Change estimation and dispersion data for each of the analysis methods (DESeq2 and edgeR) were annotated with additional data from Biomart database.

In order to control the False Discovery Rate (FDR), p-values were amended by Benjamini-Hochberg (BH) multiple testing correction. Those features showing corrected p-values values below 0.05 threshold were considered up- or down-regulated genes. In order to reinforce downstream analysis and discard false-positive over/under-expressed genes, common up- and down-regulated features were extracted from DESeq2 and edgeR tables.-

- ELISA

Serum concentrations of IL-6, IP-10, TNFα and IFNγ were measured using 50 μL of serum using ELISA kit (Thermo Scientific (IFN), R&D (others) as per manufacturer's protocol.

Supplemental references

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