Loss of MiR-7 regulation leads to α-synuclein accumulation and dopaminergic neuronal loss in vivo.

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Abstract

Abnormal α-synuclein expression and aggregation is a key characteristic of Parkinson's disease (PD). However, the exact mechanism(s) linking α-synuclein to the other central feature of PD, dopaminergic neuron loss, remains unclear. Therefore, improved cell and *in vivo* models are needed to investigate the role of α-synuclein in dopaminergic neuron loss. MiR-7 regulates α-synuclein expression by binding to the 3'UTR of the Synuclein Alpha Non A4 Component of Amyloid Precursor (*SNCA*) gene and inhibiting its translation. Here, we show that miR-7 is decreased in the substantia nigra of patients with PD and therefore may play an essential role in the regulation of α-synuclein expression. Using a lentiviral vector that contains miR-7 complementary binding sites to stably induce a loss of function phenotype, we have shown that a loss of miR-7 leads to an increase in α-synuclein expression *in vitro* and *in vivo.* We have also shown that depletion of miR-7 using our miR-decoy in mice produces a significant loss of nigral dopaminergic neurons accompanied by a reduction of striatal dopamine content. These data therefore, suggest that miR-7 has an important role in the regulation of α-synuclein and dopamine physiology and may provide a new paradigm to study the pathology of PD.

Introduction

Abnormal expression and aggregation of the α-synuclein protein is known to play an important role in the pathogenesis of PD. Missense mutations and locus multiplications in the *SNCA* gene, which encodes the protein α-synuclein, have been found to cause familial forms of PD[1-4]. Genome wide association studies (GWAS) have shown that polymorphisms in the *SNCA* gene are risk factors for developing sporadic PD [5-8] and aggregated α-synuclein is the main component of Lewy bodies, the protein inclusions found in the brains of patients with PD[9]. Furthermore, overexpression of α-synuclein has been found to be toxic to dopaminergic neurons, implicating α-synuclein as a key player in the molecular mechanisms of the disease[10, 11].

The mechanisms underlying α-synuclein toxicity are unclear but one hypothesis is that α-synuclein may be acting in the manner of a prion: transferring from cell-to-cell, thus spreading pathology. Braak and colleagues were the first to suggest that α-synuclein might be spreading throughout the brain with Lewy pathology first appearing in the dorsal motor nucleus of the vagal nerve and the olfactory bulb[12]. This idea gained further support from the discovery of Lewy pathology in the grafts of foetal mesencephalic brain tissue injected into patients with PD between the 1980s and 1990s[13, 14]. Neuronal grafts transplanted into transgenic mice overexpressing human α-synuclein have also shown α-synuclein inclusions within the graft[15, 16]. Moreover, Luk and colleagues demonstrated that injection of synthetic α-synuclein fibrils into the dorsal striatum of wild-type non-transgenic mice, led to cell-to-cell transmission of pathological α-synuclein and PD-like Lewy pathology in anatomically interconnected regions further supporting the hypothesis

that PD acts as a prion-like disorder[17]. Whilst these propagation models have highlighted that α-synuclein is important in neuron degeneration, the mechanisms controlling the expression of α-synuclein are still not fully understood.

MicroRNAs (miRs) are a class of endogenous non-coding RNAs that regulate gene expression in a sequence-specific manner[18-20]. They are single stranded and approximately 17-24 bases long. Aberrant miR activity has been associated with a number of neurodegenerative diseases including PD[21-24]. In particular, miR-7 has been shown to bind to the 3' untranslated region (UTR) of the *SNCA* gene and inhibit its translation[25, 26]. However, despite the adequate verification that miR-7 targets SNCA the physiological significance and its relevance to the pathological mechanisms in PD remain unclear[27]. In 2007 a new method to study miRs was developed by Ebert and colleagues. This involved the generation of a target sequence complementary to the miR of interest that contained multiple miR binding sites and therefore, acted as a decoy/sponge by sequestering the endogenous miR[28]. This resulted in a decrease in the amount of available miR and prevented endogenous target gene regulation. Here we use this miR sponge model in *in vitro* and *in vivo* systems and show that downregulation of miR-7 results in an upregulation of α-synuclein, leading to a downregulation of TH activity, dopaminergic neuron loss and a loss of striatal dopamine.

Results

miR-7 levels are significantly reduced in the SNpc of patients with PD

To investigate whether a decrease in miR-7 could contribute to the increase in α-synuclein in PD patients, nigral sections taken from human post mortem brain samples were collected from six patients with PD and five aged-matched healthy controls (Figure S1). The PD subjects studied were 71-87 years of age and were all taking a combination of L-DOPA medications. Neuropathological examination performed by the UK Parkinson's Disease Society Tissue Bank revealed α-synucleinopathy, which was brainstem predominant and consistent with a clinical diagnosis of PD. Immunofluorescence staining also revealed accumulation and aggregation of α-synuclein in the brain sections taken from PD patients compared to healthy aged-matched controls (Figure 1A). Furthermore, quantitative real time PCR analysis showed a significant reduction in the levels of mature miR-7 in PD patients compared to the healthy aged-matched controls ($P<0.05$) (Figure 1B,C), suggesting that miR-7 may play an important role in the increase of α-synuclein in PD patients.

miR-7 regulates α-synuclein expression *in vitro*

To explore the role of miR-7 in the neurodegenerative process of PD, we generated two lentiviral vectors, miR-7-GFP and miR-7T-AsRed (Figures S2-S4). In order to confirm effective miR-7 knockdown by the miR-7T-AsRed lentiviral vector, HEK293T cells were co-transduced with miR-7T-AsRed (MOI 2) and with varying MOIs of the miR-7-GFP lentiviral vector (0, 0.1, 1 and 5). Results show that an increase in miR-7 levels (mirrored by GFP expression) causes a decrease in the expression of the target sequence (indicated by AsRed expression) (Figure S5A). At protein level, AsRed expression was completely abolished at MOIs of 1, 5, and 10 of

the miR-7-GFP lentiviral vector (P<0.001) (Figure 5B,C), confirming that miR-7 can bind to the target sequence resulting in protein knockdown *in vitro*. MiRanda software was used to examine the potential interaction between miR-7 and the mouse *snca* gene. Three strong binding sites for miR-7 were found in the *snca* 3'UTR (Figure S6). The top predictive binding site was related to the seed sequence (119-127nt), which is highly conserved, highlighting the evolutionary importance of miR-7 in *snca* regulation (Figure S7A).

