Neurobiology of Aging 58 (2017) 30–33

Contents lists available at ScienceDirect

Neurobiology of Aging

journal homepage: www.elsevier.com/locate/neuaging

Negative results

Absence of regulator of G-protein signaling 4 does not protect against dopamine neuron dysfunction and injury in the mouse 6-hydroxydopamine lesion model of Parkinson's disease

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ARTICLE INFO

Article history:
Received 4 August 2016
Received in revised form 11 June 2017
Accepted 12 June 2017
Available online 19 June 2017

Keywords:
RGS4
Parkinson's disease
6-OHDA
Mouse model
Histopathology
Behavior
Neuroprotection
Target validation
Substantia nigra

ABSTRACT

Regulator of G-protein signaling 4 (RGS4), a member of the RGS family of proteins that inactivate G-proteins, has gained interest as a potential drug target for neurological disorders, such as epilepsy and Parkinson's disease (PD). In the case of PD, the main current options for alleviating motor symptoms are dopamine replacement therapies, which have limitations because of side effects and reduced effectiveness over the long term. Research on new nondopaminergic PD drug targets has indicated that inhibition of RGS4 could be an effective adjuvant treatment option. The effectiveness of RGS4 inhibition for an array of PD-linked functional and structural neuroprotection end points has not yet been demonstrated. Here, we use the 6-hydroxydopamine (6-OHDA) lesioning model of the nigrostriatal pathway in mice to address this question. We observe, using a battery of behavioral and pathological measures, that mice deficient for RGS4 are not protected from 6-OHDA–induced injury and show enhanced susceptibility in some measures of motor function. Our results suggest that inhibition of RGS4 as a non-dopaminergic target for PD should be approached with caution.
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1. Introduction

Parkinson's disease (PD) is a common movement disorder and, after Alzheimer's disease, the most frequent progressive neurodegenerative disorder (Shastry, 2001). It is characterized by a loss of dopaminergic neurons and debilitating motor and nonmotor symptoms (Sprenger and Poewe, 2013). No disease-modifying or preventive therapies exist, and current therapeutics only alleviate symptoms by counteracting dopamine loss (Poewe, 2009). However, the benefits of these treatments are limited by a gradual loss of efficacy and long-term adverse effects (Jankovic and Aguilar, 2008). One of the recently proposed nondopaminergic drug targets is RGS4, a GTPase accelerating protein for specific G-protein coupled receptors (De Vries et al., 2000). Increased RGS4 activity following disease-related dopamine loss has been suggested to lead to PD-associated dysfunction of neuronal projections to the globus pallidus and substantia nigra (SN) (Di Marzo et al., 2000; Lerner and Kreitzer, 2012). A first indication that RGS4 may be a target for PD was based on the observation that mice lacking the *Rgs4* gene ($Rgs4^{-/-}$ mice) were functionally less impaired than wild-type controls after 6-OHDA lesioning of their nigrostriatal pathway (Lerner and Kreitzer, 2012). To investigate if RGS4 inhibition provides protection against injury-induced loss of nigral dopaminergic neuron structure and function, focusing on the evaluation of RGS4 as a target for PD, an aspect that has not been investigated in-depth previously, we measured behavioral and neuropathological effects of intracerebral 6-OHDA administration in $Rgs4^{-/-}$ mice and their littermate wild-type controls (Grillet et al., 2005). Since mice lacking both alleles of a gene can compensate for this lack and do not always have the same phenotype or response to injury as heterozygotes (Klamer et al., 2005), we also analyzed heterozygous RGS4 mice ($Rgs4^{+/-}$). We found that motor function was

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http://dx.doi.org/10.1016/j.neurobiolaging.2017.06.008

significantly impaired by 6-OHDA in both *Rgs4*$^{-/-}$ and *Rgs4*$^{+/-}$ mice, with *Rgs4*$^{-/-}$ mice even showing motor deficiencies in tests that showed no or only slight impairment of *Rgs4*$^{+/+}$ wild-type controls. We also observed that neither tyrosine-hydroxylase (TH) positive fibers in the striatum nor TH-positive neurons in the SN were protected against 6-OHDA–induced injury. Our results contrast with those of a previous study (Lerner and Kreitzer, 2012) and indicate that *Rgs4* gene deletion does not always protect against toxin-induced nigral neuron dysfunction and injury in mice.

