Title: Systemic exosomal delivery of shRNA minicircles prevents Parkinsonian pathology

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Running title: Exosomal-shRNA minicircles stop Parkinson

Abstract:

The development of new therapies to slow-down or halt Parkinson's disease progression is a healthcare priority. A key pathological feature is the presence of alpha-synuclein aggregates and there is increasing evidence that alpha-synuclein propagation plays a central role in disease progression. Consequently the down-regulation of alpha-synuclein is a potential therapeutic target. As a chronic disease, the ideal treatment will be minimally invasive and effective in the long-term. Knockdown of gene expression has clear potential and siRNAs specific to alpha-synuclein have been designed, however the efficacy of siRNA treatment is limited by its short-term efficacy. To combat this we designed shRNA minicircles (shRNA-MC), with the potential for prolonged effectiveness, and used RVG-exosomes as vehicle for specific delivery into the brain. We optimized this system using transgenic mice expressing green fluorescent protein (GFP) and demonstrated its ability to down-regulate GFP protein expression in the brain for up to 6 weeks. RVG exosomes were used to deliver anti alpha-synuclein shRNA-MC therapy to the alpha-synuclein preformed fibrils induced model of parkinsonism. This therapy decreased alpha-synuclein aggregation, reduced the loss of dopaminergic neurones and improved the clinical symptoms. Our results confirm the therapeutic potential of shRNA-MC delivered by RVG-exosomes for long-term treatment of neurodegenerative diseases.

Introduction

Parkinson's disease (PD) is the second commonest neurodegenerative disorder worldwide, but effective disease modifying treatments are still lacking[1]. The primary cause of PD in the majority of patients is not known, however, various genetic mutations have been used to define the potential disease mechanisms involved. A number of mutations and multiplications of the alpha-synuclein gene (SNCA) are a known cause of familial PD and Genome-wide association studies have highlighted the SNCA locus as a potential PD risk factor[2]. When combined with the observation that alpha-synuclein aggregates are a predominant feature of Lewy bodies in all PD patients it is clear that alpha-synuclein plays an important role in PD pathogenesis[3]. More recently alpha-synuclein aggregation has been shown to be transmitted from pathological affected neurons to healthy unaffected neurons[4, 5] and critically the injection of alpha-synuclein fibrils into the striatum of normal mice is sufficient to recapitulate important clinical and pathological features of PD[6]. Consequently, the central role of alpha-synuclein in PD pathogenesis suggests that strategies to decrease the expression of neuronal alpha-synuclein levels are an attractive approach for the prevention and treatment of PD.

Gene therapy is a promising tool for the treatment of PD. The down-regulation of alpha-synuclein levels using siRNA[7] or shRNA delivered by AAV[8] in the central nervous system decreased alpha-synuclein aggregates and motor deficit in transgenic or toxin-based models of PD. However, one of the major challenges is designing a system that will readily deliver molecules to the brain and modify the disease by altering gene expression for prolonged periods, preferably in delivery vehicles that are not hindered by immune inhibition such as with AAV[9]. We developed modified exosomes, which specifically target the brain, by placing a brain targeting peptide (rabies virus glycoprotein, RVG peptide) on the exterior surface of the

exosome. These RVG-modified exosomes loaded with siRNAs and injected into the tail vein safely delivered the siRNAs to the brain causing an effective knockdown of BACE1[10] and alpha-synuclein[11] protein throughout the brain. While successful, the half-life of siRNAs in vivo is relatively short and the treatment of chronic diseases would require longer term gene silencing. Plasmids expressing a short-hairpin RNA (shRNA) are better for longer term gene silencing, however conventional plasmids are relatively large and have been resistant to electroporation into exosomes (unpublished observations). Minicircles (MC), however, are double stranded DNA vectors that contain the transgene expression cassette without additional bacterial sequences[12], making them smaller, but favouring higher transgene expression for longer periods[13] and, therefore, have the potential to deliver greater target depletion for longer[13]. In this way, MCs are excellent candidates for use with RVG-exosomes to deliver shRNAs and would represent an ideal combination for a disease-modifying therapy for PD and other neurodegenerative diseases.

In this study we demonstrated that shRNA-MC constructs can be readily delivered to the central nervous system (CNS) by RVG-exosomes and decrease target gene expression for prolonged periods. More specifically we have now been able to demonstrate a decrease in alpha-synuclein expression in the brain of a mouse model of PD, decreasing the level of alpha-synuclein aggregation, reducing the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and improving the clinical symptoms. These results confirm the potential of shRNA-MC RVG-exosome therapy to induce long-term down-regulation of protein expression in the brain and could be further developed as an effective PD therapy. In principle this novel approach can be applied to other neurodegenerative diseases.

Results

- Optimization and validation of shRNA-MC RVG-exosomes

RVG-exosomes were isolated from the conditioned media from primary dendritic cells transfected with RVG-Lamp2b[10]. Optimal conditions for loading the RVG-exosomes (3 μg protein) with 1 μg of anti GFP shRNA-MC constructs (Table S1) were evaluated using their efficacy at down-regulating GFP expression in SH-SY5Y cells expressing GFP. The most efficient down-regulation of GFP was obtained using electroporation with electroporation buffer, at 400 V-125 μF or 450 V-100 μF, with the latter condition giving slightly greater GFP knockdown (Fig S1a). The size profile of the exosomes after electroporation were within normal limits (Fig S1b), and DNase protection assay confirmed that the shRNA-MC was present inside exosomes after electroporation (Fig 1a and Fig S1c).

The efficiency of anti GFP shRNA-MC to down-regulate GFP expression following different delivery methods was evaluated in SH-SY5Y cells expressing GFP, relative to actin protein levels (Fig 1b). Relative to controls, cells transfected with 1 μg GFP shRNA-MC using X-tremeGENE HP transfection reagent demonstrated a 47% decrease in GFP protein (TR, p=0.046). Cells treated with 3 μg RVG exosomes electroporated with 1 μg anti GFP shRNA-MC purified by centrifugation alone demonstrated a 45% decrease in GFP protein (Exo, p=0.046) and this was similar for cells treated with RVG exosomes loaded with GFP shRNA-MC following DNase treatment to destroy un-encapsulated DNA (DNase, 50%, p=0.046) (Fig 1b).