To confirm this association, the full length 3'UTR of the mouse *snca* gene was cloned into a luciferase vector downstream of a firefly luciferase reporter (Figure S7B). HEK293T cells were co-transfected with the 3'UTR *snca* plasmid with either the miR-7-GFP plasmid, a control CMV-GFP plasmid or a control miR-449-GFP plasmid. An internal control plasmid (*Renilla* luciferase) was also co-transfected to correct for differences in transfection efficiency. miR-7-GFP decreased luciferase activity by approximately 44% (P<0.05), confirming that miR-7 can bind to the 3'UTR of the *snca* gene and inhibit its translation. This effect was specific to miR-7, as miR-449-GFP had no effect on luciferase activity (Figure S7C). To confirm that miR-7 regulates α-synuclein expression, HEK293T cells were transduced with the miR-7-GFP or miR-7T-AsRed vectors (MOI 0, 0.1, 1 and 5). miR-7-GFP significantly decreased α-synuclein expression in a dose-dependent fashion (61% at an MOI of 5, P<0.05) (Figures 2A and B) whilst miR-7T-AsRed significantly increased α-synuclein expression by 513% at an MOI 5 (**, P<0.01) (Figures 2C,D).

miR-7 knock down induces α-synuclein overexpression in the SNpc

We next tested our miR-7T-AsRed lentiviral vector *in vivo.* In order to titrate the amount of tissue transduced we compared a single injection site with a double injection site in the SNpc (Figure S8). We found that a single injection site of the miR-7T-AsRed into the SNpc was not sufficient to induce a significant upregulation of α-synuclein expression by 16 or 24 weeks (Figure S9A). However, the number of TH positive neurons in the injected SNpc was decreased by 27% 24 weeks post-injection, when compared to the control hemisphere (Figure S9B,C). Behavioural analysis showed no signs of motor impairments (Figure S9D,E) and HPLC analysis revealed no significant difference in the striatal levels of DA or its metabolites DOPAC or HVA (Figure S10A-C). In contrast animals that had a unilateral double injection showed a moderate increase in α-synuclein expression at 16 weeks with further accumulation at 24 weeks ($P<0.05$) (Figure 3A,B). This effect was specific to miR-7T overexpression as animals injected with a control CMV-AsRed lentiviral vector showed no change in α-synuclein levels (Figure 3B). Given that a double site miR-7T injection was able to target a larger SNpc area leading to miR-7 knockdown and α-synuclein overexpression, this approach was used in subsequent studies.

Oligomeric forms of α-synuclein are known to play an important role in the pathology of PD [29]. Therefore, to establish whether the unilateral double injection of the miR-7T vector also increased the oligomerisation of α-synuclein, a proximity ligation assay (PLA) was used to detect α-synuclein oligomers. α-synuclein-PLA has been shown to preferentially label oligomers compared to monomers and fibrils (Supplementary Figure 11A,B) [30]. The number of α-synuclein-PLA puncta was quantified in the injected SNpc and contralateral non-injected SNpc in three miR-7T injected animals from the 24-week post-surgery cohort plus two control animals.

MiR-7T increased α-synuclein-PLA signal by 50% compared to the control non-injected SNpc (Figure 3C, D). Furthermore, control animals injected with the CMV-AsRed vector showed no difference in PLA signal (Figure 3E) illustrating that the miR-7T is causing an accumulation and aggregation of α-synuclein *in vivo.*

miR-7 knockdown causes TH cell loss in the SNpc and reduction in striatal DA

The number of TH-positive neurons was quantified in both the injected SNpc and contralateral non-injected SNpc to determine whether overexpression of miR-7T-AsRed could induce dopaminergic neuron death in the SNpc. The number of TH neurons in the miR-7T-AsRed injected SNpc was significantly decreased by 39% at 16 weeks and by 73% at 24 weeks post-injection ($P<0.05$) (Figure 4A, B). No significant difference in TH expression was found in control CMV-AsRed injected animals (Figure 4C) indicating that the reduction in TH expression is due to miR-7T expression. However, HPLC analyses only detected a small difference in striatal DA and HVA levels at 24-weeks post-surgery ($P<0.05$) (Figure 5A-C), which was not consistent with the profound loss of dopaminergic neurons. Therefore, to detect whether this decrease in TH expression was due to cell death or a downregulation of activity, nigral sections from the 24-week cohort were also stained for the vesicular monoamine transporter (VMAT2), which is needed to transport monoamines (including dopamine) into synaptic vesicles[31]. The number of VMAT2 positive neurons was decreased by approximately 30% in the MiR-7T injected SNpc compared to the contralateral non-injected SNpc (Figure 5D) suggesting that whilst there was some cell death many of the dopaminergic neurons had downregulated their activity accounting for the profound loss of TH expression.

Overexpression of miR-7T-AsRed is not sufficient to induce locomotor deficits

To establish whether overexpression of the miR-7T-AsRed lentiviral vector could induce PD-like motor impairment, behavioural analyses were carried out. Locomotor activity and amphetamine-induced rotations of the 24-week miR-7T-AsRed and control-injected animals were measured at 2, 10 and 20 weeks post-surgery. There was no significant difference in locomotor activity (Figure 6A,B) or in the number of amphetamine-induced rotations (Figure 6C-E) after injection of the miR-7T lentiviral vector at any of the three time points compared to the control animals, indicating that the loss of dopaminergic neurons and striatal DA was not sufficient to induce a behavioural phenotype. Interestingly, there was trend towards a decrease in distance moved and velocity as well as an increase in the number of amphetamine-induced rotations in the miR-7T-AsRed injected animals compared to the control animals (Figure 6A-E), suggesting that the development of motor deficits in this model requires more than the 20 weeks analysed in this study.