2. Methods

All experiments were approved by the institutional animal experimentation ethics committee and by appropriate government agencies. Heterozygote *Rgs4* (*Rgs4*$^{+/-}$) mice were purchased from Jackson (#005833; https://www.jax.org/strain/005833) and bred with C57BL/6J wild-type mice from the same vendor to obtain study cohorts (see Supplementary Material, Materials and Methods section, for details on genotyping). Mice were used at 12–18 weeks of age (equal numbers of males and females) and euthanized after the in-life procedures. Standard protocols were used according to the reference publications for stereotactical unilateral intrastriatal 6-OHDA injections (Bagga et al., 2015), motor behavior measurements [cylinder test (Glajch et al., 2012), pole test (Matsuura et al., 1997), and grip strength (Ferguson et al., 2015)], TH immunostaining, and quantitative image analysis of striatal and nigral TH-positive neurons (Masliah et al., 2000), except for minor modifications as detailed in the Supplemental Material. The morphometric quantitation of TH-positive nigral neurons was validated by stereological assessment on a separate set of tissues (see Supplementary Material and Supplementary Fig. 2). For statistical evaluation, all variables were first tested for normality (Shapiro-Wilk, D'Agostino-Pearson, and Kolmogorov-Smirnov). Since for each type of measurement, the normality assumption was rejected at least once, the nonparametric Kruskal-Wallis test was used for multiple comparisons, followed by the Mann-Whitney test for pairwise group comparisons. Control groups were either wild-type mice before surgery or the wild-type mice after injection with vehicle. The *p*-values below 5% were considered significant.

3. Results

We observed no gross neurological, developmental, or organ deficiencies in heterozygous (*Rgs4*$^{+/-}$) or homozygous (*Rgs4*$^{-/-}$) mice compared to their wild-type littermate controls (*Rgs4*$^{+/+}$). The segregation of genotypes in the study cohorts was Mendelian. Weights of mice in all 3 genotypes were similar (Supplementary Fig. 1A and B). In 3 different behavioral motor assessments (pole, grip strength, and cylinder tests), *Rgs4*$^{-/-}$ and *Rgs4*$^{+/-}$ mice showed no significantly different performance in comparison to *Rgs4*$^{+/+}$ controls (Supplementary. Fig. 1A–F). The integrity of their nigrostriatal pathway, measured by TH immunostaining, was also similar to that of their *Rgs4*$^{+/+}$ littermates (Supplementary Fig. 1G). This allowed us to use these mice to study the effects of RGS4 deficiency on experimental PD-related disease outcomes. After determining

