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Research report

Interaction between subclinical doses of the Parkinson's disease associated gene, α-*synuclein*, and the pesticide, rotenone, precipitates motor dysfunction and nigrostriatal neurodegeneration in rats

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HIGHLIGHTS

- Various risk factors may interact to precipitate presymptomatic Parkinson's disease.
- We investigated the interaction between α-synuclein and the pesticide, rotenone.
- Dual exposure to α-synuclein and rotenone precipitated Parkinsonism in rats.
- Rats exposed to both factors had profound motor dysfunction and neurodegeneration.
- This highlights the importance of these interactions in Parkinson's disease.

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ABSTRACT

In most patients, Parkinson's disease is thought to emerge after a lifetime of exposure to, and interaction between, various genetic and environmental risk factors. One of the key genetic factors linked to this condition is α-*synuclein*, and the α-synuclein protein is pathologically associated with idiopathic cases. However, α-synuclein pathology is also present in presymptomatic, clinically "normal" individuals suggesting that environmental factors, such as Parkinson's disease-linked agricultural pesticides, may be required to precipitate Parkinson's disease in these individuals. In this context, the aim of this study was to assess the behavioural and neuropathological impact of exposing rats with a subclinical load of α-synuclein to subclinical doses of the organic pesticide, rotenone. Rats were randomly assigned to two groups for intra-nigral infusion of $AAV_{2/5}$-GFP or $AAV_{2/5}$-α-synuclein. Post viral motor function was assessed at 8, 10 and 12 weeks in the Corridor, Stepping and Whisker tests of lateralised motor function. At week 12, animals were performance-matched to receive a subsequent intra-striatal challenge of the organic pesticide rotenone (or its vehicle) to yield four final groups (Control, Rotenone, $AAV_{2/5}$-α-synuclein and Combined). Behavioural testing resumed one week after rotenone surgery and continued for 5 weeks. We found that, when administered alone, neither intra-nigral AAV-α-synuclein nor intra-striatal rotenone caused sufficient nigrostriatal neurodegeneration to induce a significant motor impairment in their own right. However, when these were administered sequentially to the same rats, the interaction between the two Parkinsonian challenges significantly exacerbated nigrostriatal neurodegeneration which precipitated a pronounced impairment in motor function. These results indicate that exposing rats with a subclinical α-synuclein-induced pathology to the pesticide, rotenone, profoundly exacerbates their Parkinsonian neuropathology and dysfunction, and highlights the potential importance of this interaction in the etiology of, and in driving the pathogenesis of Parkinson's disease.

1. Introduction

Despite decades of research, the etiology of idiopathic Parkinson's disease is still poorly understood [30]. The most widely accepted hypothesis is that the disease arises as a result of complex

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interactions between a person's genetics and their environmental exposures [1,9,27]. Although this gene-environment hypothesis of idiopathic Parkinson's disease is widely accepted, the disease is still most commonly modelled in experimental animals using single neurotoxic insults, such as MPTP or 6-hydroxydopamine, which are not etiologically relevant to human Parkinson's disease reviewed in [31]. Moreover, these catecholaminergic neurotoxic models are also limited in that they do not replicate some of the characteristic neuropathological features of the disease such as a slowly emerging motor dysfunction underpinned by progressive nigrostriatal neurodegeneration and α-synuclein pathology. Indeed, this lack of etiological and neuropathological relevance has been suggested by some as one of the factors underlying the lack of clinical translation of preclinically-effective experimental anti-Parkinsonian therapies [2,13,18].

The limitation of existing models has led to a considerable drive to develop gene-environment interaction models of the disease with improved etiological and neuropathological relevance. These gene-environment models have the potential 1) to elucidate relevant gene-environment interactions in Parkinson's disease, 2) to determine the pathological consequences of gene-environment interactions in Parkinson's disease, 3) to determine the mechanisms underlying gene-environment interactions in Parkinson's disease, and 4) to provide relevant models for testing of novel therapies for the condition [12]. In this regard, we have recently embarked on a series of studies to develop animal models of Parkinson's disease that are triggered by genetic and/or environmental factors relevant to the human condition [20,22,21,11,24]. For the gene-environment interaction studies, we selected risk factors that are known to be associated with the human condition, namely, the α-*synuclein* gene and the organic pesticide, rotenone [21,20].

Interestingly, α-synuclein pathology, and even Lewy body formation, is not in itself sufficient to induce an overtly manifest clinical syndrome with both having being identified in the normal aged human brain [19,17]. However, in the [17] study, the "normal" patients with α-synuclein pathology were suggested to represent a population with preclinical or presymptomatic Parkinsonism. Given that Parkinson's disease is thought to emerge after interaction between genetic and environmental risk factors, it follows that sufficient exposure of such presymptomatic individuals to environmental risk factors (or conversely, insufficient exposure to environmental protective factors) could result in clinical manifested Parkinson's disease.

