



Gene therapy using synthetic microRNA directed against GAD67 has beneficial effect on motor behaviour in 6-OHDA lesioned rats

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Abstract

Background: Modification of the glutamic acid decarboxylase (GAD) level is a promising future treatment tool for Parkinson's disease (PD). GAD is the rate-limiting enzyme in the synthesis of the inhibitory neurotransmitter gamma amino butyric acid (GABA) and modification of the GAD level could therefore potentially be used to alter the GABA output from a nucleus.

Methods: We have previously developed a synthetic microRNA (smiRNA1550) designed to knockdown the GAD isoform GAD67 and shown that it can efficiently knockdown the target protein. In this study we have further evaluated the therapeutic potential of smiRNA1550 in treating motor symptoms using the 6-OHDA rat model of PD and lentiviral vectors. The smiRNA was evaluated in striatum and substantia nigra pars reticulata (SNpr), two nuclei with increased GAD levels and GABA output in PD. The result was evaluated by behaviour tests and western blot. Differences between groups were assessed using analysis of variance (ANOVA) followed by the Tukey's multiple comparison test for group comparisons.

Results: There was a beneficial effect in apomorphine-induced rotations after injection of LV-smiRNA1550 into SNpr.

Conclusion: These findings suggest that manipulation of the GABA system could be a valid approach for development of novel gene therapies for PD that warrant further study.

Keywords: Glutamic acid decarboxylase, synthetic microRNA, striatum, substantia nigra pars reticulata, parkinson's disease, rat

Introduction

Glutamic acid decarboxylase (GAD) is the rate-limiting enzyme in the production of the inhibitory neurotransmitter gamma amino butyric acid (GABA). Aberrant GAD expression and GABAergic signalling is involved in several diseases, such as Parkinson's disease (PD) [1,2]. In PD loss of dopamine causes an imbalance in the signalling pathways throughout the basal ganglia and ultimately difficulties in initiating movements. Normalisation of the GABA signalling could potentially alleviate some of these symptoms and this could be achieved by modifying the level of GAD.

Modification of the GAD level by gene therapy has been shown to be a promising future treatment option for PD. The studies performed so far have used adeno-associated vectors expressing GAD. Beneficial effects have been reported after

injection into the subthalamic nucleus (STN), a glutamatergic nucleus that is over-active in PD, in rats with experimental PD, macaques with experimental PD and parkinsonian patients [3-8]. Modification of GAD in other nuclei may however also be beneficial.

Increased expression of GAD has previously been reported in striatum and substantia nigra pars reticulata (SNpr) following dopamine depletion [9,10] and modification of GAD in these nuclei may have beneficial effect on parkinsonian symptoms. This could be achieved by using RNA interference (RNAi). Studies have shown that highly efficient RNAi can be achieved by embedding a small interfering RNA (siRNA), complementary to a target mRNA, into a miR30 scaffold [11]. These synthetic microRNA (smiRNA) have several advantages over frequently used short-hairpin RNA (shRNA), both in terms of safety [12,13]

and in terms of versatility in the vector design [11,14].

We have recently constructed a smiRNA; smiRNA1550, directed against GAD67 [9]. GAD67 is one of two GAD isoforms found in the CNS [15]. We found that smiRNA1550 was able to efficiently knock down GAD67 protein levels *in vitro* to 20% of untreated cells. Moreover, smiRNA1550 was also able to normalise the GAD67 protein level in striatum of 6-OHDA lesioned rats without any signs of toxicity. These results show that smiRNA1550 is safe and that it is possible to knockdown endogenous GAD67 levels both *in vitro* and *in vivo*. However, it is still unclear if knockdown of GAD67 using smiRNA has any beneficial effect on PD symptoms. In this study we have evaluated possible beneficial effects of GAD67 down-regulation on motor behaviour in the 6-OHDA rat model of PD. We report a beneficial effect after injection of lentiviral vectors (LV) expressing smiRNA1550 into the SNpr, but not after injection into the striatum.

