Genetic activation of Nrf2 signaling is sufficient to ameliorate neurodegenerative phenotypes in a Drosophila model of Parkinson’s disease

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SUMMARY
Parkinson’s disease (PD) is the most common neurodegenerative movement disorder. Oxidative stress has been associated with the etiology of both sporadic and monogenic forms of PD. The transcription factor Nrf2, a conserved global regulator of cellular antioxidant responses, has been implicated in neuroprotection against PD pathology. However, direct evidence that upregulation of the Nrf2 pathway is sufficient to confer neuroprotection in genetic models of PD is lacking. Expression of the PD-linked gene encoding α-synuclein in dopaminergic neurons of Drosophila results in decreased locomotor activity and selective neuron loss in a progressive age-dependent manner, providing a genetically accessible model of PD. Here we show that upregulation of the Nrf2 pathway by overexpressing Nrf2 or its DNA-binding dimerization partner, Maf-S, restores the locomotor activity of α-synuclein-expressing flies. Similar benefits are observed upon RNA-interference-mediated downregulation of the prime Nrf2 inhibitor, Keap1, as well as in conditions of keap1 heterozygosity. Consistently, the α-synuclein-induced dopaminergic neuron loss is suppressed by Maf-S overexpression or keap1 heterozygosity. Our data validate the sustained upregulation of the Nrf2 pathway as a neuroprotective strategy against PD. This model provides a genetically accessible in vivo system in which to evaluate the potential of additional Nrf2 pathway components and regulators as therapeutic targets.

INTRODUCTION
Parkinson’s disease (PD) is the second most frequent neurodegenerative disorder. Among other clinical features, PD is characterized by motor deficits, progressive and irreversible loss of dopaminergic (DA) neurons in the substantia nigra pars compacta, and formation of intracellular proteinaceous aggregates referred to as Lewy bodies. The current treatments for PD are symptomatic and no preventive interventions are yet available, in part owing to an incomplete understanding of disease etiology. It is clear, however, that age, environmental stressors and genetic predisposition can contribute to the clinical manifestation of PD (Hindle, 2010; Horowitz and Greenamyre, 2010; Jomova et al., 2010).

Accumulating evidence suggests that the ability to elicit an adaptive response to oxidative stress is a key survival mechanism for neurons affected by PD. Specifically, pharmacological treatments and genetic manipulations that increase the cellular antioxidant defenses can confer neuroprotection in a number of different PD models; examples include the oral administration of the antioxidant N-acetyl-cysteine and the overexpression of several detoxifying enzymes (Asanuma et al., 1998; Callio et al., 2005; Thiruchelvam et al., 2005; Whitworth et al., 2005; Barkats et al., 2006; Ridet et al., 2006; Botella et al., 2008; Trinh et al., 2008; Oien et al., 2009; Clark et al., 2010; Zhang et al., 2010).

The Nrf2 signaling pathway plays a central role in the regulation of the cellular antioxidant response (Motohashi et al., 2002; Johnson et al., 2008; Clark and Simon, 2009; Jung and Kwak, 2010; Sykiotis and Bohmann, 2010). Nrf2 (NFE2-related factor 2) belongs to the conserved Cnc (Cap’n’collar) family of leucine zipper transcription factors and regulates the expression of genes encoding antioxidant and detoxifying proteins such as thioredoxins, glutathione synthetase, glutathione S-transferases and NAD(P)H:quinone oxidoreductase. Under basal conditions, Nrf2 is sequestered in the cytoplasm via its interaction with the repressor Keap1 (Kelch-like ECH-associating protein 1), which facilitates its polyubiquitylation and proteasome-mediated degradation (Fig. 1A). Exposure to oxidants, electrophiles or xenobiotics disrupts the Keap1–Nrf2 interaction, thereby stabilizing Nrf2 and allowing it to accumulate in the nucleus. Nrf2 activates the transcription of its target genes via antioxidant response elements (AREs) in their promoter regions, to which it binds as a heterodimer with a member of the small Maf (musculoaponeurotic fibrosarcoma oncogene) protein family.

Previous studies suggested that increased activity of the Nrf2 pathway might be protective in PD. Notably, a haplotype of the human Nrf2 gene that includes a promoter polymorphism known to confer higher transcriptional activity (Marzec et al., 2007) was recently associated with decreased risk for or later disease onset of PD (von Otter et al., 2010). In mice, astrocyte-specific overexpression of Nrf2 protects DA neurons from toxins such as MPTP or 6-hydroxydopamine that can elicit Parkinson’s-like neurodegenerative symptoms (Jakel et al., 2007; Chen et al., 2009). In several models of PD, the pharmacological activation of Nrf2...
has been proposed to underlie the neuroprotection conveyed by a variety of treatments (Burton et al., 2006; Hara et al., 2006; Wruck et al., 2007; Yamamoto et al., 2007; Hwang and Jeong, 2008; Trinh et al., 2008; Minelli et al., 2009; Siebert et al., 2009; Yang et al., 2009; Niso-Santano et al., 2010; Trinh et al., 2010). Yet, there is no direct evidence that activation of Nrf2 in genetic models of PD and specifically in DA neurons is sufficient to protect against PD pathology.