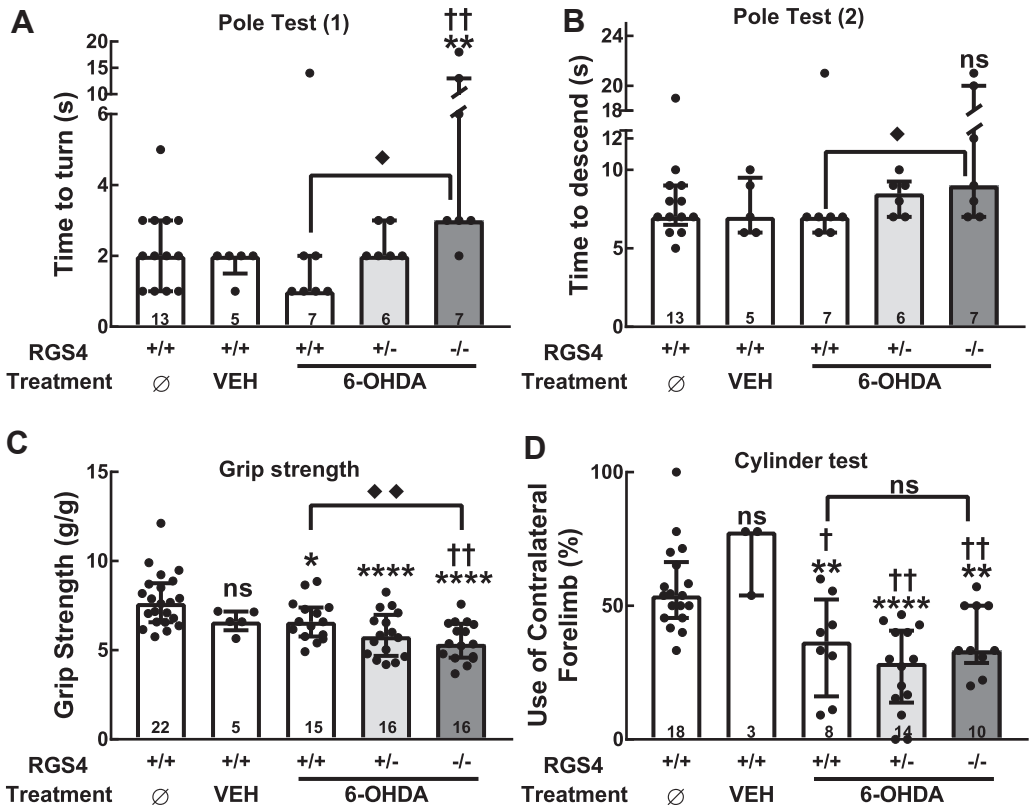

Fig. 1. RGS4 deficiency in mice does not protect against motor impairment induced by intrastriatally administered 6-OHDA. (A) Pole test (1): time to turn on pole top; (B) pole test (2): time to descend from pole; (C) grip strength test; and (D) cylinder test. The bars represent the group medians +/− the interquartile ranges (data were not normally distributed). Individual data points are shown as dots. The numbers at the bottom of the bars represent the numbers of mice in each group. Ø represents all the wild-type mice (*Rgs4*$^{+/+}$) at baseline (before or without lesioning), VEH indicates vehicle injected *Rgs4*$^{+/+}$ mice. Then, 2 μg of 6-OHDA was administered to mice of all 3 genotypes. Two mice in the VEH wild-type group did not perform the cylinder test and had to be excluded, hence the small group size. See main text and Supplementary Material for details. *, **, and **** represent $p <$ 0.05, <0.01, and <0.0001 compared to baseline *Rgs4*$^{+/+}$ (Ø); † and †† represent $p < 0.05$ and <0.01 compared to VEH-treated *Rgs4*$^{+/+}$; ◆ and ◆◆, represent $p < 0.02$ and <0.01 6-OHDA–treated *Rgs4*$^{+/+}$ versus *Rgs4*$^{-/-}$, ns = not significant; all comparisons by Mann-Whitney test.

that a dose of 2 μg 6-OHDA induced limited motor deficits (significant in 1 of 4 tests, the cylinder test) and unilateral TH neuron loss of approximately 50% in wild-type mice (Figs. 1 and 2), we observed that neither *Rgs4*$^{+/-}$ nor *Rgs4*$^{-/-}$ mice were protected against 6-OHDA–induced motor deficits (Fig. 1). Remarkably, we noticed that lesioned *Rgs4*$^{-/-}$ but not *Rgs4*$^{+/-}$ or *Rgs4*$^{+/+}$ mice were significantly impaired in a measure of motor coordination (time to turn at top of the pole, Fig. 1A), while also showing a tendency toward impairment in another such measure (time to climb down the pole, Fig. 1B). Lesioned *Rsg4*$^{-/-}$ mice also showed a worse outcome in muscle coordination (grip strength, Fig. 1C). Lesioned mice of all 3 genotypes were similarly impaired in the cylinder test. (Fig. 1D). We also found that 6-OHDA administration induced a similar loss of striatal positive TH fibers (Fig. 2A and B) and of nigral TH-positive neurons (Fig. 2A and C) in mice of all genotypes in their ipsilateral brain sides. Our results indicate that loss of one or both *Rgs4* alleles in mice does not protect against PD toxin–induced dysfunction and injury.