Material and methods

Production of lentiviral vectors

The LV-CMV-smiRNA1550 [9], LV-CMV-GFP [16] and LV-EF1 α -GFP [16] vectors have been published elsewhere. The LV-EF1 α -smiRNA α GFP and LV-EF1 α -smiRNA1550 vectors were constructed using Gateway cloning (Invitrogen, Carlsbad, CA). Briefly, the dsRed-smiRNA α GFP fragment was amplified from a LV-CMV-smiRNA α GFP vector [17] and cloned into a pDONR221 vector, according to the manufacturers protocol, creating a pDONR221-smiRNA α GFP vector. A pDONR-smiRNA1550 vector was created by annealing the oligonucleotides coding for smiRNA1550 [9], cutting out smiRNA α GFP from pDONR-smiRNA α GFP using SpeI/XmaI (New England Biolabs, Ipswich, MA) and ligating the annealed oligonucleotides into the SpeI/XmaI site. The resulting vectors were combined with a pDONRP4P1R-EF1 α and a pHg backbone to create the expression vectors LV-EF1 α -smiRNA α GFP and LV-EF1 α -smiRNA1550. Schematic maps of the vectors are shown in **Figure 1**. Lentivirus was produced and the viral titres were determined, as previously described [17]. The control vectors; LV-CMV-GFP, LV-EF1 α -GFP and LV-EF1 α -smiRNA α GFP, had a viral titre of 5×10^7 - 5×10^8 TU/ml. The smiRNA1550 vectors all had a viral titre $> 10^9$ TU/ml.

Surgical procedures

A total of 54 adult female Sprague-Dawley rats were included in this study. The animals were housed in groups of 2-5 per cage under a 12 hour light-dark cycle with ad libitum access to food and water. All procedures were approved and performed according to the guidelines of the ethical committee for use of laboratory animals at Lund University. Animals were anesthetized using 6.2 ml/kg of a fentanyl-citrate (Fentanyl, B. Braun Melsungen AG, Melsungen, Germany, 50 μ g/ml) and medetomidine hydrochloride (Domitor, Orion Pharma, Espoo, Finland, 1 mg/ml) mixture (20:1) injected ip. After all surgeries 0.9 ml/kg Atipamezol hydrochloride (Antisedan, Orion Pharma, 5mg/ml) in sterile water (1:15) and 1.3 ml/kg buprenorphine hydrochloride (Temgesic, Shering-Plough Europe, Heist-Op-Den-Berg, Belgium, 0.3 mg/ml) in sterile saline (1:9) were administered sc.

A subgroup of animals (n=13) was used as intact controls. Remaining animals (n=41) were unilaterally lesioned using 6-hydroxydopamine (6-OHDA). Lesioned animals received an injection of 14 μ g 6-OHDA (Sigma-Aldrich, St. Louis, MO) diluted to 3.5 μ g/ μ l in a 0.02% ascorbic acid/saline solution, into the medial forebrain bundle (A/P -4.4, M/L -1.2, D/V -7.8, measured from the bregma and dural surface, with the tooth bar set to -2.4) using a 10 ml Hamilton syringe (Hamilton, Bonaduz, Switzerland). 3 weeks after the lesion, the animals were randomized into different groups and a subgroup of animals (n=28) were re-anesthetized and injected with virus using a 5 ml Hamilton syringe fitted with a glass capillary. Remaining animals (n=13) were used as lesion only controls. Animals were injected with LV-CMV-smiRNA1550 (n=5) or LV-CMV-GFP (n=3) into the striatum (3 sites with 2 μ l virus/site: A/P +0.6, M/L -2, D/V -5 and -4 (1 μ l/depth); A/P +0.6, M/L -3.2, D/V -5 and -4 (1 μ l/depth); A/P +1.2, M/L -2.5, D/V -5 and -4 (1 μ l/depth); all measured from the bregma and dural surface, with the tooth bar set to 0). Other animals were injected with a low dose of LV-EF1 α -smiRNA1550 (n=6) or LV-EF1 α -GFP (n=4) into the SNpr (1 site with 1 μ l virus; A/P -5.3, M/L -2, D/V -7.6; all measured from the bregma and dural surface, with the tooth bar set to -2.3). Some were injected with a high dose of LV-EF1 α -smiRNA1550 (n=6) or LV-EF1 α -smiRNA α GFP (n=4) into the SNpr (2 sites with 1.5 μ l virus/site; A/P -5.2, M/L -2.4,