To investigate the ability of shRNA-MC RVG-exosomes to down-regulate gene expression in the CNS we evaluated the intravenous (iv) injection of 150 μg RVG exosomes electroporated with two different anti GFP shRNA-MC concentrations (100 μg or 150 μg MC) into a transgenic mouse over-expressing EGFP protein. After 30 days GFP protein levels were significantly

decreased in the olfactory bulb and midbrain regions, and the decrease in GFP was always higher with 150 μg MC loaded exosomes (Fig S2a, S2b). Extraction of the anti GFP shRNA-MC construct from the electroporated RVG-exosomes (150 μg shRNA-MC with 150 μg RVG-exosomes), revealed a 19 ± 5% recovery of the shRNA-MC DNA in the RVG-exosomes (30 μg shRNA-MC/150 μg exosomes) as quantified by nanodrop analysis.

Our results demonstrated that anti GFP shRNA-MC can be successfully loaded into RVG-exosomes which can down-regulate GFP protein levels in vitro and for at least 30 days in selected brain regions after iv administration.

- Evaluation of alpha-synuclein down-regulation by shRNA-MC RVG-exosomes.

An anti alpha-synuclein shRNA-MC construct was generated using a sequence that targets both mouse and human alpha-synuclein mRNA (Table S1) based upon previous siRNA down-regulation studies[11]. RVG-exosomes (3 μg) were loaded with anti alpha-synuclein shRNA-MC construct (1 μg) using the optimized conditions used above, and their ability to down-regulate alpha-synuclein protein was evaluated in SH-SY5Y cells overexpressing S129D alpha-synuclein. Relative to controls, S129D alpha-synuclein protein levels were significantly decreased 72 hours after lipofection with anti alpha-synuclein shRNA-MC (MC-TR, 1 μg, decreased by 44%, p=0.009), with the shRNA alpha-synuclein parental plasmid (Plasmid-TR, 1 μg, decreased 41.3%, p=0.014) or with the previously used anti alpha-synuclein siRNA (siRNA-TR, 100 nM decreased 37%, p=0.009) (Fig 1c)[11]. Alpha-synuclein protein levels were decreased to a similar extent after incubation with 3 μg RVG-exosomes containing either alpha-synuclein siRNA (siRNA Exo, 100 nM, decreased 48.3%, p=0.025) or anti alpha-synuclein shRNA-MC (MC Exo, 1μg, decreased 47%, p=0.014) (Fig 1c) suggesting that delivery by RVG-exosomes to SH-SY5Y cells was at least as efficient as transfection.

The efficacy of the iv delivery of RVG-exosomes containing anti alpha-synuclein shRNA-MC to down-regulate alpha-synuclein in vivo was studied using a transgenic mouse model expressing the phospho-mimic human S129D alpha-synuclein-HA under the prion promoter[11]. This model exhibits alpha-synuclein aggregates throughout the brain from 3 months of age. 10-14 week old transgenic mice were injected (iv) with 150 μg RVG-exosomes electroporated with 150 μg anti alpha-synuclein shRNA-MC (n=10) or 150 μg RVG-exosomes loaded with 150 μg anti GFP shRNA-MC as a control group (n=5) and compared with mice injected with vehicle alone (n=10). Mice were sacrificed 45 days after injection and were evaluated for alpha-synuclein mRNA and protein levels. Administration of anti alpha-synuclein shRNA-MC RVG-exosomes resulted in the down-regulation of S129D alpha-synuclein-HA mRNA levels in all regions studied, which were significantly lower in the midbrain (decreased by 45%, p=0.001), cortex (decreased by 28%, p=0.043), striatum (decreased by 30%, p=0.042) and brainstem (decreased by 32%, p=0.04), but with no consistent changes in the group treated with anti GFP shRNA-MC RVG-exosomes (Fig 1d) . In the group treated with alpha-synuclein shRNA-MC the S129D alpha-synuclein-HA protein levels were lower than the controls in all regions studied, and these were significantly lower in olfactory bulb (decreased 33%, p=0.034) and midbrain (decreased 54%, p=0.003) (Fig 1e), confirming the potential of this therapy for longer term down-regulation of alpha-synuclein levels in the CNS.

The serum levels of TNFα, IFNγ, IL-6, IP-10 in the alpha synuclein or GFP shRNA-MC RVG-exosomes treated mice 45 days post-treatment were not increased to levels indicting they had induced immune activation and were not significantly different to the controls (Table S2).

- Alpha-synuclein shRNA-MC RVG-exosomal therapy prevents the neurodegeneration in the Syn PFF mouse model of PD.

While the in vivo RVG-exosome delivery of anti alpha-synuclein shRNA-MC therapy was able to decrease brain alpha-synuclein protein levels over a prolonged period it is important to identify if this can influence the clinical, pathological and degenerative changes typical of Parkinsonism. The progressive alpha-synucleinopathy mouse model based on the intrastriatal injection of alpha-synuclein preformed fibrils (Syn PFF) exhibits the progressive spread of alpha-synuclein aggregation, loss of dopaminergic neurons and clinical defects affecting motor functions[6] and is ideal to evaluate the efficacy of this therapy on key clinical and pathological features associated with PD.

Initial studies aimed to both establish the Syn PFF model and confirm the efficacy of the RVG-exosomal delivered anti alpha-synuclein shRNA-MC therapy in this model were performed. 24 normal C57BL6/C3H mice received unilateral intrastriatal injection of murine alpha-synuclein PFF. After two days mice were injected (iv) with 150 μg RVG-exosomes loaded with alpha-synuclein shRNA-MC (150 μg, n=8), or with 150 μg RVG-exosomes loaded with anti GFP shRNA-MC (150 μg, n=8), or an injection (iv) of vehicle (glucose 5%, n=8). Mice were sacrificed 30 days after the treatment.

After 30 days in the Syn PFF injected mice there were no detectable changes in alpha-synuclein mRNA levels in the ipsilateral (Fig 2a) or contralateral (Fig 2c) brain regions relative to control mice. There was a significant increase in alpha-synuclein protein levels in the striatum reflecting alpha-synuclein aggregation (Fig 2b), which was observed as S129 phospho-alpha-synuclein positive neurites in striatum and inclusions in the midbrain (Fig S3), consistent with alpha-synuclein aggregation as previously reported[6]. In the Syn PFF injected mice, 30 days after treatment with anti alpha-synuclein shRNA-MC RVG-exosome therapy there was evidence that alpha-synuclein mRNA levels remained down-regulated in the ipsilateral midbrain (decreased by

29%, not sig), striatum (decreased by 36%, p= 0.046) and cortex (decreased by 29%, not sig) relative to control but unaffected in the mice treated with RVG-exosomes loaded with anti GFP shRNA-MC (Fig 2a). At this time point the RVG-exosome alpha-synuclein shRNA-MC therapy was associated with a decrease in alpha-synuclein protein levels in all 3 ipsilateral brain regions analysed (Fig 2b), in particular the significant increase in alpha-synuclein levels in the striatum with Syn PFF injection was reduced to values approaching the controls. However, with RVG-exosome anti GFP shRNA-MC therapy alpha-synuclein levels were similar to PFF treated mice, confirming the specificity of the alpha synuclein shRNA-MC (Fig 2b). The S129 phospho-alpha-synuclein positive inclusions evident in the midbrain of the Syn PFF injected mice (Fig S3) were also present in the GFP shRNA-MC treated (Fig S3) and the alpha-synuclein shRNA-MC treated mice, although this preliminary study suggested aggregates were mildly lower in the latter (decreased 20%, not sig, Fig S3).