Discussion

The basic pathophysiology of PD is the progressive loss of nigral DA neurons leading to a loss of striatal DA and the appearance of aggregated α-synuclein. Many models of PD have been generated to date, including neurotoxin models, transgenic mice, viral vector models and more recently induced pluripotent stem cell (iPSCs) models. Whilst available models display some cardinal PD features and have helped to

elucidate many of the molecular pathways involved, none of these have been able to fully recapitulate PD pathology. This has hindered our understanding of how α-synuclein accumulation and aggregation drive neurodegenerative processes leading to DA neuron loss in patients with PD. In this study, we have generated a mouse model that develops α-synuclein accumulation, due to the depletion of miR-7 in dopaminergic neurons. This results in a loss of nigral dopaminergic neurons leading to a loss of striatal DA, thereby providing a novel model to study the pathophysiology of PD. Furthermore, we have shown that miR-7 levels are decreased in the nigra of patients with PD compared to aged matched healthy controls suggesting that miR-7 plays a critical role in the regulation of α-synuclein expression.

To deplete miR-7 we exploited the technology developed by Ebert and colleagues to design a miR-7 decoy. The miR target sequence contains multiple binding sites and therefore, acts as a decoy/sponge by sequestering away the endogenous miR[28]. This results in a decrease in the amount of available miR, which prevents the appropriate regulation of downstream targets, thereby providing a promising new approach for investigating the role of miRs in the pathology of PD.

Junn and colleagues showed that miR-7 could bind to the 3'UTR of the *SNCA* gene between bases 119 and 127 and inhibit its translation in a dose dependent manner. This sequence is conserved across several species suggesting that miR-7 may play a critical evolutionary role in the regulation of α-synuclein[25, 26]. Using a miR-7-GFP-expressing lentiviral vector we confirmed the binding between miR-7 and the 3'UTR of the mouse *snca* gene and shown that miR-7 inhibits its expression resulting in a functional knockdown of α-synuclein protein. Furthermore, we have shown that

the miR-7 target sequence competes for the miR-7 sequence *in vitro* and *in vivo* resulting in a loss of function and an increase in α-synuclein protein expression.

There has been much controversy about the morphological differences between disease associated α-synuclein and which forms are toxic to the cell. Furthermore, oligomerisations of α-synuclein as well as truncations and post-translational modifications have been shown to play an important role in neuronal toxicity[32]. Using the new method PLA to detect α-synuclein oligomers [30], we have shown that the miR-7T-AsRed target vector not only affects the monomeric form of α-synuclein but also increases its oligomerisation thereby mirroring the pathology seen in patients with PD.

Lentiviral vectors expressing human wild type or mutant forms of α-synuclein have been shown to cause a progressive loss of nigral dopaminergic neurons by 24-35% in rats over 5 months[33] whereas adenoviruses expressing human α-synuclein have been shown to decrease nigral dopaminergic neurons by up to 80% in as short as 6 weeks, however these results are less consistent[10, 11, 34-37]. In our model, miR-7T-AsRed significantly reduced TH expression by one-third at week 16 and two thirds at week 24. However, staining with VMAT2, another neuronal marker showed that there was only a 30% loss of neurons at 24 weeks suggesting that the nigral neurons had downregulated their activity. This was consistent with the mir-7T injected animals only showing a small deficit in striatal DA production as well as being comparable to other animal models using lentiviral vectors expressing α-synuclein. Downregulation of TH activity has previously been reported after overexpression of human α-synuclein as a protective mechanism of the cell[34].

296 Interestingly aggregated human α-synuclein has also been shown to reduce TH
297 activity in dopaminergic neurons [38].

298 As miRs have many targets it is impossible to rule out that miR-7 may be affecting
299 TH expression via another method other than α-synuclein. The absence of strong
300 binding sites for miR-7 in the TH 3'-UTR (data not shown) suggests that miR-7 does
301 not affect TH expression directly but an interesting future experiment would be to
302 look at miR-7T in an α-synuclein knock out animal. This would conclude whether
303 the downregulation of TH activity and loss of nigral neurons in our mouse model is
304 a consequence of the increase in accumulation and oligomerisation of α-synuclein.

305 MiR-7T-AsRed injected animals displayed a trend towards decreased LMA and
306 increased amphetamine-induced rotations compared to control animals but these
307 effects were not found to be significant suggesting that the dopaminergic neuron
308 loss was not sufficient to cause a major loss of striatal DA and thereby motor deficit
309 at the time points studied. It is however, known that the nigro-striatal system has
310 remarkable plasticity as patients only present clinically with motor symptoms once
311 their striatal DA levels have decreased by approximately 70%[39]. In addition,
312 previous studies have shown that motor impairments only appear in animals with
313 over 50-60% loss of nigral neurons thus, explaining the lack of motor impairment in
314 our model [34, 40, 41].

315 Non-coding regions of the genome such as miRs have been suggested to play a vital
316 role in the pathological mechanisms underlying PD. Kim and colleagues found that
317 deleting Dicer in murine ES cell lines led to a near complete loss of production of
318 dopaminergic neurons. Concurrently, mice lacking Dicer in postmitotic
319 dopaminergic neurons developed a 90% loss of midbrain dopaminergic neurons

after 8 weeks suggesting that miRs play an important role in neurodegeneration[42]. This loss of dopaminergic neurons is significantly higher than in our study, which can be explained by the lack of Dicer causing a complete loss of miR-7 in these neurons as well as the loss of other miRs that may be playing a role in dopaminergic survival. It has been suggested that increasing the number of miR complementary binding sites or using imperfect complementary binding sites within the sponge target vector can be more effective at supressing the miR target[28, 43, 44]. Whilst the miR-7T-AsRed target vector used in our model was sufficient to induce α –synuclein accumulation and aggregation it might be that the miR-7 levels are not 100% suppressed. Therefore, it would be interesting to see whether a complete loss of miR-7 would result in a higher level of nigral degeneration.

