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Smirna Directed Against GAD67 Effects on Behavior in Rats with Unilateral Dopamine-Depletions

Stalin Reddy Challa [1*], Swathi Chilukala [1]

Jangaon Institute of Pharmaceutical Sciences, Depot. Of Pharmaceutics, Jangaon, India.

***Corresponding Author:** Stalin Reddy Challa, Jangaon Institute of Pharmaceutical Sciences, Depot. Of Pharmaceutics, Jangaon, India.

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Abstract

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.

Keywords: Glutamic acid decarboxylase; synthetic microRNA; striatum; parkinson's disease

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.

Methods and Meterials

Production of Lentiviral Vectors

The LV-CMV-smiRNA1550, LV-CMV-GFP] and LV-EF1α- GFP vectors have been published elsewhere. The LVEF1α- smiRNAαGFP and LV-EF1α-smiRNA1550 vectors were constructed using Gateway cloning (Invitrogen, Carlsbad, CA). Briefly, the dsRed-smiRNAαGFP fragment was amplified from a LV-CMV-smiRNAαGFP vector.

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 12hour 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 µg/ml) and medetomidine hydrochloride (Domitor, Orion Pharma, Espoo, Finland, 1 mg/ml) mixture (20:1) injected ip. After all surgeries 0.9 ml/kg Atipamelzol 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 µg 6-OHDA (Sigma-Aldrich, St. Louis, MO) diluted to 3.5 µg/µl 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 µl virus/ site: A/P +0.6, M/L -2, D/V -5 and -4 (1 µl/depth); A/P +0.6, M/L -3.2, D/V -5 and -4 (1 µl/depth); A/P +1.2, M/L -2.5, D/V -5 and -4 (1 µl/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-EF1α-smiRNA1550 (n=6) or LV-EF1α-GFP (n=4) into the SNpr (1 site with 1 µl 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-EF1α-smiRNA1550 (n=6) or LV-EF1α-smiRNAαGFP (n=4) into the SNpr (2 sites with 1.5 µl virus/site; A/P –5.2, M/L -2.4, 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 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 counterclockwise 360° turns/min.

Corridor ***Test***

The corridor test was performed essentially as described by Dowd et al. 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.

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.

Discussion

In this study we have used a smiRNA based on miR30 to knockdown 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. A recent study by Lau et al., 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, 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. 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 and in the liver. 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. 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. 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. 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 .

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. 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.

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. 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. No effect on the GAD67 protein level could be seen in any of the groups in this study. It is however possible is 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 shows 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 and important role.

Competing Interests

The authors declare that they have no competing interests.

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