In a second cohort of mice the RVG-exosome anti alpha-synuclein shRNA-MC therapy was extended to 90 days after Syn PFF injections to explore the impact of the therapy on the neurodegenerative process. The mice were treated as above except a second therapeutic injection (iv) was administered after 45 days and the mice were analyzed 90 days after Syn PFF injection. At 90 days, striatal Syn PFF injections were associated with S129 phospho-alpha-synuclein positive inclusions in the SNpc, frontal, somatosensory, somatomotor cortex, amygdala and striatum (Fig 3a, b) which were not observed in control mice (data not shown), consistent with the PFF induction and spread of alpha-synuclein aggregation. After 90 days of the treatment with RVG-exosomes containing anti alpha-synuclein shRNA-MC therapy, there was a significant decrease in phospho-synuclein positive aggregates in the frontal (decreased 58% p=0.045), somatosensory (decreased 65% p=0.02), somatomotor cortex (decreased 80% p=0.014),

amygdala (decreased 37% p=0.048) and SNpc (decreased 37% p=0.046) (Fig 3a, b, c), but in the striatum the change was not significant. After 90 days the alpha-synuclein mRNA levels remained lower in all 3 brain regions analyzed in both the ipsilateral and contralateral parts of the brain (Fig 4a, b). This reduction was statistically significant in the ipsilateral midbrain (decreased 37 % compared to controls, p=0.039) and cortex (decreased 47 % compared to controls, p<0.001), and in the contralateral midbrain (decreased 42 % compared to controls, p=0.027) (Fig 4a, b). The prolonged decrease in alpha-synuclein mRNA levels in the contralateral regions were associated with lower levels of alpha-synuclein protein in all 3 regions and this was statistically significant in the midbrain (decreased 60 % compared to controls, p=0.033) and cortex (decreased 55 % compared to controls, p=0.039) (Fig 4d). Of the ipsilateral regions, however, only alpha-synuclein protein in the midbrain remained significantly decreased (decreased 54 % compared to controls p=0.043). In the striatum and cortex, the alpha synuclein protein levels were similar to controls (Fig 4d) which may reflect the alpha-synuclein aggregation evident in the ipsilateral brain (Fig 3a, b) which maybe more resistant to the effects of shRNA therapy.

The mice injected with Syn PFF demonstrated a unilateral loss of dopaminergic innervation with a decrease in TH staining in the anterior, medium and posterior stratum of 21% (p<0.001), 41% (p<0.001) and 56% (p<0.001) respectively (Fig 5a). Immunostainig for dopamine transporter (DAT) confirmed the loss of dopaminergic innervation with a decreased staining in the anterior, medium and posterior stratum of 13% (p=0.025), 27% (p<0.001) and 48% (p<0.001) respectively (Fig S5a, b). Stereological evaluation of TH positive staining neurons in the midbrain demonstrated Syn PFF injected mice had a unilateral 30% loss of dopaminergic neurons in the SNpc (p=0.002) (Fig 5b). Motor performance was evaluated at 30, 60 and 90 days using the negative geotaxis and wire hang tests. In agreement with previous studies[6], after 90

days the performance of the Syn PFF treated mice was compromised significantly on the negative geotaxis test (p=0.047) and the wire hang test (p=0.036) (Fig 5c, d).

The anti alpha-synuclein shRNA-MC RVG-exosomal treatment reduced the dopaminergic neuronal loss associated with Syn PFF treatment with levels similar to the controls (75% inhibition of cell loss relative to Syn PFF mice, p=0.028) (Fig 5b). This was associated with a significant protection against the Syn PFF induced loss of dopaminergic terminals in the striatum (Fig 5a, Fig S5a, b). These improvements in pathology were associate with improved clinical parameters at 90 days, with the performance of the Syn PFF treated mice that received the anti alpha-synuclein shRNA-MC RVG-exosome therapy being indistinguishable from the control mice (Fig 5c, d).

To assess if anti alpha-synuclein shRNA-MC RVG-exosome therapy affects the inflammatory response, brain sections from mice 90 days after Syn PFF injection were stained with anti-Iba1 antibodies and assessed for the number and morphology of positive cells. Activated microglia are usually increased in number and size with shortened and clumpy processes and can be qualitatively evaluated by the Colburn scale (Table S6). Striatal injection of Syn PFF was associated with a modest non-significant increase in the number and average size of Iba1-positive cells in those areas exhibiting S129 phospho-alpha-synuclein inclusions (Fig S4a, b, c and d). The qualitative analysis of microglia using the Colburn scale suggested that microglial cells were demonstrating features indicating mild activation at 90 dpi (score≥1) in all the areas analyzed (Fig S4e), whereas the Colburn scores approached the control levels following anti alpha-synuclein shRNA-MC RVG-exosomal treatment.

To evaluate the impact of repeated therapeutic applications on inflammatory markers, TNFα, IFNɣ, IL-4, IL-5, IL-6, IL-12p70 levels were analyzed in serum after alpha-synuclein shRNA-MC RVG-exosome treatment. The levels of these cytokines were not significantly elevated in either the Syn PFF or Syn PFF and RVG-exosome shRNA-MC treated mice (Table S3). This confirms that two doses of anti alpha-synuclein shRNA-MC RVG-exosome therapy did not activate the immune response in mice, nor have off-target effects assessed by transcriptomic analysis compared to Syn PFF mice (Table S4).

To analyze whether the prolonged downregulation of alpha-synuclein associated with this therapy had any detrimental effects, we studied 3 groups of control mice sham injected intrastriatally and treated with 2 doses of; RVG-exosomes loaded with alpha-synuclein shRNA-MC , RVG-exosomes loaded with anti GFP shRNA-MC or vehicle. Mice were analyzed 100 days after the first iv treatment. The RVG-exosome alpha-synuclein shRNA-MC therapy was associated with a significant decrease in alpha-synuclein mRNA and protein levels in midbrain, which was not apparent in the RVG-exosome anti GFP shRNA-MC therapy (Fig S6a). There was no evidence that the decrease in alpha-synuclein levels was associated with detectable changes in: striatal dopaminergic innervation (Fig S6b), number of TH positive staining neurons in the midbrain (Fig S6c) or in wire hang test of motor performance (Fig S6d).