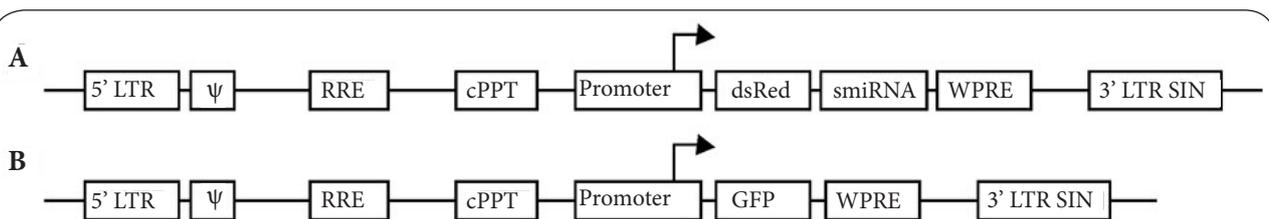


Figure 1. Schematic maps of the vector constructs.

A map of the constructs containing smiRNA1550 or smiRNA α GFP is shown in (A).

A map of the constructs containing GFP is shown in (B).

A CMV promoter was used in the smiRNA and GFP constructs in the striatal injections while an EF1 α promoter was used in the smiRNA and GFP constructs in the striatal injections.

D/V -8; A/P -5.6, M/L -2.2, D/V-8; all measured from the bregma and dural surface, with the tooth bar set to -2.4). The animals belonging to the intact and lesion only groups were also anesthetized, an incision was made on the head and the wound was closed in order to make them indistinguishable from the virus-injected animals in the subsequent behaviour tests.

Behavioural tests

All behaviour tests and scoring of results were performed by an observer blind to the treatment given to the animal.

Drug-induced rotations

Drug induced rotations were assessed essentially as described by Ungerstedt and Arbuthnott [18] in automated rotometer bowls (Accuscan Instrument Inc, Columbus, Ohio). Rotations were monitored for 90 min following ip injection of D-amphetamine sulphate (2.5 mg/kg, Apoteksbolaget, Sweden) or for 40 min following sc injection of apomorphine-HCl (0.2 mg/kg, Sigma-Aldrich). Amphetamine rotations were used to assess the 6-OHDA lesion before viral injection. All animals included in the study achieved >4.3 turns/min after injection of D-amphetamine. The scores shown from the apomorphine-HCl experiments are expressed as counter-clockwise 360° turns/min.

Corridor test

The corridor test was performed essentially as described by Dowd et al [19]. Briefly, food restricted rats was first placed in an empty corridor (150*7*32 cm), to reduce exploratory behaviour, and then in a test corridor (150*7*32 cm) with 10 adjacent pairs of plastic lids. The animals were habituated in the 2 corridors for 5 min the first day. Some sugar pellets (Test Diet, Richmond, VA) were sprinkled in on the empty side and in some of the lids on the test side. On test days, all lids were filled with sugar pellets and the number of retrievals from the right and left side was counted. A retrieval was counted for each time the rat poked its head into a lid, regardless if any sugar pellets were eaten or not. Repeated retrievals from the same lid were not counted unless another lid was visited in between. Each rat was kept in the test corridor until 20 retrievals were achieved or 5 min had elapsed. All rats were tested for 4 consecutive days. The scores shown is the percentage of contralateral retrievals. Days when the animal did not achieve 20 retrievals were excluded.

Spontaneous rotations

Spontaneous rotations were assessed in automated rotometer bowls for 40 min. The scores shown from the experiments are expressed as clockwise 360° turns/min. Time periods when the rat was not moving was excluded.