Kim and colleagues revealed that miR-133b regulates the maturation and function of midbrain dopaminergic neurons and is deficient in the midbrain tissue of patients with PD. Furthermore, miR-34b/c downregulation has been coupled to a decrease in the expression of DJ1 and Parkin, two proteins found to be important in familial forms of PD [24]. In addition, miR-205 has been shown to regulate Leucine-rich repeat kinase (LRRK2) another protein linked to sporadic and familial forms of PD[45]. miR-34b/c and miR-205 also have decreased expression in patients with PD further suggesting an important role for miRs in the pathology of PD[45]. This is the first study, however to reveal that miR-7 is also deficient in the dopaminergic neurons of patients with PD. GWAS data has shown that several polymorphisms of the *SNCA* gene associated with an increased risk of developing PD are found in the 3'UTR. These polymorphisms could affect miR binding sites and cause dysregulation

of *SNCA* expression [5]. Furthermore, miR-7 has been shown to be protective against impaired proteasome function and cytotoxicity caused by overexpression of α-synuclein *in vitro* and downregulated by 50% in an MPTP mouse model of PD [25, 41]. In addition, toxicity studies using MPTP in dopaminergic neuronal SH-SY5Y cells resulted in a 40% increase in α-synuclein mRNA levels and an equivalent decrease in miR-7 levels [25]. α-synuclein expression also increases following inhibition of miR-7 *in vitro,* [26] further suggesting that miR-7 may provide a candidate therapeutic target for modulating α-synuclein levels in PD.

In conclusion, this is the first study to use a lentiviral-mediated target sponge approach to knockdown miR-7 *in vitro* and *in vivo.* We have recapitulated key pathological hallmarks of PD by injecting the miR-7 decoy lentiviral vector into the SNpc of mice, namely increased α-synuclein expression and oligomerisation, dopaminergic neuron death and a reduction in striatal DA. Furthermore, in human nigral samples from patients with PD, we report decreased miR-7 levels compared to healthy aged matched controls. Our data highlights the significance of the evolutionary conserved regulation of α-synuclein by miR-7 and the importance of this miR for dopaminergic cell biology. Moreover it provides a new model to study the pathology of PD and to develop novel therapeutic interventions.

Methods

Real time quantitative PCR analysis of miR-7 levels in the nigra of patients with PD

Nigral sections were obtained from six patients with clinical diagnosis of PD patients

and five aged matched healthy controls from the UK Parkinson's Disease Society Tissue Bank at Imperial College London, UK (Figure S1). To quantify the expression of miR-7 in these sections, RNA was extracted using the mirVana miR isolation kit (Thermo Fisher) according to manufacturer's instructions. A Taqman microRNA assay (Applied Biosystems) was used to detect and quantify miR-7 in these RNA.

Generation of miR-7 and miR-7T-expressing lentiviral vectors

A miR-7 lentiviral vector was generated using high fidelity PCR to amplify the mouse microRNA-7-a1 sequence using a forward (5'ACG TTC TAG ACC TTA ACC AAG CAA ACT TC 3') and reverse primer (5' CGA TGG ATC CAA TGA AAC TGG AAG CTG 3'). The sequence was then cloned into the lentiviral vector backbone pRRL-sincppt.CMV-GFP.wpre upstream of the GFP reporter tag (Figure S2 and S3). To produce the miR-7 target sequence (miR-7T) a four-times repeated sequence complementary to the miR-7 was designed to compete for the miR-7 binding site. Four oligonucleotides (sense 1 strand: 5' AAT TAT AAA CAA CAA AAT CAC TAG TCT TCC ACG ATA CAA CAA AAT CAC TAG TCT TCC AAC GCG 3'; sense 2 strand: 5' TAC AAC AAA ATC ACT AGT CTT CCA TCA CAC AAC AAA ATC ACT AGT CTT CCA ACG TAC 3'; antisense 1 strand: 5' TTG GAA GAC TAG TGA TTT TGT TGT ATC GTG GAA GAC TAG TGA TTT TGT TGT TTA T 3'; and antisense 2 strand: 5' TGG AAG ACT AGT GAT TTT GTT GTG TGA TGG AAG ACT AGT GAT TTT GTT GTA CGC G 3') were annealed together by heating to 95˚C for 5 minutes and then cooling for 1 hour. The target sequence (Figure S2) was then cloned into the lentiviral vector backbone pRRL-sincppt.CMV-AsRed.wpre downstream of the AsRed reporter tag (Figure S4). Viral titres were determined using fluorescence activated cells sorting (FACS Calibur, BD).

HEK293T cell culture and transductions

Lentiviral vector production and validation were performed in the human embryonic kidney cells, baring the large T antigen of SV40 virus immortalised cell line (HEK293T). The cells were maintained in Dulbecco's Modified Eagle medium (DMEM, Sigma) supplemented with 10% foetal bovine serum (FBS, Sigma), 2mM L-glutamine, 100-units/ml penicillin, 100 µg/ml streptomycin and 1X non-essential amino acids. HEK293T cells were seeded at 7.5 x 10^4 cells/well in a 12 well plate 24 hours prior to transduction with lentiviral vectors. The lentiviral vectors at different multiplicity of infections (MOI) were added to the wells in a reduced culture volume (500 µl). After 3-4 hours the volume was increased to 1 ml and the cells incubated at 37˚C for 48 hours. Transduced cells were visualised using a fluorescence and bright field microscope (Leica DMRB). If cell nuclear staining was needed, 1mg/ml of Hoechst was added to the cells before imaging.