Discussion

In this study we have demonstrated the potential of shRNA-MC RVG-exosomal therapy to induce the long term down-regulation of targeted genes in the CNS. More specifically we have been able to use this therapeutic approach to prevent dopaminergic cell death and motor abnormalities in a progressive mouse model of PD. Previous studies confirmed that iv injection of un-targeted exosomes[10], empty exosomes[10] or exosomes loaded with inactive modified

siRNA[11] did not significantly affect mRNA or protein expression in the brain. The present results confirm the capability of RVG-exosomes to deliver nucleic acids safely and specifically to the CNS in agreement with previous reports[11, 14, 15]. The exosomal delivery of minicircles expressing shRNA now extends the duration of knock-down achieved to over 7 weeks, compared to previous strategies using siRNAs[11, 14, 16] or plasmids[16] that required re-administration every few days. This longer down-regulation represents a clear clinical advantage for the prevention and treatment of chronic neurodegenerative diseases.

Alpha-synuclein is increasingly recognized as an important target for PD therapy with a suggestion that its down-regulation may delay or halt disease progression[17]. To date several therapeutic approaches designed to combat the progressive increase in alpha-synuclein aggregates in PD have been reported. These include strategies to prevent its aggregation, such as the use of intrabodies[18] or hsp70 over-expression[19], or to promote the removal of alpha-synuclein by immunization[20, 21]. Weekly intraperitoneal administration of alpha-synuclein immunotherapy for 30 days has been reported to decrease alpha-synuclein aggregates, improve motor behavior and reduce dopaminergic cell death in the alpha-synuclein PFF model[21]. However, the immunotherapy approach has some important issues to resolve regarding the antibody brain penetration, the timing of intervention in the course of the disease and the targeting of the extracellular alpha-synuclein. Moreover, LRRK2 antisense oligonucleotides have also been reported to reduce alpha-synuclein aggregation and cell death in this model[22], however this treatment required intraventricular administration and high doses of the gene therapy molecule and its influence was relatively local.

The approach we report here to decrease alpha-synuclein levels by exosomal delivery of shRNA-MC has the advantage that it is minimally invasive and acts on the intra-cellular protein levels

and could lead to a decrease in monomeric protein preventing its aggregation or post-translational modification. Previous studies reported dopaminergic alpha-synuclein down-regulation using intra-cerebral administration of AAV2 viral vectors and confirmed the therapeutic potential of this intervention in transgenic[23] and toxin[8] based models of PD. In agreement with all these reports our results confirmed that in a model demonstrating the spread of alpha-synuclein aggregation, loss of DA neurons and movement defects, decreasing alpha-synuclein expression was an effective therapy, strengthening this approach as a PD treatment. A clear advantage of the exosomal approach is that it enables a more widespread down-regulation of alpha-synuclein within the brain with aggregation decreasing in most regions analysed. Moreover, the prolonged down-regulation of alpha-synuclein levels allows fewer administrations which have economic and therapeutic benefits. Some studies reported a 25-50% loss of dopaminergic neurons over 21 days associated with a dramatic 45-85 % reduction in alpha-synuclein and an associated inflammatory response[24, 25]. However, other studies reported a decrease in alpha-synuclein expression using AAV in toxic and transgenic models[8, 23] without an associated decrease in TH expression. These results have been supported in a recently study using a transgenic mouse models treated with an intravenous injection of AAV9, delivered to the brain using FUS in combination with microbubbles[26]. The lack of toxicity associated with down-regulated alpha-synuclein levels reported in these studies, and confirmed in our expeiments could be related with a lower level of alpha-synuclein down-regulation. Moreover, SNCA knockout mice[27, 28] have a relatively mild phenotype which supports the potential redundancy of alpha-synuclein especially if only mildly decreased. The degree of alpha-synuclein down-regulation may be a critical factor in determining any detrimental effects, and care may be required to obtain alpha-synuclein reduction enough to halt or delay the neurodegenerative

process but sufficient to maintain the normal function of the protein fundamental for neuronal function.

In this study we used the alpha-synuclein PFF intra-striatal injected mouse model, where the injection of the PFFs has been shown to lead to the spread of LB-like aggregates and dopaminergic cell death and behavioral and motor changes[6]. The role of neuroinflammation and glial cell activation in PD has gained increased attention in recent years and are now recognized as important features of PD pathogenesis[29]. In this study we assessed the microglia immuno-reactivity in the Syn PFF model at the stage when intraneuronal alpha-synuclein inclusions were linked to DA neuron death and motor impairments. Our results demonstrated in the Syn PFF treated mice at 90dpi there was evidence for modest microglia activation in the areas affected by alpha-synuclein pathology and there was a tendency for this to decrease to control levels following RVG-exosomes shRNA-MC therapy. Mild inflammatory changes in this model are in agreement with data recently described in a rat model involving intrastriatal injection of Syn PFF[30]. These authors observed an increase in immunoreactivity during the initial accumulation of intracellular alpha-synuclein prior to dopaminergic cell death but a significant decrease in microglial immunoreactivity during the period of degeneration. Authors suggested that increase in MHC-II microglia may be a first-response mechanism to initial accumulation of intracellular alpha-synuclein, the RVG-exosomes shRNA-MC therapy reduced alpha-synuclein aggregation and could decrease microglia activation.

Our strategy involved treating the animals immediately after induction of the pathological process and therefore at the start of the spread of the pathology, prior to the development of the degenerative process and resulting clinical symptoms. Consequently, the benefits we observed may be limited to therapies applied very early in the disease process, and therefore it is important

to address if it can influence disease progression after the appearance of the pathology, and after the onset of motor symptom, corresponding to the initial clinical stage of PD.

The results of this study highlight the in vivo therapeutic potential of the RVG exosomal delivery of MC constructs. The combination of shRNA-MC to down-regulate gene expression with the specific delivery by targeted-exosomes is a potential treatment not only for PD but also for other neurodegenerative diseases (Alzheimer's disease, Amyotrophic Lateral Sclerosis) as well as other tissue specific pathologies potentially amenable to gene therapy. This new therapy will offer a completely different approach to therapy for these progressive neurodegenerative illnesses and, hopefully will change the lives of those who live with these conditions.

Material & Methods

- Cell culture

The human SH-SY5Y neuroblastoma cell line (American Type Culture Collection) clones constitutively expressing human S129D alpha-synuclein with a C-terminal haemagglutinin (HA) tag or GFP-alpha-synuclein were cultured using standard conditions[11].