Western blot

Animals were sacrificed using 1.5 ml sodium pentobarbital (Apoteket, Lund, Sweden, 60 mg/ml). The brains were briefly put

in ice-cold saline and then coronally sectioned into three 1 mm and one 2 mm (most posterior part) sections to include the whole striatum or 1mm of striatum and 2mm of SNpr. The individual striatal and SNpr pieces were then dissected and placed in individual tubes. 3 ml/g (minimum 100µl) of RIPA buffer with protease inhibitor (Roche, Basel, Switzerland) was added to each sample and the samples were homogenized by sonication. The samples were left on ice for 30 min and were then centrifuged at 10,000xg for 10 min at 4°C to remove debris.

The protein concentration was determined using the BioRad DC Protein assay kit (BioRad, Hercules, CA) according to the manufacturer's protocol. 5 µg of protein was loaded on the gel and Western blot was performed essentially as previously described [9]. Antibodies used: mouse anti-GAD67 (1:3000, sc-58531, Santa Cruz, Santa Cruz, CA), mouse anti-β-actin HRP (1:50000, A3854-200UL, Sigma Aldrich), mouse anti-TH (1:5000, MAB318, Millipore, Billerica, MA) and goat anti-mouse HRP (1:5000, sc-2005, Santa Cruz). The staining was detected by ECL prime (GE Healthcare, Waukesha, WI) and photographed using the Versadoc MP 4000 or ChemiDoc MP system (BioRad). The images were analyzed using Image lab (BioRad). The values were normalized first to actin (striatum) or the total amount of protein loaded (SNpr) and secondly to the untreated left side.

Statistical analysis

Differences between groups were assessed using analysis of variance (ANOVA) followed by the Tukey's multiple comparison test for group comparisons. Significance was accepted at a 95% probability level.

Results

Injection of smiRNA1550 into rat striatum

The ability of smiRNA1550 to alleviate motor symptoms was evaluated using 6-OHDA lesioned rats and LV. Lesioned rats were injected with LV-CMV-smiRNA1550 or LV-CMV-GFP at 3 sites (2 depths/site) in the striatum. There was no beneficial effect on behaviour following injection of LV-CMV-smiRNA1550 (data not shown). Analysis of the GAD67 protein level by Western blot showed an up-regulation of GAD67 protein level to 163±22% of the intact untreated side on the lesioned side of the "lesion only" control animals (Figure 2). By contrast, a maximal down-regulation to 45-108 % of the intact untreated side could be seen after injection of LV-CMV-smiRNA1550 into the lesioned side. These results show that smiRNA1550 has effect on the GAD67 protein level after injection into lesioned animals. The effect of smiRNA1550 was however limited to areas close to the injection site, targeting only a fraction of the total neurons in the striatum (Figure 2). We therefore hypothesised that we targeted a too small portion of the affected neurons to achieve any significant behavioural effect and the therapy could potentially be more beneficial in a smaller nucleus where a larger portion of the total cells could be targeted.

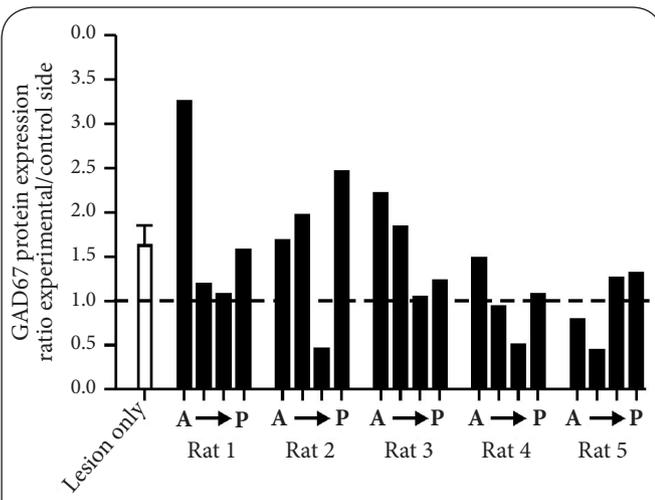


Figure 2. The effect of smiRNA1550 gene therapy in striatum on GAD67 protein levels was evaluated by Western blot. The striatum was dissected into 4 pieces per striata; anterior striatum (A) to the posterior striatum (P). White bar represent the lesion only animals (n=3, mean±SEM). The black bars represent the GAD67 level in the individual striatal pieces after injection of LV-CMV-smiRNA1550 into the striatum of 5 animals.