Here we address this question using a genetic model of Parkinson’s disease that relies on the transgenic expression of the PD-associated gene encoding α-synuclein in the fruit fly Drosophila melanogaster. α-synuclein is the major structural component of the Lewy body in both sporadic and familial PD (Spillantini et al., 1997). The physiological role of α-synuclein is still under study; however, it is clear that a major cause of α-synuclein toxicity is its propensity to misfold, forming toxic aggregates (Auluck et al., 2010). This property is further exacerbated by high expression levels of the wild-type protein, mutations and oxidative- and/or nitrosative-stress-induced post-translational modifications (Ischiropoulos, 2003; Auluck et al., 2010). Experimental models of α-synuclein toxicity have been developed in yeast, worms, fruit flies and mice (Dawson et al., 2010; Franssens et al., 2010). The gene encoding α-synuclein (SNCA) is not conserved in Drosophila; however, expression of human α-synuclein in flies can lead to the formation of intracellular aggregates and cell death (Feany and Bender, 2000). Importantly, the overexpression paradigm developed in Drosophila is the only one that exhibits all three hallmarks of PD, i.e. progressive loss of DA neurons, L-DOPA-responsive motor deficits and Lewy-body-like inclusions. It has therefore been suggested to be particularly suitable for testing disease-modifying therapies (Dawson et al., 2010). The Drosophila PD model employed here utilizes the promoter of the gene encoding tyrosine hydroxylase (TH), the rate-limiting enzyme in the synthesis of dopamine, to express α-synuclein specifically in DA neurons (Trinh et al., 2008). Previous studies have shown that, in this PD model, a protective effect is obtained by overexpression of fly genes that are homologous to target genes of mouse Nrf2 that are involved in the regulation of glutathione conjugation and metabolism (Trinh et al., 2008). In addition, it has been shown that the Nrf2-activating compounds DL-sulforaphane and allyl sulfide can protect DA neurons from α-synuclein toxicity. Similarly, the protective effects of decaffeinated coffee and nicotine-free tobacco are suppressed in the presence of a double-stranded (ds)RNA that targets all transcripts encoded by the Drosophila cnc locus (Trinh et al., 2010). This finding correlates with the observation that cafeisol, a sterol that is present in coffee, can activate a gstD1-GFP reporter of Nrf2 activity (Trinh et al., 2010). Although this set of observations supports a requirement of Nrf2 for compound-induced neuroprotective mechanisms, it remains unknown whether genetic upregulation of Nrf2 signaling is sufficient to counteract α-synuclein toxicity. Recent work has shown that Drosophila possesses a conserved Nrf2 pathway, consisting of the transcription factor CncC (the protein encoded by the largest splice form of the cnc gene) and by the repressor Keap1. Similar to its mammalian counterpart, CncC can orchestrate the cellular defenses against oxidative stress (Sykiotis and Bohmann, 2008). Moreover, Drosophila has a single member of the small Maf gene family, maf-S (CG9954), whose protein product can form DNA-binding heterodimers with the CncB isoform and is required for its biological function during fly development (Veraska et al., 2000). We have observed that overexpression of Maf-S can also stimulate CncC target gene expression and promote acute stress resistance in adult flies (Mohammed Mahidur Rahman and D.B., unpublished). Thus, the core of the Nrf2 pathway is fully conserved and composed of non-redundant gene products in Drosophila. By genetically manipulating the individual pathway components, we demonstrate that activation of Nrf2 signaling is sufficient to counteract α-synuclein toxicity in vivo.

**RESULTS**

**Upregulation of the Nrf2 pathway rescues α-synuclein-induced locomotor deficits**

Transgenic expression of α-synuclein under the control of the pan-neuronal promoter elav or the DA-specific promoter of the TH gene causes age-dependent defects in the negative geotaxis response of fruit flies (i.e. their innate tendency to walk upwards after being tapped to the bottom of their containing vial), providing a tool to study the effect of α-synuclein neurotoxicity on locomotor behavior (Feany and Bender, 2000; Du et al., 2010).