- Exosome isolation

Murine dendritic cells were harvested from bone marrow and cultured ($3x10^6$ cells per well, 6 well/plate) in DMEM Glutamax (Gibco-BRL)[10], 10% Foetal Calf Serum (FCS) depleted of exosomes by centrifugation at 120,000 g for 60 minutes, and penicillin/streptomycin, supplemented with 10 ng/mL murine GM-CSF (MP Biomedicals). Cells were transfected after 4 days with 5 µg of RVG-Lamp2b plasmid and 5 µL of TransIT LT1 transfection reagent (Mirus Bio) as per manufacturer's instructions. Cell culture medium was changed on Day 7. After 24 hours the medium was removed and exosomes harvested by centrifuged at 12,000 g for 30 min

to remove cell debris, and the supernatant centrifuged again at 120,000 g for 1 hour to pellet exosomes. Exosomes were resuspended in 0.1 M ammonium acetate with a 27G needle.

- Loading of RVG-exosomes with shRNA-MC constructs

To determine the optimal conditions to load exosomes with shRNA-MC constructs, 1 μg shRNA-MC (see supplemental information for details) and 3 μg RVG-exosomes were mixed in 100 μL electroporation buffer (1.15 mM potassium phosphate pH 7.2, 25 mM KCl, 21% Optiprep), PBS or Minimum Essential Medium (MEM) and electroporated using 3 different settings (450 V, 100 mA; 450 V, 250 mA; 400 V, 125 mA;) in a 4 mm cuvette using GenePulse Xcell electroporator BioRad. The exosome samples were treated with 1U DNase (Promega) for 30 minutes at 37 ºC and purified by ultracentrifuged at 120,000 g for 1 hour. Exosomes were resuspended in RPMI medium.

- Exosome analysis and shRNA-MC content

Extracellular vesicle size distribution was assessed by nanoparticle tracker analysis using a NS500 instrument (Nanosight Ltd.) as described previously[11]. To evaluate the quality and concentration of shRNA-MC in RVG-exosomes following electroporation and DNAse treatment, ultracentrifuge purified exosomes were used to purify the shRNA-MC constructs .using the Qiaprep Spin miniprep kit. DNA was qualitatively analysed by ethidium bromide stained agarose gel electrophoresis and quantified using Nanodrop (Thermo) spectrophotometry. The percentage of shRNA-MC loaded into RVG-exosomes by electroporation was quantified by qPCR and compared to the same amount of shRNA-MC not electroporated. The DNAse protection assay involved incubation the samples with 1U DNAse at 37 ºC for 30 minutes.

- Treatment of cells with siRNAs, shRNA plasmid and shRNA minicircle

Cells were transfected with 100 nM siRNAs (see supplemental information for details) (Eurogentec) using HiPerfect transfection reagent (Qiagen) or with 1 μg of alpha-synuclein shRNA plasmid, or 1 μg anti GFP or anti alpha-synuclein shRNA-MC with 1 μL of X-tremeGENE HP transfection reagent (Roche) .

- Experimental design

Normal male C57BL6/C3H F1 mice (8-9-week-old) and male EGFP (C57BL/6-Tg(CAG-EGFP)131Osb/LeySopJ) mice (8-10-week-old) were purchased from Charles River. Male S129D alpha-synuclein transgenic mice (10-14-week-old)[11] were generated at University College London (UCL).

Normal C57BL6/C3H F1 (8-9 week-old) mice received an injection of sonicated murine alpha-synuclein PFFs (5 μg) into the dorsal striatum as previously described[6]. All animal experiments were designed to minimize the suffering and pain of the animals and were conducted according to the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals. Sample size was calculated using an online programme

(http://powerandsamplesize.com/Calculators/Compare-k-Means/1-Way-ANOVA-Pairwise). All animals were randomly distributed to the cages by a technician of the animal facilities and before any procedure; the cages were randomized to each group by a person not involved in the study. All the in vivo experiments were blinded and the investigators responsible for data collection and analysis were blinded. The experiments with the EGFP and the S129D alpha-synuclein transgenic mice were carried out in the animal unit, Royal Free Campus, University College London, London, UK according to procedures authorized by the UK Home Office. The experimentation involving the alpha-synuclein PFF intra-striatal injection model were approved

by Spanish Local Board for Laboratory Animals and performed in accordance with the ethical permission.

- RVG-Exosome treatment of Mice

For the therapeutic preparation of RVG-exosomes containing shRNA-MCs, 150 μg of shRNA-MC and 150 μg of RVG-exosomes were electroporated (450 V, 100 mA) in 5000 μL of electroporation buffer (1.15 mM potassium phosphate pH 7.2, 25 mM potassium chloride, 21% optiprep) and treated with 100U DNase (Promega) at 37 ºC for 30 minutes. After ultracentrifugation (120,000 g for 1 hour) the exosomes (150 μg) were resuspended in 100 μL of 5% glucose immediately before tail vein injection. Animals were sacrificed 30, 45 or 90 days after injection.

-Clinical evaluation

To evaluate motor function (see supplemental information for details), mice were tested using the wire hang and negative geotaxis tests. These were conducted before treatment and at 30 day intervals during the study and prior to sacrifice.

- Immunohistochemistry

Animals were perfused with PBS followed by 4% paraformaldehyde in PBS, the brains were post-fixed in 4% paraformaldehyde, cryoprotected in 30% sucrose, frozen and stored at -80 °C. 30-μm-thick coronal sections were prepared using a cryostat. Slices were washed with PBS and were incubated with 3% hydrogen peroxidase in PBS to inactivate the endogenous peroxidase. The slices were washed in PBS and were incubated with a blocking solution PBS containing 5% normal goat serum, 0.04% Triton X-100) for 1 hour. Slices were incubated for 24 or 48 hours at 4 ºC with the primary antibodies (Table S5): anti-TH (Millipore), anti-alpha-synuclein (phospho

S129, Abcam), anti-DAT (Millipore), anti-Iba1 (Wako). Slices were washed three times with PBS and were then incubated with fluorescent or biotinylated secondary antibody of the appropriate species. All the samples were processed simultaneously to allow comparison.

The total number of DA (TH-positive) neurons in the SNpc was estimated by sterological cell counting using the optical fractionator method which is unaffected by changes in the volume of reference of the structure sampled[31, 32]. We used an interactive computer system consisting of an Olympus microscope equipped with a digital camera (Lumenera, MBF). The interactive test grids and the motorized stage were controlled by Stereo Investigator software (MicroBrightField, VT, USA). TH-positive stained neurons were counted in the SNpc throughout the entire rostro-caudal axis of the SNpc (10 sections with a 4-section interval). The total numbers of TH-positive stained neurons in the SNpc were calculated using the formula described by West[33].