Generation of the 3'UTR of the *snca* gene and Luciferase assay

A luciferase assay was performed to investigate whether miR-7 was binding to the 3'UTR of the *snca* gene. The mRNA sequence of the 3'UTR of the mouse *snca* gene (NCBI: NM_001042451.2) and the binding sites of miR-7 were confirmed using Miranda software (microRNA Target Scanning Algorithm). A forward (5' CCG CTC GAG AAT GTC ATT GCA CCC AAT CT 3') and reverse primer (5' CCC AAG CTT GGT GCA TAG TCT CAT GCT CACA 3') was then used to clone the 3'UTR sequence into the luciferase backbone vector pMIR-Tac-1Reprot-Luc (Figure S7C). HEK293T cells were plated in a 24 well plate (4.0 x 10^4 cells/well) in 500 µl media 24 hours prior to transfection. HEK293T cells were co-transfected using Fugene transfection 6 reagent (Promega) with 100 ng of the *snca* gene 3'UTR plasmid and an internal control *Renilla* Luciferase plasmid (pRL-SV40, Promega) in the presence of either 300 ng of the miR-7-GFP-expressing plasmid or a control plasmid (miR-449-GFP or

GFP). The cells were then lysed and luciferase activity measured using a dual luciferase assay kit (Promega) and a luminometer (Glomax multi+, Promega). Renilla activity was used to normalise luciferase activity to account for any difference in transfection efficiency between the conditions. Three independent experiments were performed.

Animals

The present study was conducted in adult male C57BL/6J mice (Harlan, UK) weighing between 20-30 g. All animals were housed in individually ventilated cages (IVCs) with free access to food and water, under standard conditions (6 animals per IVC cage, 07:00 to 19:00 light phase, constant temperature and humidity). All procedures were carried out with the approval of Lilly Ethical Review Board and in accordance to UK Home Office regulations.

Stereotactic Surgery

The determine whether the miR-7T-AsRed lentiviral vector could cause overexpression of α-synuclein protein and produce PD pathology *in vivo,* two experiments were completed. The first experiment involved stereotactic injection of 2 µl of the miR-7T-AsRed lentiviral vector ($2x10^9$ TU/ml) into a single site in the right SNpc (AP: -2.7, ML: +1.0, DV: -4.5) and 2 µl of a control vector (CMV-AsRed, $2x10^9$ TU/ml) into the same site in the left SNpc (AP: -2.7, ML: -1.0, DV: -4.5) of C57Bl/6J mice (Figure S8A). Animals were then left for 8, 16 and 24 weeks with some used for immunohistochemistry and others used for biochemical analyses. Behavioural analysis at 2, 10 and 20 week's post-surgery was carried out in the 24-week animal cohort. The second experiment involved stereotactic injection of 2 µl of the miR-7T-AsRed lentiviral vector ($2x10^9$ TU/ml) into two separate sites of the right SNpc of C57Bl/6J mice (1^{st} injection site = AP: -2.7, ML: +1.0, DV: -4.5; 2^{nd}

injection site = AP: -2.7, ML: +1.5, DV -4.3) (Figure S8B). The miR-7T-AsRed injected animals were again left for 8, 16 and 24 weeks with some used for immunohistochemistry and others used for biochemical analyses. The 24-week cohort was also subject to behavioural analyses at 2, 10 and 20 weeks post-surgery. A control group of mice received two injections of the control CMV-AsRed lentiviral vector and were left for the full 24 weeks for biochemical analysis with behavioural analyses carried out at 2, 10 and 20 weeks post-surgery.

Locomotor activity (LMA)

LMA was measured in complete darkness in clear Perspex boxes (40x40x30 cm^3, Eli Lilly) based on infrared fields. Four boxes were placed on each field, which were monitored using overhead infrared cameras. The cameras input data into a computer via a Quad compressor unit. The image analysis application Ethovision (Noldus Information technology, Netherlands) digitised the path made by animals and used this to calculate various parameters such as distance moved, velocity and number of rotations. Mice were weighed and placed in the LMA boxes. Exploratory behaviour was assessed for 30 minutes. Following this, they were dosed with 2.5 mg/kg d-amphetamine sulphate (Sigma, A-5880 LY2800792) by intraperitoneal (i.p.) injection. LMA was assessed for a further 90 minutes. When the experiment was complete the data were analysed using Microsoft Excel and the total distance moved (cm), velocity (cm/s) and ipsilateral rotations calculated for each animal.

Dissection of striatal and nigral tissue

For biochemical analysis animals were culled by schedule 1 and their brains removed and placed on ice. Using a mouse matrix (Zivic Instruments, USA) the ST and SN from the right and left hemisphere were removed. The ST sections were weighed and transferred onto dry ice before being stored at -80˚C for high

performance liquid chromatography (HPLC) analysis. The SN sections were processed for Western blotting analysis.

Transcardial perfusions

For immunofluorescence analysis animals were terminally anaesthetised with 200 mg/kg i.p pentobarbital sodium (Ayrton Suader Ltd, UK) before transcardial perfusions were performed through the ascending aorta using PBS followed by 4% paraformaldehyde (PFA) (Sigma). The animals were then decapitated, the brain removed and post-fixed in 4% PFA at 4˚C for 24 hours. The following day the brain was immersed in 30% sucrose (Sigma) solution for at least 24 hours.

Sectioning tissue using a cryostat

Before brains were sectioned, they were snap frozen using isopentane (Fisher Scientific) and dry ice. Brains were stored at -80˚C until ready to use. Brains were mounted into OCT mounting matrix (Fisher Scientific) and placed in a -20˚C Leica CM190 Cryostat and 20 μm coronal sections prepared. These were transferred onto glass slides (Thermoscientific), and air-dried before storing at 4˚C.