Thus, to test this hypothesis, in the present study, we sought to determine if subclinical α-synuclein pathology could be precipitated into overt motor dysfunction by subclinical rotenone exposure. Not only would this shed further light on subclinical α-synuclein-rotenone interactions in the context of the etiology of Parkinson's disease, but would also provide a novel gene-environment model for testing potential neuroprotective and disease-modifying therapies for this condition.

2. Materials & methods

2.1. Animals

All procedures were approved by the Animal Care and Research Ethics Committee of the National University of Ireland, Galway, were completed under licence by the Irish Department of Health and Children and the Irish Health Products Regulatory Authority, and were carried out in accordance with European Union Directive 2010/63/EU and S.I. No. 543 of 2012. Animals were housed in groups of four per cage, on a 12:12 h light/dark cycle, at 19–23 °C, and at humidity levels maintained between 40 and 70%. Throughout the experiment, rats were allowed water *ad libitum,* and were fed 15–20 g of standard rat chow each per day to maintain their body weight at ~90% of free feeding weight (to ensure they were motivated to perform the food-motivated Corridor Test). All behavioural testing and quantitative immunohistochemistry were completed blind to the treatment of the rats.

2.2. Experimental design

Thirty nine male Sprague Dawley rats were used in this experiment (weighing 225–250 g at the start of the study). Rats were randomly assigned to two groups for intranigral infusion of $AAV_{2/5}$-GFP ($n = 19$) or $AAV_{2/5}$-α-synuclein ($n = 20$). Post viral motor function was assessed at 7, 8, 10 and 12 weeks in the Corridor, Stepping and Whisker tests. Twelve weeks post viral vector administration, animals were performance-matched (matching can been seen in Figs. 1 and 2) to receive a subsequent intrastriatal challenge of rotenone or its corresponding vehicle to yield four final groups (Table 1). Post rotenone behavioural testing resumed one week later and continued for another four week period during which the animals also underwent amphetamine-induced rotational testing. The animals were then sacrificed by transcardial perfusion-fixation and their brains were processed for *post mortem* quantitative assessment of nigrostriatal neurodegeneration (via tyrosine hydroxylase immunohistochemistry) and α-synuclein overexpression (via α-synuclein immunohistochemistry). An overview of the experimental timeline is shown in Fig. 1A.

2.3. AAV preparation

The gene for GFP was cloned into pTRUF plasmid flanked by AAV_2 inverted terminal repeats and the gene for normal human α-synuclein was cloned into pSnaSw plasmid flanked by AAV_2 inverted terminal repeats. These plasmids and the AAV helper plasmid, pDG-5 were purified for subsequent transfection. The $AAV_{2/5}$-GFP and $AAV_{2/5}$-α-synuclein viral vectors were produced by co-transfecting HEK-293T cells with the relevant AAV2 plasmid and pDG-5, as described previously [10] for 48 h, by JetPEI (Polyplus Transfection Ltd). Viral vectors were then purified by the treatment of the transfected cell pellet with a DNA endonuclease to degrade any non-encapsulated DNA. For purification, the virus was concentrated using iodixanol gradient ultracentrifugation. Further concentration of the virus via centrifugal filter units resulted in a final volume of 400–600 μl. Viral titres were established using real time PCR, expressed as vector genomes (vg)/μl. Viruses were aliquoted in 20 μl aliquots and stored at −80 °C. The final viral titres were 1×10^{10} vg/μl for $AAV_{2/5}$-α-synuclein and 8×10^{7} vg/μl for $AAV_{2/5}$-GFP (note that vg is equivalent to DNase-resistant particles (drp), and such relatively small differences in viral titres do not affect experimental outcome due to saturation of viral binding sites).

Table 1
Groups used in this study. At week 0, rats were randomly divided into two groups to receive either $AAV_{2/5}$-GFP or $AAV_{2/5}$-α-synuclein. Twelve weeks later, they were performance matched to receive either rotenone (1.8 μg in 3 μl at 2 points across the rostro-caudal axis of the striatum) or its corresponding vehicle to yield 4 final groups as shown.

Group	Virus injection	Rotenone injection	*n*
Control	$AAV_{2/5}$-GFP	Vehicle	10
Rotenone	$AAV_{2/5}$-GFP	Rotenone	9
α-Synuclein	$AAV_{2/5}$-α-synuclein	Vehicle	10
Combined	$AAV_{2/5}$-α-synuclein	Rotenone	10