Striatal optical density (OD) of TH and DAT immunostaining was used as an index of the density of striatal dopaminergic innervation. This was determined by computer-assisted image analysis using the Image J program (National Institutes of Health, USA). All samples were processed at the same time and digital images were captured under the same exposure settings for all experimental analyses. Briefly, six representative rostro-caudal sections (at three levels of the striatum) were examined for each animal and regions of interest in the striatum were delineated and pixel densities were estimated using ImageJ. Background staining was quantified by measurement of pixel intensities in the white matter and subtracted from striatal regions for normalization.

Numbers of phospho-alpha-synuclein inclusions were manually quantified at 20 X magnification on coronal sections (120 µm intervals between sections) at multiple rostrocaudal levels corresponding to SNpc, frontal cortex, striatum and amygdala from 8 animals per group. SNpc

aggregates were assessed in sections that covered the full extent of SNpc, double labelled using antibodies against S129 phospho-alpha-synuclein and TH to quantify intra-DA neuron alpha-synuclein inclusions. Aggregates in other regions were quantified in sections stained for S129 phospho-alpha-synuclein. The regions were defined using the Paxinos and Franklin atlas (Paxinos and Franklin, 2013).

For evaluation of microglia activation, representative rostro-caudal sections corresponding to SNpc, frontal cortex, striatum and amygdala were stained with Iba1 antibody (Chemicon). Estimation of numbers of Iba1-positive cells and their average size was calculated using Image J software (NIH). Microglial cells were also semi-quantitatively analyzed by using a 4 point categorical rating scale developed by Colburn and colleagues[34] which provide an evaluation of microgliosis based on morphological and immunoreactivity changes. The rating criteria are explained in table S6.

Immunofluorescent images were captured with a Zeiss LSM 800 confocal scanning laser microscope and images taken under identical conditions were analyzed with FIJI.

- Western blot

Cell and brain samples were homogenised in buffer containing; 10 mM Tris/HCl (pH 7.4), 0.1% sodium dodecyl sulfate, protease inhibitor mixture (Thermo scientific), and DNAse (Promega). Samples were solubilised in LDS buffer and reducing agent and separated on NuPAGE Novex 4%-12% Bis-Tris Gels (Invitrogen), blotted onto PVDF membrane and analysed by Western blot as previously described11 using anti alpha-synuclein (Abcam) and anti beta-actin (Abcam) antibodies. Horseradish peroxidise-conjugated anti mouse IgG secondary antibody was detected using ECL Western Blot Substrate (Pierce) and Hyperfilm ECL (GE Healthcare). Films were

scanned and signals in the linear range were quantified using Image J and normalized to beta-actin levels.

- Quantitative PCR

Total RNA was isolated using the RNeasy kit (Qiagen) as per manufacturer's protocol. Reverse transcription (RT) was performed with qSCRIP Reverse Transcriptase kit (Primer Design, Southampton, England) as per manufacturer's instruction. qPCR experiments were performed on a StepOneTM Real-Time PCR system (Applied Biosystems) using Precision qPCR Mastermix (Applied Biosystems). Values were calculated using the standard delta-delta Ct method.

- Statistics

Statistical analyses of the data were performed using SPSS, program 21.0, using the non-parametric Kruskall-Wallis and Mann-Whitney U test for in vitro experiments and parametric one-way ANOVA followed by the Tukey HSD test or two-tailed t-Test for in vivo studies.

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Author contributions

J.M.C and L.A.E. designed the study. L.A.E. performed and analyzed cell and transgenic mouse experiments. M.I. and R.F. performed and analyzed alpha-synuclein PFFs mouse experiments. F.J.B. and A.R.S. performed stereological analysis. S.E. performed the intravenous injections of transgenic mouse. R.P. and V.B. synthetized and prepared alpha-synuclein PFF. R.A. and J.P.S. generated the S129D transgenic mouse model. Y.S. generated the parental alpha-synuclein

shRNA plasmid. M.S. and M.Sc. generated and produced shRNA MC. C.G. performed the NTA analysis. M.D.T. performed primary and differential analysis of the RNA-seq data. M.I., J.M.C. and L.A.E. wrote the manuscript. All authors contributed to the manuscript and its amendments.

Relevant conflicts of interest/financial disclosures: L.A.E. has filed a patent application related to the work in this paper.

References

1. Olanow CW, Kieburtz K, Katz R. Clinical approaches to the development of a neuroprotective therapy for PD. Exp Neurol. 2017; S0014-4886: 30156-5.

2. Simón-Sánchez J, Schulte C, Bras JM, Sharma M, Gibbs JR, Berg D, et al. Genome-wide association study reveals genetic risk underlying Parkinson's disease. Nat Genet 2009; 41: 1308-12.

3. Lee VM, Trojanowski JQ. Mechanisms of Parkinson's disease linked to pathological alpha-synucleinuclein: new targets for drug discovery. Neuron 2006; 52: 33-8.

4. Li JY, Englund E, Holton JL, Soulet D, Hagell P, Lees AJ, et al. Lewy bodies in grafted neurons in subjects with Parkinson's disease suggest host-to-graft disease propagation. Nat Med. 2008; 14: 501-3.

5. Kordower JH, Chu Y, Hauser RA, Freeman TB, Olanow CW. Lewy body-like pathology in long term embryonic nigral transplants in Parkinson's disease. Nat Med 2008; 14: 504-6.

6. Luk KC, Kehm V, Carroll J, Zhang B, O'Brien P, Trojanowski JQ, et al. Pathological α-alpha-synucleinuclein transmission initiates Parkinson-like neurodegeneration in nontransgenic mice. Science. 2012; 338: 949-53.

7. Lewis J, Melrose H, Bumcrot D, Hope A, Zehr C, Lincoln S, et al. In vivo silencing of alpha-alpha-synucleinuclein using naked siRNA. Mol Neurodegener 2008; 3: 19.

8. Zharikov AD, Cannon JR, Tapias V, Bai Q, Horowitz MP, Shah V, et al. shRNA targeting α-alpha-synucleinuclein prevents neurodegeneration in a Parkinson's disease model. J Clin Invest 2015; 125:2721-35.

9. Mingozzi F, High KA. Immune responses to AAV vectors: overcoming barriers to successful gene therapy. Blood. 2013; 122: 23-36.

10. Alvarez-Erviti L, Seow Y, Yin H, Betts C, Lakhal S, Wood MJ. Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. Nat Biotechnol 2011; 29: 341-5.