Immunofluorescent staining of mouse brain sections

Non-specific binding was blocked using 10% normal goat serum (Vector Labs) diluted in 0.1% TritonX-100/PBS (blocking solution) and left to incubate overnight at 4˚C. Sections were transferred to primary antibody solution (either Tyrosine hydroxylase (TH) (ab152, Millipore), or vesicular monoamine transporter (VMAT2) (ref, abcam) in blocking solution) and incubated overnight at 4˚C. The secondary antibody (Alexafluor 488; A11008, Invitrogen) diluted in blocking solution was then added and incubated for 1 hour at room temperature. If required, cell nuclei were counterstained with 1 mg/ml Hoechst. The slides were mounted in PBS and glycerol

(1:1, Sigma) and imaged using a Fluorescence microscope (Leica DMRB). The number of TH positive cells was counted in the injected and control/non-injected SN in three sections per mouse at the site of injection using Image J and the 'cell counter' plug-in.

Proximity ligation assay

α-synuclein proximity ligation assay experiments were carried out using Duolink kits supplied by Sigma according to the manufacturer's instructions. Briefly, the conjugates were prepared with the anti-*α-synuclein* 4D6 antibody (Abcam ab1903) as previously described [30]. 20 μm thick coronal sections were dried at 37C, immersed in PBS and then antigen retrieved in citrate buffer pH 6 (Abcam 93678) by microwave heating for a total of 10 min. All samples were incubated in Duolink block solution at 37C for 1 h, followed by the conjugates diluted in Duolink PLA diluent (1:2000) overnight at 4C. After washing in TBS + 0.05% Tween 20, samples were incubated with Duolink ligation solutions and ligase for 1 h at 37C, before washing and incubation with Duolink amplification reagents and polymerase for 2.5 h at 37C. Samples were then washed in TBS in the dark and counterstained and mounted with FluorSave (Merck). Preparation and analysis of *α-synuclein* fibrils was carried out as previously described [30]. After preparation samples were centrifuged at 13000 g for 10 min; the supernatant was taken as oligomeric fraction and the precipitate was taken as fibrillar fraction. PLA and electron microscopy were performed as previously described [30]. Quantitation of the PLA signal was performed averaging 4 blinded random fields per sample at 20x magnification on an EVOS flAUTO (Thermo) and counting the resulting puncta on ImageJ.

HPLC analysis

Striatal samples were sonicated for 10 seconds in 500 µl of ice-cold homogenising buffer (0.1 M Perchloric acid, 0.1 mM EDTA, 2.5 mg/L ascorbate). The samples were then centrifuged at 20,000xg for 15 minutes at 4°C. The supernatant was collected and filtered through a syringeless filtration device (UniPrep) and samples stored at -80°C until use. 20 µl of the supernatant were analysed by HPLC to detect dopamine (DA) and its metabolites 3,4-hydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). The HPLC system consisted of a Jasco HPLC pump, triathlon autosampler (Spark Holland) and a BAS LC4C detector (BAS Analytical) coupled to an Empower data system (Waters). To separate the samples, a Hypersil BDS column (150 x 3.0 mm C18 3u) was used. The mobile phase (pH 2.8) consisted of 0.1 M monosodium phosphate (Fisher), 350 mg/L Octanesulfonic acid (Fisher), and 14 % methanol and the flow rate set to 400 µl/minutes with a column oven temperature of 40°C. Oxidation settings were +775 mV, 20 nA/V whilst the reduction settings were +50 mV, 10 nA/V.

Immunoblotting

Protein samples were isolated from HEK293T cells or brain tissue using RIPA lysis buffer (1% Igepal CA-630 (Sigma), 0.5% sodium deoxycholate (BDH laboratories supplies) and 0.1% sodium dodecyl sulphate (SDS, Sigma) in PBS) supplemented with 1X protease inhibitor (Complete Mini, Roche). Protein levels were determined using a bicinchoninic acid (BCA) assay (Pierce, ThermoScientific). Proteins (10-50 µg) were resolved by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis; transferred onto polyvinylidene difluoride (PVDF) membrane and incubated with primary antibodies as follows: AsRed (1:500, Clontech), α-synuclein (1:500 610786, BD) and TH (1:1000 ab81855, Abcam). Horseradish-peroxidase

(HRP) conjugated secondary antibodies (1:5,000-10,000) were added and proteins visualised using enhanced chemiluminescence (34080, Pierce). Alpha tubulin (1:2000 T9026, Sigma) was used as a protein loading control.

Data handling and statistical analysis

Statistical analysis was performed using Graph Pad Prism 5.0 software (Graph Pad Software Inc., CA). A one-way ANOVA followed by a Dunnetts post hoc test was performed, accepting $P<0.05$ as statistically significant. For *in vivo* experiments all data were normalised to a mean of all of the control hemispheres for every animal within that time point. Student T-tests were used when only two groups were analysed. For more than two groups and only one factor as the source of variance a one-way ANOVA was used followed by a Newman-Keuls post hoc test. The figures show the mean +/- SEM with $P<0.05$ accepted as statistically significant.

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Figure 1 | MiR-7 levels are significantly decreased in the nigra of patients with PD

*A \ Representative images of α-synuclein staining (green) and nuclear counterstain (blue) in a PD brain section compared to a healthy age-matched control. Scale bar: ?μm.. B | RNA was extracted using the mirVana miR isolation kit from frozen nigral sections collected from six patients with PD and five healthy age-matched controls. MiR-7 levels were detected by qPCR using a taqman miR-7 assay. MiR-7 levels were significantly decreased in the PD patients compared to the healthy controls. Data were analysed using an unpaired t-test and expressed as mean ± SEM. *, P<0.05 for difference between PD and control samples. C | Age range of the six patients with PD and five healthy age-matched controls. PD: Parkinson's disease.*

Figure 2 | miR-7 regulates α-synuclein protein expression

*A | Representative Western blot of α-synuclein protein expression after transduction of miR-7-GFP (MOI, 0, 0.1, 1 and 5). α-tubulin was used as a protein loading control. B | Mean levels of α-synuclein protein in A following transduction with miR-7-GFP. Data were expressed as a percentage of the untransduced control and analysed by a one-way ANOVA followed by a Dunnett post hoc test. Data are shown as mean ± SEM from three independent experiments. *, P<0.05 for difference between untransduced control and miR-7 treated samples. C | Representative Western blot of α-synuclein expression after transduction of miR-7T-AsRed (MOI, 0, 0.1, 1 and 5). α-tubulin was used as a protein loading control. D | Mean levels of α-synuclein in C following transduction with miR-7T-*