Injection of smiRNA1550 into rat substantia nigra pars reticulata

The smiRNA was further evaluated in the SNpr of 6-OHDA lesioned rats. We changed the promoter from CMV to EF1α since previous studies have shown that CMV is mainly expressed in glial cells in the midbrain [20]. Two experiments were performed: a low dose of LV-EF1α-smiRNA1550 or LV-EF1α-GFP was used in the first experiment while a higher dose of LV-EF1α-smiRNA1550 or LV-EF1α-smiRNAαGFP was used in the second experiment.

The effect of the gene therapy was evaluated using several behaviour tests: the corridor test [19], spontaneous rotations and apomorphine-induced rotations (Figure 3). The control groups, intact animals and lesion only animals, were similar and not significantly different in the two experiments and thus the results from these groups were combined to form one intact group and one lesion only group. Comparison between the intact control animals and lesion only animals showed that the 6-OHDA lesion induced a significant sensory motor neglect and rotational behaviour (Figure 3).

There was no significant beneficial effect in the corridor test (Figure 3A), although a trend towards increased left paw retrievals could be seen in the smiRNA1550 groups

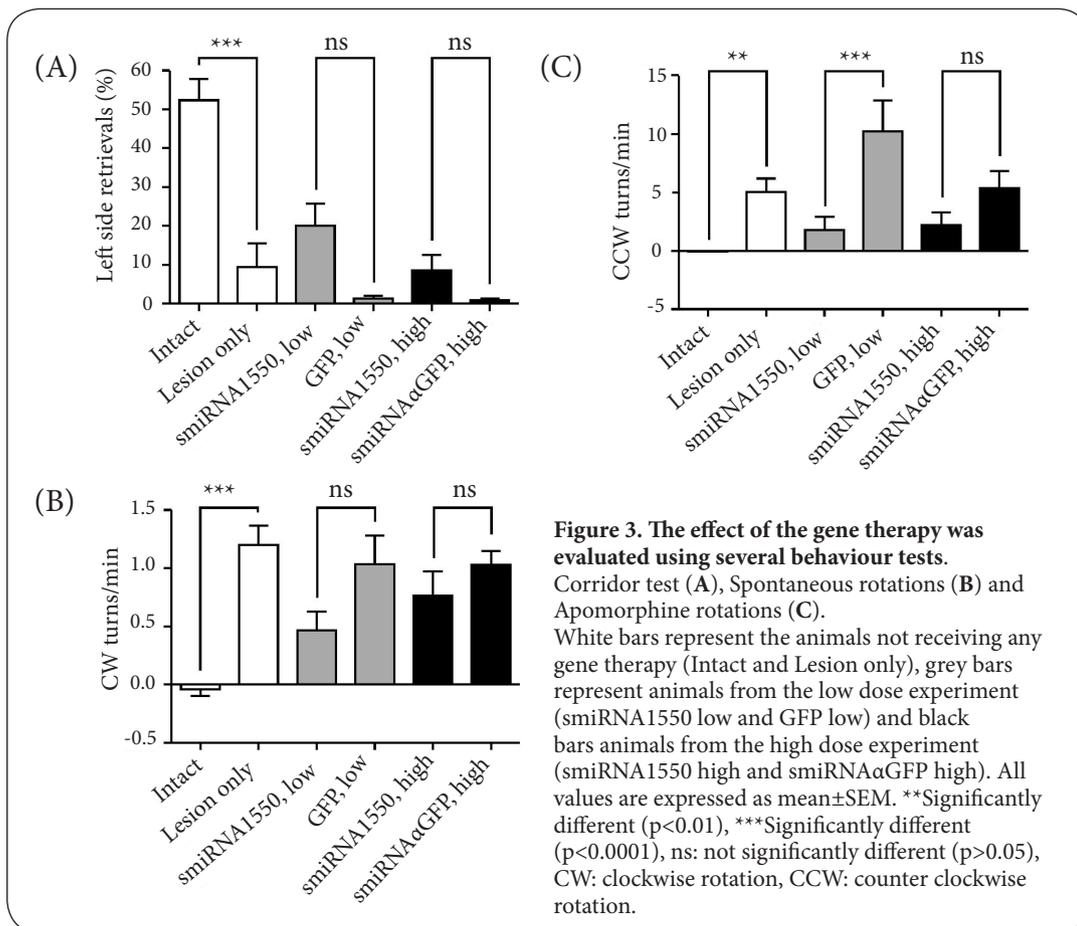


Figure 3. The effect of the gene therapy was evaluated using several behaviour tests. Corridor test (A), Spontaneous rotations (B) and Apomorphine rotations (C). White bars represent the animals not receiving any gene therapy (Intact and Lesion only), grey bars represent animals from the low dose experiment (smiRNA1550 low and GFP low) and black bars animals from the high dose experiment (smiRNA1550 high and smiRNAαGFP high). All values are expressed as mean±SEM. **Significantly different (p<0.01), ***Significantly different (p<0.0001), ns: not significantly different (p>0.05), CW: clockwise rotation, CCW: counter clockwise rotation.