11. Cooper JM, Wiklander PB, Nordin JZ, Al-Shawi R, Wood MJ, Vithlani M, et al. Systemic exosomal siRNA delivery reduced alpha-alpha-synucleinuclein aggregates in brains of transgenic mice. Mov Disord 2014; 29: 1476-85.

12. Darquet AM, Cameron B, Wils P, Scherman D, Crouzet J. A new DNA vehicle for nonviral gene delivery: supercoiled minicircle. Gene Ther 1997; 4: 1341-9.

13. Yew NS, Zhao H, Przybylska M, Wu IH, Tousignant JD, Scheule RK, Cheng SH. CpG-depleted plasmid DNA vectors with enhanced safety and long-term gene expression in vivo. Mol Ther 2002; 5: 731-8.

14. Liu Y, Li D, Liu Z, Zhou Y, Chu D, Li X, et al. Targeted exosome-mediated delivery of opioid receptor Mu siRNA for the treatment of morphine relapse. Sci Rep. 2015; 5:17543.

15. Didiot MC, Hall LM, Coles AH, Haraszti RA, Godinho BM, Chase K, et al. Exosome-mediated delivery of hydrophobically modified siRNA for Huntingtin mRNA silencing. *Mol Ther*. 2016; 24:1836-1847.

16. Kamerkar S, LeBleu VS, Sugimoto H, Yang S, Ruivo CF, Melo SA, Lee JJ, Kalluri R. Exosomes facilitate therapeutic targeting of oncogenic KRAS in pancreatic cancer. Nature 2017; 546: 498-503.

17. Dehay B, Bourdenx M, Gorry P, Przedborski S, Vila M, Hunot S, Singleton A, Olanow CW, Merchant KM, Bezard E, Petsko GA, Meissner WG. Targeting α-synuclein for treatment of

Parkinson's disease: mechanistic and therapeutic considerations. Lancet Neurol. 2015 ; 14: 855-66.

18. Joshi SN, Butler DC, Messer A. Fusion to a highly charged proteasomal retargeting sequence increases soluble cytoplasmic expression and efficacy of diverse anti-alpha-synucleinuclein intrabodies. MAbs. 2012; 4:686-93.

19. Moloney TC, Hyland R, O'Toole D, Paucard A, Kirik D, O'Doherty A, et al. Heat Shock Protein 70 Reduces α-Alpha-synucleinuclein-Induced Predegenerative Neuronal Dystrophy in the α-Alpha-synucleinuclein Viral Gene Transfer Rat Model of Parkinson's Disease. CNS Neurosci Ther 2014; 20: 50-58.

20. Spencer B, Valera E, Rockenstein E, Overk C, Mante M, Adame A, et al. Anti-α-alpha-synucleinuclein immunotherapy reduces α-alpha-synucleinuclein propagation in the axon and degeneration in a combined viral vector and transgenic model of alpha-synucleinucleinopathy. Acta Neuropathol Commun 2017; 5:7.

21. Tran HT, Chung CH, Iba M, Zhang B, Trojanowski JQ, Luk KC, et al. α-Alpha-synucleinuclein immunotherapy blocks uptake and templated propagation of misfolded α-alpha-synucleinuclein and neurodegeneration. Cell Rep 2014; **7**: 2054-2065.

22. Zhao HT, John N, Delic V, Ikeda-Lee K, Kim A, Weihofen A, et al. LRRK2 Antisense Oligonucleotides Ameliorate α-Alpha-synucleinuclein Inclusion Formation in a Parkinson's Disease Mouse Model. Mol Ther Nucleic Acids 2017; 8: 508-519.

23. Kim YC, Miller A, Lins LC, Han SW, Keiser MS, Boudreau RL, et al. RNA Interference of Human α-Alpha-synucleinuclein in Mouse. Front Neurol 2017; 8:13.

24. Benskey MJ, Sellnow RC, Sandoval IM, Sortwell CE, Lipton JW, Manfredsson FP. Silencing Alpha Synuclein in Mature Nigral Neurons Results in Rapid Neuroinflammation and Subsequent Toxicity. Front Mol Neurosci. 2018; 11: 36.

25. Gorbatyuk OS, Li S, Nash K, Gorbatyuk M, Lewin AS, Sullivan LF, Mandel RJ, Chen W, Meyers C, Manfredsson FP, Muzyczka N. In vivo RNAi-mediated alpha-synuclein silencing induces nigrostriatal degeneration. Mol Ther. 2010; 18:1450-7.

26. Xhima K, Nabbouh F, Hynynen K, Aubert I, Tandon A. Noninvasive delivery of an α-synuclein gene silencing vector with magnetic resonance-guided focused ultrasound. Mov Disord. 2018; 33: 1567-79.

27. Abeliovich A, Schmitz Y, Fariñas I, Choi-Lundberg D, Ho WH, Castillo PE, Shinsky N, Verdugo JM, Armanini M, Ryan A, Hynes M, Phillips H, Sulzer D, Rosenthal A. Mice lacking alpha-synuclein display functional deficits in the nigrostriatal dopamine system. Neuron. 2000 ; 25: 239-52.

28. Klivenyi P, Siwek D, Gardian G, Yang L, Starkov A, Cleren C, Ferrante RJ, Kowall NW, Abeliovich A, Beal MF. Mice lacking alpha-synuclein are resistant to mitochondrial toxins. Neurobiol Dis. 2006; 21: 541-8.

29. Gelders G, Baekelandt V, Van der Perren A. Linking Neuroinflammation and Neurodegeneration in Parkinson's Disease. J Immunol Res. 2018; 2018: 4784268.

30. Duffy MF, Collier TJ, Patterson JR, Kemp CJ, Luk KC, Tansey MG, et al. Lewy body-like alpha-synuclein inclusions trigger reactive microgliosis prior to nigral degeneration. J Neuroinflammation. 2018; 15: 129.

31. Gonzalez-Reyes LE, Verbitsky M, Blesa J, Jackson-Lewis V, Paredes D, Tillack K, Phani S, Kramer ER, Przedborski S, Kottmann AH. Sonic hedgehog maintains cellular and neurochemical homeostasis in the adult nigrostriatal circuit. Neuron. 2012; 75: 306-19.

32. Kett LR, Stiller B, Bernath MM, Tasset I, Blesa J, Jackson-Lewis V, Chan RB, Zhou B, Di Paolo G, Przedborski S, Cuervo AM, Dauer WT. α-Synuclein-independent histopathological and motor deficits in mice lacking the endolysosomal Parkinsonism protein Atp13a2. J Neurosci. 2015; 35: 5724-42.