We used this assay to test the effect of genetic Nrf2 activation in the α-synuclein fly PD model. One of the key steps in the activation of the Nrf2 response upon oxidative insult is its escape from proteolysis and the resulting accumulation of Maf–Nrf2 heterodimers in the nucleus. We thus reasoned that increasing the levels of the Drosophila Nrf2 homolog CncC in DA neurons might be sufficient to elicit an antioxidant response and protect from the behavioral consequences of α-synuclein toxicity. To avoid any developmental effects of CncC overexpression that might mask its putative protective role later in the fly life cycle, experimental animals were reared at 18°C, conditions in which the UAS-Gal4 transgenic system is only weakly active (Duffy, 2002). At 2-5 days after eclosion, flies were transferred to the permissive temperature of 25°C and tested 1, 2, 4 and 5 weeks later. Whereas CncC overexpression alone had no detrimental effect on the age-associated decline in negative geotaxis, it could prevent the deficit in locomotor performance induced by α-synuclein overexpression (Fig. 1B). Thus, transgenically activated Nrf2 function in the TH-positive neurons is sufficient to counteract or delay the loss of motor performance in this Drosophila model of α-synuclein toxicity.

Next, we asked whether a similar effect might be achieved by genetically increasing the activity of endogenous Nrf2. To this end, we decreased the levels of Keap1, which in basal conditions limits Nrf2 activity by targeting the protein for proteasomal degradation (Sykiotis and Bohmann, 2010). Keap1 loss-of-function conditions were generated using two different approaches. First, we examined the phenotype of TH-Gal4-driven α-synuclein expression in a keap1 heterozygous background. keap1P551A flies have been shown to exhibit increased resistance to acute oxidative stress, as well as increased life span (Sykiotis and Bohmann, 2008), in line with the notion that moderate activation of the endogenous Nrf2-mediated antioxidant response can be beneficial. Second, we downregulated keap1 specifically in DA neurons by expressing a dsRNA targeting this gene under the control of the TH promoter (keap1PNA). Unlike flies expressing α-synuclein in an otherwise wild-type background, 5-week-old flies expressing α-synuclein in a keap1P551A background or together with keap1PNA displayed locomotor activity similar to
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that of wild-type flies. These genetic manipulations neither affected
the negative geotaxis response in younger (1-week old) flies nor
delayed the normal aging-associated decline of mobility observed
in control flies not expressing α-synuclein (Fig. 1C,D). These results
suggest that mobilizing the endogenous Nrf2 response can
specifically defend DA neurons against α-synuclein toxicity,
supporting the notion that Nrf2 activation is sufficient for
protection against PD-related pathologies.

Finally, we investigated whether ectopic expression of Maf-S, the
obligatory DNA-binding partner of Nrf2 (Blank, 2008), could also
restore normal locomotor activity in α-synuclein-expressing flies.
In mammals, small Maf proteins have been implicated in the
regulation of biological functions such as stress response, CNS
function and inflammation (Blank, 2008), all of which are relevant
to PD (McGeer et al., 1988; Dexter et al., 1989; Yoritaka et al., 1996;
Jenner, 1998). Consistent with the effects observed in keap1
flies and keap1RNAi flies, the ectopic expression of Maf-S conferred
complete protection from α-synuclein toxicity in old flies (Fig. 1E).

Partial loss of Keap1 function and ectopic expression of Maf-S
rescue α-synuclein-induced loss of DA neurons

In addition to the motor defects, the α-synuclein fly PD model
recaptulates the selective and age-dependent loss of DA neurons.
Specifically, Trinh et al. observed a small but significant loss of TH-
positive neurons from the posterior protocerebral lateral cluster 1
(PPL1) (Trinh et al., 2008). We investigated whether this α-
synuclein-induced decrease in neuronal number could be reversed
by genetically activating the endogenous Nrf2 antioxidant response.
Brains from 2-day- and 4-week-old flies expressing α-synuclein
under the control of the TH promoter in a keap1+/− or keap1
background were dissected and immunostained for TH to visualize
DA neurons, and the number of neurons in the PPL1 cluster was
assessed as described in the Methods. In the brains of the younger
flies, the number of PPL1 neurons was not affected by the genotype
(supplementary material Fig. S1A,B). In brains from aged α-
synuclein-expressing flies, we detected an average loss of 1.5-2
neurons per PPL1 cluster relative to age-matched controls,
consistent with previous reports (Trinh et al., 2008; Trinh et al.,
2010). In partial loss-of-function conditions for keap1, the neuronal
number was restored to wild-type levels (Fig. 2A,B). This protective
effect of keap1 heterozygosity in the preservation of PPL1 neurons
is in agreement with the results of the behavioral assays (Fig. 1).