when compared to their respective control groups. No beneficial effect was seen in spontaneous rotations when the smiRNA1550 groups were compared to their respective control groups (**Figure 3B**). It is however worth noting that the smiRNA1550 low dose group was not significantly different from the intact group (0.466 ± 0.166 vs -0.043 ± 0.056 CW turns/min, $p > 0.05$) and significantly different from the lesion only group (0.466 ± 0.166 vs 1.198 ± 0.169 CW turns/min, $p < 0.05$). A beneficial effect could be seen in apomorphine-induced rotations (**Figure 3C**) where the smiRNA1550 low dose group was statistically different to the GFP low dose control group (1.770 ± 1.144 vs 10.223 ± 2.634 turns/min $p < 0.0001$). Additionally, neither of the smiRNA1550 groups was statistically different from the intact control group (1.770 ± 1.144 (low dose) and 2.191 ± 1.079 (high dose) vs -0.038 ± 0.018 CCW turns/min, $p > 0.05$). These results indicate that expression of smiRNA1550 has beneficial, i.e., normalising, effect on motor behaviour in 6-OHDA lesioned rats.

The GAD67 protein levels in SN was analysed by Western blot (**Figure 4**). Both the SNpr and the substantia nigra pars compacta (SNpc) were included in the dissected piece due technical limitations and included in the Western blot samples. There was no significant difference after lesion or following injection of either of the smiRNA1550 doses, GFP or smiRNA α GFP (lesion only: $111 \pm 18\%$; smiRNA1550 low: $98 \pm 18\%$; GFP: 77% (average of 2 animals); smiRNA1550 high: $78 \pm 12\%$; smiRNA α GFP: $86 \pm 20\%$ of untreated intact side) when compared to intact untreated animals ($100 \pm 10\%$). We chose to normalise the values to the amount of protein loaded instead of actin since we frequently noted an increased actin level in the samples taken from the lesioned side. The 6-OHDA lesion was validated by Western blot, using antibodies directed against tyrosine hydroxylase (TH). All animals included in this study were well lesioned (**Figure 4**).