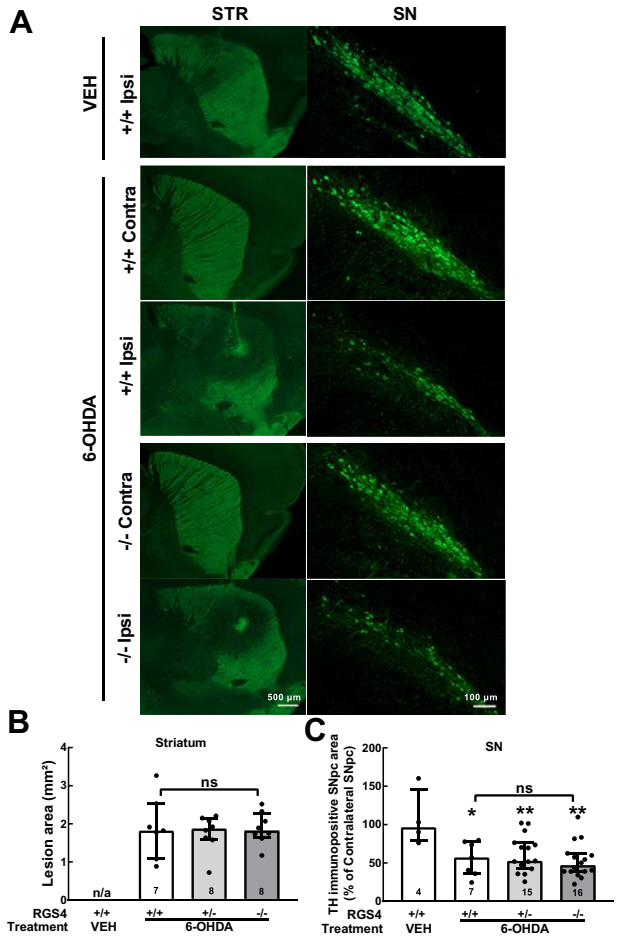

Fig. 2. RGS4-deficient mice are not protected against 6-OHDA–induced injury to the nigrostriatal pathway. The upper 5 rows (A) show microphotographs of TH-immunostained sections at the level of the injection into the striatum (STR) and substantia nigra (SN) for VEH-injected *Rgs4*$^{+/+}$ mice (upper row) and for 6-OHDA–injected *Rgs4*$^{+/+}$ and *Rgs4*$^{-/-}$ mice (ipsi = ipsilateral [injected] side; contra = contralateral [uninjected] side). All microphotographs were pseudocolored in green for illustration. Scale bars in (A): 500 μm (STR, first column), 100 μm (SN, second column). The bottom row shows the quantification of the striatal lesion area (B) and the number of nigral TH neurons for mice of all 3 genotypes (C). For the panels in (B) and (C), the bars represent the group medians +/− the interquartile ranges (data was not normally distributed). The numbers at the bottom of the bars represent the numbers of mice in each group. Individual data points are represented by dots. See main text and Supplementary Material for details. * and ** represent $p < 0.05$ and 0.01 compared to VEH-treated *Rgs4*$^{+/+}$ wild-type; ns = not significant, all comparisons by Mann-Whitney test. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

RGS4 is considered a potential nondopaminergic drug target for PD. *Rgs4*$^{-/-}$ mice were reported to be functionally less impaired than wild-type controls after 6-OHDA administration (Lerner and Kreitzer, 2012), *Rgs4* expression inhibition was shown to reduce L-Dopa–induced dyskinesias in rats (Ko et al., 2014), and inhibition of RGS4 activity was shown to counteract parkinsonism-related bradykinesia induced by the toxin raclopride in mice (Blazer et al., 2015). Here however, using a battery of behavioral and histological measurements on the 6-OHDA mouse model of parkinsonism, we fail to observe a protective effect of lowered or absent *Rgs4* expression. Moreover, we observe that 6-OHDA induced impairments in some motor tests in the complete absence of *Rgs4* expression but not in its presence. Our findings show that reducing RGS4 is not protective in a toxin model of PD, and even suggest that extended treatment with an RGS4 inhibitor may lead to adverse effects, in particular in the presence of an already existing pathology. These observations are important because, in drug discovery research, multiple assessments are required to enable a robust target validation. We conclude that although it is still possible that RGS4 inhibition may be useful for the management of PD-related brady- or dyskinesias (Blazer et al., 2015; Ko et al., 2014), or for other neurological disease indications (Gu et al., 2007), for actual neuroprotection in PD, it should be approached with caution.

Disclosure statement

The authors declare no actual or potential conflicts of interest.

Acknowledgements

The authors thank Kirsten Marlen Kleemann for help with breeding up mouse cohorts. This work was supported by the Fonds Nationale de la Recherche, Luxembourg (grant C13/BM/5782168 and through the National Centre of Excellence in Research (NCER) on Parkinson's disease, I1R-BIC-PFN-15NCER). KS was supported through intramural grants from the Helmholtz-Association (Program Infection and Immunity) and through a contractual agreement ("Knowledge Transfer") with the Luxembourg Centre for Systems Biomedicine. EG received support by the Fonds Nationale de la Recherche (FNR), Luxembourg, as part of the project MitoPD, under the auspices of the bilateral e:Med program by the German Federal Ministry of Education and Research and the FNR (INTER/BMBF/13/04).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neurobiolaging.2017.06.008.

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