*AsRed. Data were expressed as a percentage of the untransduced control and analysed by a one-way ANOVA followed by a Dunnett post hoc test. Data are shown as mean ± SEM from three independent experiments. **, P<0.01 for difference between untransduced control and miR-7T treated samples. MOI: multiplicity of infection; UT: untransduced control.*

Figure 3| α-synuclein quantification in the SNpc at 16 and 24 weeks after double site injection of the miR-7T-AsRed lentiviral vector

*A| Representative Western blots of α-synuclein expression in the miR-7T injected SNpc and the control non-injected left SNpc at 16 and 24 weeks post surgery. α-tubulin was used as a protein loading control. B | Mean levels of α-synuclein expression after double site injections of either the miR-7T (16 weeks n=6, 24 weeks n=4) or control cmv-AsRed lentiviral vector (24 weeks n=3). Data were expressed as a percentage of the control non-injected hemisphere and analysed by a paired t-test for each time point. Data are shown as mean ± SEM. *, P<0.05 **, P<0.01 for a difference between the miR-7T injected SNpc and the control non-injected SNpc. C | Representative images of α-synuclein-PLA (red) and nuclear counterstain (blue) of miR-7T injected mice 24 weeks after injection. Scale bar: 25μm D | Mean levels of α-synuclein-PLA expression after double site injection of the miR-7T 24 weeks post-surgery (n=3). Data were expressed as a percentage of the control non-injected hemisphere and analysed by a paired t-test. Data are shown as mean ± SEM. *, P<0.05 for a difference between the miR-7T injected SNpc and the control non-injected SNpc. E | Representative images of α-synuclein-PLA (red) and nuclear counterstain (blue) of control injected mice 24 weeks after injection. Scale bar: 25μm SNpc: substantia nigra pars compacta*

Figure 4 | MiR-7T and TH expression in the SNpc at 16 and 24 weeks after double site injection of the miR-7T-AsRed lentiviral vector

*A | Representative images of TH (green) and miR-7T-AsRed (red) expression in the right SNpc compared to the non-injected control SNpc at 16 and 24 weeks post surgery. Scale bar: 100μm. B | Number of TH positive cells after double site injections of the miR-7T-AsRed lentiviral vector into the right SNpc. Data were expressed as a percentage of the control non-injected SNpc and analysed by a paired t-test for each time point. Data are shown as mean ± SEM. ***, P<0.001, **, P<0.01, *, P<0.05 for a difference between the miR-7T injected SNpc and the control non-injected SNpc. C | Mean levels of TH protein expression after double site injections of the control cmv-AsRed lentiviral vector into the right SNpc. Data were expressed as a percentage of the control non-injected SNpc and analysed by a paired t-test. Data are shown as mean ± SEM. Representative western blot is also shown of TH expression in the CMV-AsRed injected SNpc (AsRed) and the control non-injected SNpc (control) at 24 weeks post surgery. α-tubulin was used as a protein loading control. SNpc: substantia nigra pars compacta; TH: Tyrosine hydroxylase.*

Figure 5 | HPLC analysis of striatal samples and VMAT2 SNpc quantification after double site injection of the miR-7T-AsRed lentiviral vector

Striatal levels of A | Dopamine (DA), B | 3,4-Dihydroxyphenylacetic acid (DOPAC), C | Homovanillic acid (HVA) after injection of miR-7T or control CMV-AsRed. Data were

*analysed by a paired t-test for each time point (MiR-7T 16 weeks n=6, 24 weeks n=3, CMV-Asred 24 weeks n=3). Data are shown as mean ± SEM. *, P<0.05 for a difference between the miR-7T-AsRed injected SNpc and the control non-injected SNpc. D | Number of VMAT2 positive cells after double site injections of the miR-7T-AsRed lentiviral vector into the right SNpc. Data were expressed as a percentage of the control non-injected SNpc and analysed by a paired t-test for each time point. Data are shown as mean ± SEM. *, P<0.05 for a difference between the miR-7T injected SNpc and the control non-injected SNpc. SNpc: substantia nigra pars compacta.*

Figure 6 | Locomotor activity in mice injected with miR-7T-AsRed or control cmv-AsRed lentiviral vector 2, 10 and 20 weeks post-surgery

A | Total distance moved in cm over 30 minutes (n=10). B | Total velocity over 30 minutes (n=10). C | Number of amphetamine induced ipsilateral rotation after injection of the miR-7T-AsRed lentiviral vector at 5 minute intervals (n=10). D | Number of amphetamine induced ipsilateral rotations after injection of control cmv-AsRed lentiviral vector at 5 minute intervals (n=5). E | Total number of amphetamine induced ipsilateral rotations after 90 minutes. Data were analysed by a two way ANOVA followed by a Bonferroni post hoc test. Data are shown as mean ± SEM. No significant difference was found.

Figure S1 | Human brain tissue samples

Figure S2 | MiR-7 and miR-7T sequences

A | MiR-7 sequence. B | MiR-7T sequence. The MiR-7T sequence contains four complementary binding sites for miR-7, which are represented by the four blue boxes.

Figure S3 | MiR-7 lentiviral construct

MiR-7 sequence was inserted between the XbaI and BamHI restriction sites upstream of a GFP reporter gene in the pRRL-sincppt.CMV-GFP.wpre lentiviral backbone.

Figure S4 | MiR-7T lentiviral construct

MiR-7T sequence was inserted between the EcoRI and KpnI restriction sites downstream of an As-Red reporter gene in the pRRL-sincppt.cm-AsRed.wpre lentiviral backbone.