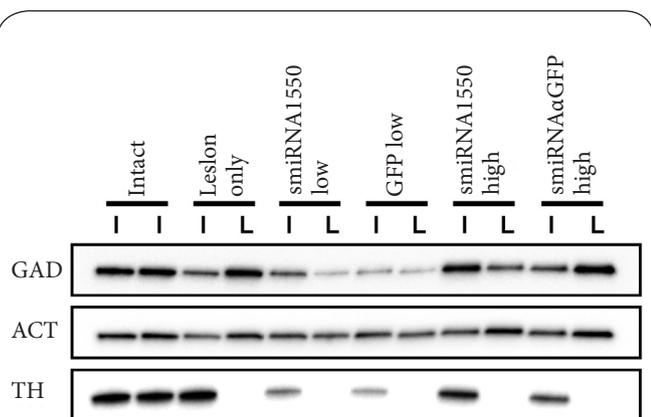


Figure 4. The effect of smiRNA1550 gene therapy in SNpr on GAD67 protein levels and validation of the lesion by loss of TH in striatum was evaluated by Western blot. GAD: GAD67 antibody (SNpr), Act: Actin antibody (SNpr), TH: Tyrosine hydroxylase antibody (striatum), I: Intact side, L: Lesioned side.

Discussion

In this study we have used a smiRNA based on miR30 to knock-down the endogenous GAD67 level. Furthermore, we have evaluated its effects on behaviour in the 6-OHDA rat model of PD after injection into the striatum or the SNpr. GAD67 has been shown to play a pivotal role in the synthesis of GABA and amount of vesicular GABA [21,22]. A recent study by Lau et al., [22] has shown that GAD67 expression is upregulated by neuronal activity and down-regulated by neuronal inactivity. These changes were strongly correlated to a corresponding increase or decrease in the synthesis and vesicular filling of GABA, indicating that GAD67 regulate GABA levels in an activity-dependent manner. Since smiRNA1550 has been shown to regulate the level of endogenous GAD67 protein [9], it is possible that the GABA output from a smiRNA-expressing nucleus is modified. The use of miR30 based silencing constructs has several advantages over similar constructs using shRNA. Several studies have noted an increased cytotoxicity when expressing high levels of shRNA [12,13,23]. This could potentially be detrimental for therapeutic use of this type of RNAi, especially when using viral vectors *in vivo* where it may not be possible to control the multiplicity of infection. This has indeed been noted in studies using shRNA for example in the brain [12,13] and in the liver [23]. The cause of this toxicity has been suggested to involve saturation of the miRNA system and disruption in the biogenesis of endogenous miRNAs. No such toxicity has been noted when using a smiRNA, even when dosed to silence as efficiently as shRNA [12,13]. The design of the gene therapy vector is also more flexible when using a smiRNA.

Transcription of shRNA using a pol II promoter can occur but the spacing of the hairpin to the transcription start site has to be carefully optimised to ensure production of functional shRNA [24]. A pol III promoter is therefore generally used for shRNA since this type of promoter provides a strict control of the transcription initiation and termination sites. By contrast, smiRNA has no such requirements and can therefore be readily transcribed by both pol II and pol III promoters [11,14]. This enables easy use of cell specific or regulatable promoters that further enhance the specificity and safety of the gene therapy. A reporter, such as GFP or dsRed, can also be easily included in the smiRNA vector to allow tracking of cells containing the smiRNA [11,17].

We started by evaluating the effect of smiRNA1550 in the striatum of 6-OHDA lesioned rats. The lowest GAD67 protein levels obtained after injection of smiRNA1550 into the lesioned side ranged from 45-108% of the intact untreated side, showing an efficient down-regulation of the lesion-induced upregulation of GAD67 seen after injection of 6-OHDA. This level of down-regulation is similar to the levels found around the injection site in the striatum on the lesioned side in our previous study [9]. The effect of smiRNA1550 on GAD67 protein levels was however limited to an area close to the injection site in this study and this partial striatal down-regulation was

not sufficient to induce any behavioural benefits. We therefore hypothesised that smiRNA1550 could be more beneficial in a smaller nucleus, such as SNpr.

Previous studies have used deep brain stimulation (DBS), lesions, glutamate receptor antagonists or GABAA receptor agonists to evaluate the therapeutic effect of SNpr manipulation in rodent and primate PD models and parkinsonian patients. The main beneficial effect found in these studies was on axial symptoms of PD [25-27], such as posture and gait, while the effect on arm and leg akinesia and bradykinesia has been more varied with some studies showing a positive effect and some showing no effect [27-29]. Modification of SNpr is not as efficient as modification of STN in treatment of parkinsonian symptoms [26]. Recent studies on parkinsonian patients have however reported that combined DBS of STN and SNpr has some additional beneficial effects on gait when compared to DBS of STN alone [30,31]. To our knowledge, this is the first study using gene therapy in an attempt to modify SNpr activity in a parkinsonian model.