33. West MJ. New stereological methods for counting neurons. Neurobiol Aging. 1993; 14: 275-85.

34. Colburn RW, DeLeo JA, Rickman AJ, Yeager MP, Kwon P, Hickey WF. Dissociation of microglial activation and neuropathic pain behaviors following peripheral nerve injury in the rat. Journal of Neuroimmunology 1997; 79: 163-75.

Figure legends

Figure 1. Optimization of exosomal loading and influence on alpha-synuclein levels in SH-SY5Y cells and in the brain of S129D alpha-synuclein transgenic mice a) Agarose gel electrophoresis of anti GFP shRNA-MC DNA (GFP MC), or DNA isolated from GFP MC incubated with RVG-exosomes with (Elect) or without (No elect) electroporation followed by DNAse treatment. b) GFP actin levels in SH-SY5Y cells over-expressing GFP after delivery of anti GFP shRNA-MC by; 3 μg RVG-exosomes after centrifugation (Exo); after centrifugation and treatment with DNase (+ DNAse) or transfection of 1 μg using XtremeGENE transfection reagent (TR) (n=4). GFP and actin protein levels were quantified by Western blot. Values mean ± SEM (n=4) c) SH-SY5Y cells over-expressing S129D alpha-synuclein were transfected (TR) with 100 nM anti alpha-synuclein siRNA, 1 μg anti alpha-synuclein shRNA MC (MC) or anti-synuclein shRNA plasmid (Plas) or treated with 3 μg RVG-exosomes (Exo) electroporated with 100 nM anti alpha-synuclein siRNAs (siRNA), or 1 μg anti alpha-synuclein shRNA-MC (MC). Quantitation of alpha-synuclein protein levels normalised to control cells, a typical Western blot is shown. Data expressed as mean ± SEM (n=3). Non-parametric Kruskal-Wallis test, statistical analyses compared with untreated control group, *$p<0.05$, ** $p<0.01$. d) S129D alpha-synuclein transgenic mice treated with 150 μg RVG-exosomes containing anti GFP (GFP-Exo) or anti alpha-synuclein shRNA-MC (Syn-Exo). Olfactory bulb (OL), mid brain (MB), striatum (St), cortex (Ctx) and brain stem (BS) samples analysed for (b) alpha-synuclein mRNA qPCR and (e) protein levels by Western blot. Typical Western blot of the midbrain is shown. Data expressed as mean ± SEM (n=10). One-way Anova test, statistical analyses compared with untreated control group, *$p < 0.05$, **$p<0.01$.

Figure 2. Effect of anti alpha-synuclein shRNA-MC RVG-exosome treatment 30 days after administration in PFF treated mice. Normal C57BL6/C3H F1 mice intra-striatally injected with alpha-synuclein PFFs (PFF) were treated with injections (iv) of 150 μg RVG-exosomes electroporated with 150 μg anti alpha-synuclein shRNA-MC (PFF + Syn MC) or 150 μg anti GFP shRNA-MC (PFF + GFP MC) for 30 days. Analyses of alpha-synuclein (a) mRNA expression and (b) protein levels normalized to actin in ipsilateral mid brain (MB), striatum (St), and cortex (Ctx). Values expressed as mean ± SEM (n=5). Typical Western blot of the midbrain is shown. c) Analyses of alpha-synuclein mRNA levels normalized to actin in the contralateral midbrain (MB), striatum (St) and cortex (Ctx). Data expressed as mean ± SEM (n=4). Non-parametric Kruskal-Wallis test, $*p < 0.05$, $**p < 0.01$. Scale bars: 200 μm, 100 μm.

Figure 3. Influence of 90 day anti alpha-synuclein shRNA-MC RVG-exosome treatment on alpha-synuclein aggregation. a) Brain sections were stained for S129 phospho-synucelin and the number of positive aggregates per section quantified in the: frontal cortex (F Ctx), somatosensory cortex (SS Ctx), somatomotor cortex (SM Ctx), striatum (St) and amygdala (Amyg) and substatia nigra (SNpc) sections. Typical immunohistochemical image of frontal cortex, striatum and amygdala stained S129 phospho-alpha-synuclein are shown. Data expressed as mean ± SEM (n=8). Non-parametric Kruskal-Wallis test, $*p < 0.05$. b) Immunofluorescent staining of midbrain sections with antibodies to S129 phospho-alpha-synuclein (green) and tyrosine hydroxylase (red). Magnified regions of the SNpc shown on the right, arrows indicate alpha-synuclein aggregates. Scale bars: 100 μm (left), 10 μm (right).

Figure 4. Effect of 90 day anti alpha-synuclein shRNA-MC RVG-exosome treatment on alpha-synuclein mRNA and protein. Quantitation of alpha-synuclein (a, b) mRNA levels and (c, d) protein levels in the (a, c) ipsilateral and (b, d) contralateral midbrain (MB), striatum (St),

and cortex (Ctx) from alpha-synuclein PFFs treated mice (PFF) after 90 days following anti alpha-synuclein shRNA-MC RVG-exosome treatment (PFF + Syn MC). mRNA and protein levels were normalized to actin, typical Western blots of the midbrain are shown. Data expressed as mean ± SEM (n = 10). One-way Anova test, statistical analyses compared with control group, *p < 0.05, **p < 0.01, ***p<0.001.

Figure 5. Anti alpha-synuclein shRNA-MC delivered by RVG-exosomes reduces the dopaminergic denervation, dopaminergic neuronal loss and motor abnormalities caused by intra-striatal injection of alpha-synuclein PFFs. a) TH immunoreactivity was analysed in coronal sections of the forebrain from: control mice, mice 90 days after alpha-synuclein PFF treatment alone (PFFs, ipsilateral striatum black arrow) and following anti alpha-synuclein shRNA-MC RVG-exosome (PFF + Syn MC) treatment. Striatal TH was quantified by optical density in the ipsilateral sections normalized to the contralateral striatum. b) Quantitation of nigral dopaminergic neurons on each side of the brain by unbiased stereology. Data expressed as mean ± SEM (n=8). Non-parametric Kruskal-Wallis test, statistical analyses compared with untreated control group, *p < 0.05, ***p<0.001. Clinical evaluations of the influence of alpha-synuclein PFFs intra-striatal injection (PFFs mice) and treatment with alpha-synuclein shRNA-MC RVG-exosomes (PFF + Syn MC). c) Negative geotaxis and d) wire hanging tests compared to control mice. Data expressed as mean ± SEM (n=18). One-way Anova test, statistical analyses compared with untreated control group, *p < 0.05. Scale bars: 500 μm