Figure S5 | Knockdown of miR-7T-AsRed expression by miR-7-GFP

*A | HEK293T cells were co-transduced with miR-7T-AsRed (MOI 2) and miR-7-GFP (MOIs 0, 0.1, 1 and 5) lentiviral vectors and the expression visualised by fluorescence microscopy. Hoechst staining of the nuclei is shown in blue. Scale bar: 100μM. Green: GFP; red: AsRed. B | Representative Western blot using the AsRed antibody to detect miR-7T expression (MOI 2) after co-transduction with the miR-7-GFP lentiviral vector (MOI 0,1,5 and 10). α-tubulin was used as a protein loading control. C | Mean levels of AsRed protein from B calculated from 3 independent experiments. Data were expressed as a percentage of the untransduced control and analysed by a one-way ANOVA followed by a Dunnett post hoc test. Data are shown as mean ± SEM from three independent experiments. ***, P<0.001 for difference between control and miR-7 treated samples. MOI: multiplicity of infection; UT: untransduced control.*

Figure S6 | MiR-7 binding sites on the 3'UTR snca

Predictive binding sites of miR-7 on the 3'UTR snca using miRanda software. All binding sites show a score of >80 indicating a strong binding site.

Figure S7 | Validation of miR-7-GFP binding to the 3'UTR of the mouse snca gene

*A | The conserved miR-7 binding site on 3'UTR snca gene. The seed match is indicated in red on the human, mouse and rat snca gene. B | Schematic diagram of the mouse snca 3'UTR luciferase construct. C | HEK293T cells were co-transfected with the 3'UTR snca plasmid and the miR-7, control miR (449) or GFP control plasmid. Cells were harvested 48h later and the luciferase activity measured. Data were expressed as a percentage of the GFP control and analysed by a one-way ANOVA followed by a Dunnett post hoc test. Data are shown as mean ± SEM from three independent experiments. *, P<0.05 for difference between GFP control and miR-7 treated samples.*

Figure S8 | Design of in vivo experiments

A | Design of experiment 1. Animals were injected with the miR-7T-AsRed lentiviral vector into one site of the right substantia nigra (RSN) whereas a control cmv-AsRed lentiviral vector was injected into the same site in the left substantia nigra (LSN). Animals were left for 16 and 24 weeks before being sacrificed for immunohistochemistry and biochemical analysis. The 24 weeks animals were also tested for behavioural analysis at 2, 10 and 20 weeks post surgery. B | Design of experiment 2. Animals were injected with the miR-7T-AsRed or control cmv-As Red lentiviral vectors into two sites of the right substantia nigra (RSN). MiR-7T-AsRed injected animals were left for 16 and 24 weeks before being sacrificed for immunohistochemistry and biochemical analysis. Control injected animal were left for the full 24 week before being taken for biochemical analysis. The 24 weeks animals were also tested for behavioural analysis at 2, 10 and 20 weeks post surgery. RSN: right substantia nigra; LSN: left substantia nigra; AP: anterior/posterior; ML: medial/lateral; DV: dorsal/ventral; SNpc: substantia nigra pars compacta; HPLC: high performance chromatography.

Figure S9 | α-synuclein and TH quantification 16 and 24 weeks post-surgery after single site injection of the miR-7T-AsRed lentiviral vector

*A | Mean levels of α-synuclein expression after single site injection of the miR-7T-AsRed lentiviral vector into the right SNpc and the control cmv-AsRed lentiviral vector into the left SNpc at 16 weeks (n=4) and 24 weeks post surgery (n=5). Data were expressed as a percentage of the control cmv-AsRed injected hemisphere and analysed by a paired t-test for each time point. Data are shown as mean ± SEM. No significant difference was found. Representative western blot are also shown of α-synuclein expression in the miR-7T-AsRed injected SNpc and the control cmv-AsRed injected left SNpc at 16 and 24 weeks post surgery. α-tubulin was used as a protein loading control. B | Number of TH positive cells after injection of the miR-7T-AsRed lentiviral vector into the right SNpc and the control cmv-AsRed lentiviral vector into the left SNpc at 16 weeks (n=3) and 24 weeks post surgery (n=4). Data were expressed as a percentage of the control cmv-AsRed injected SNpc and analysed by a paired t-test for each time point. Data are shown as mean ± SEM. *, P<0.05 for a difference between the miR-7T-AsRed injected SNpc and the cmv-AsRed injected SNpc.* C *| Representative images of TH (green) and miR-7T-AsRed (red) expression in the right SNpc compared to the control cmv-AsRed (red)*

injected left SNpc at 24 weeks post surgery. Scale bar: 100µm. D | Number of amphetamine induced ipsilateral rotations at 5 minute intervals. E | Total number of amphetamine induced ipsilateral rotations after 90 minutes. Data were analysed by a one-way ANOVA followed by a Newman Keuls post hoc test. Data are shown as mean ± SEM. No significant difference was found. SNpc: substantia nigra pars compacta; TH: Tyrosine hydroxylase.

Figure S10 | HPLC analysis 16 and 24 weeks post surgery after single site injection of the miR-7T-AsRed Lentiviral vector

Striatal levels of A | Dopamine (DA), B | 3,4-Dihydroxyphenylacetic acid (DOPAC), C | Homovanillic acid (HVA). Data were expressed as a percentage of the control injected hemisphere of the brain and each time point analysed by a paired t-test (16 weeks n=5, 24 weeks n=6). Data are shown as mean ± SEM. No significant difference was found.

Figure S11 | Detection of oligomeric forms of α-synuclein using PLA

*A | Top: electron microscopy analysis of monomeric, oligomeric and fibrillar α-synuclein, at 23000 magnification. Scale bars = 500 nm. Bottom: α-synuclein -PLA analysis of monomeric, oligomeric and fibrillar α-synuclein. Scale bars = 25 um. Representative images of three independent experiments are shown. B | Quantitation of three independent experiments is shown. Error bars are + SD. *, P<0.05. One-way ANOVA with Turkey's multiple comparisons test.*