We could not detect any beneficial effect on sensorimotor neglect in the group receiving smiRNA1550 when assessed by the corridor test. This is in line with previous studies that have shown no effect on sensorimotor neglect following SNpr manipulation in PD animal models [25,27]. As reported in previous studies, the 6-OHDA lesion caused a small spontaneous ipsilateral rotational bias [18]. Injection of a GABA agonist or a glutamate antagonist into the SNpr of 6-OHDA lesioned rats has previously been shown to induce spontaneous contralateral rotations [32,33]. There was no statistically significant beneficial effect on spontaneous rotation following injection of smiRNA1550 into the SNpr in this study. There was however a trend towards a reduced rotational behaviour in the smiRNA1550 low dose group. Injection of apomorphine induced a strong contralateral rotation in 6-OHDA lesioned rats in this study, which is in line with previous studies [34]. In contrast to the spontaneous rotations, the apomorphine-induced rotations were significantly reduced in animals expressing a low dose of smiRNA1550. Rats with both a 6-OHDA lesion and a lesion of the SNpr has previously been shown to have an altered rotational behaviour when compared to 6-OHDA lesioned rats after injection of apomorphine [34,35]. In these previous studies, the additional lesion in SNpr was shown to reduce the contralateral rotations induced by the 6-OHDA after injection of apomorphine. Furthermore, increased doses of apomorphine or repeated injections over several days further attenuated the rotations and at high doses or several repeated doses even reversed the direction of the rotations. It is possible that smiRNA1550 has a similar, but milder, effect as a GABA agonist, glutamate antagonist or SNpr lesion and is therefore able to reduce some types of rotational behaviour. In the high dose experiment there was no significant difference between the groups. The main difference in the data consisted of a lower rotational response in the control group. This could be due to toxicity of the vector

itself, a phenomenon that has been described previously (see e.g., [12]). Some studies have reported adverse effects following manipulations of SNpr. Acute tonic-clonic seizures has been reported after kainic acid lesion of the mid-SNpr in naïve and 6-OHDA lesioned rats [25] and acute depression or hypomania has been reported in patients following DBS in SNpr [36,37]. No severe adverse effects, such as seizures, were noted following injection of LV-EF1 α -smiRNA1550 into the SNpr in this study, but this needs to be further evaluated since no tests assessing non-motor symptoms were performed.

We chose to normalise the GAD67 protein level obtained from the Western blot to the amount of protein loaded instead of actin in the SN samples, since we frequently noted an increased actin level on the lesioned side compared to the intact side. This increased actin level may be caused by astrogliosis and/or infiltrating microglia [38,39]. These cell types have both been shown to increase in SNpc following injection of 6-OHDA into the medial forebrain bundle (MFB). Since the SNpr is very small to dissect, both the reticulata and the compacta part of the SN were included in the dissections and it is therefore possible that these infiltrating cells could influence the result. Previous studies have shown an increase of GAD67 in SNpr of 6-OHDA lesioned rats [10]. No effect on the GAD67 protein level could be seen in any of the groups in this study. It is however possible that the effect of the therapy is masked by the extra tissue included in the dissection. The effect of smiRNA1550 on the GAD67 protein level in SNpr therefore needs to be further evaluated.

Conclusion

The present report show that injection of smiRNA directed against GAD67 can have beneficial effects on behaviour in rats with unilateral dopamine-depletions. This validates further investigations in models of Parkinson's disease as well as in other disorders where GABA is known to play an important role.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	EEW	LQ	CL
Research concept and design	✓	--	✓
Collection and/or assembly of data	✓	--	--
Data analysis and interpretation	✓	✓	✓
Writing the article	✓	--	✓
Critical revision of the article	✓	✓	✓
Final approval of article	✓	✓	✓
Statistical analysis	--	✓	--

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