To investigate the effect of Nrf2 activation specifically in DA
neurons, we co-expressed α-synuclein together with Maf-S and
examined the number of PPL1 neurons in brains from 2-day- and 4-week-old flies (Fig. 2A,C; supplementary material Fig. S1A,C). The neuronal overexpression of Maf-S did not affect the number of PPL1 neurons in either the young or the old flies, but was sufficient to rescue α-synuclein-induced neurotoxicity in old flies. To exclude the possibility that this observation might be due to a direct effect of Maf-S-dependent Nrf2 activation on the TH-Gal4 driver used to control transgenic expression in the model, we examined the effect of ectopic Maf-S on a GFP transgene expressed under the control of the TH promoter. Indeed, under the experimental conditions employed, the expression of GFP was not affected by the presence of transgenically expressed Maf-S (Fig. 3).

DISCUSSION
In this study we investigated whether genetic manipulations aimed at increasing Nrf2 activity are sufficient to protect the organism from α-synuclein toxicity. To this end we exploited the advantages of Drosophila as both a genetic model of PD and an experimental paradigm for studying Nrf2 signaling in vivo. We found that genetic upregulation of the Nrf2 pathway by manipulating its individual core components is sufficient to counteract the deleterious effects of α-synuclein accumulation on two distinct phenotypes: the number of PPL1 DA neurons and the locomotor activity of the fly.

Our genetic data are a necessary and logical complement to previous studies in this model that had shown that: (1) drugs that can activate Nrf2, such as DL-sulforaphane and allyl sulfide, are protective (Trinh et al., 2008); (2) Cnc is required for the neuroprotective effects of coffee and tobacco, which are associated with decreased PD risk (Trinh et al., 2010); and (3) upregulation of a subset of putative Nrf2 target genes that regulate glutathione metabolism can protect DA neurons in this model of PD (Trinh et al., 2008). Our study differs from these prior investigations in at least three major ways. First, our genetic data demonstrate that upregulation of the Nrf2 pathway is sufficient – as opposed to merely required – to counteract α-synuclein toxicity in vivo. Demonstration of sufficiency is essential for the validation of the Nrf2 pathway as a therapeutic target in PD. Second, the beneficial effects of Nrf2 activation are apparent not only on the maintenance of PPL1 neuronal numbers but also on the maintenance of normal locomotor activity (negative geotaxis) response. This provides an

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**Fig. 2. Partial Keap1 loss of function or overexpression of small Maf rescues α-synuclein-induced loss of DA neurons.** (A-C) The brains of 4-week-old flies in which the indicated transgenes were expressed under the control of the TH-Gal4 driver were dissected and immunostained with an anti-TH antibody. (A) Representative micrographs (z-stack projections). Scale bar: 10 μm. (B,C) Quantitation. Data are mean ± s.e.m.; n represents the number of independent samples; significance was assessed with the Student’s t-test (*P<0.05). Genotypes: see Fig. 1 legend.
It has been previously reported that overexpression of Nrf2 in astrocytes can protect DA neurons of the mouse brain substantia nigra from MPTP toxicity (Jakel et al., 2007; Chen et al., 2009). Given the profound differences between genetic and intoxication models of PD (Dawson et al., 2010), the finding that Nrf2 activation is sufficient to achieve neuroprotection independent of the cause of degeneration indicates that this could be a promising, wide-spectrum and disease-modifying strategy. An important difference between our Drosophila study and the mouse (MPTP) studies is the activation of Nrf2 in different cell types (neurons versus astrocytes). Our observation is that activation of Nrf2 in DA neurons is sufficient to confer protection from α-synuclein toxicity, even though effects due to neuron-glial communication are also compatible with our results. Interestingly, Nrf2 can activate different sets of genes in neurons versus glia (Kraft et al., 2004). Future studies should characterize the downstream target genes of Nrf2 that are required for protective effects in neurons and glia cells, as well as the specific cell types involved in the neuroprotection mechanism.

The Nrf2 pathway has recently become a major focus in the quest for new therapeutic targets in PD (van Muiswinkel and Kuiperij, 2005; de Vries et al., 2008; Clark and Simon, 2009; Cuadrado et al., 2009). The discovery of a PD protective haplotype in the human Nrf2 gene (von Otter et al., 2010), which includes a polymorphism previously associated with higher transcriptional activity of the Nrf2 promoter (Marzec et al., 2007), suggests that upregulating the Nrf2-dependent program is a plausible strategy against PD in humans. Previous studies in Drosophila have shown that this model organism can be used not only to test pharmacological treatments but also to identify new therapeutic targets for PD (Auluck and Bonini, 2002; Auluck et al., 2005; Outeiro et al., 2007; Trinh et al., 2008; Shaltiel-Karyo et al., 2010; Trinh et al., 2010). To this end, our finding that neuroprotection in the α-synuclein PD model can be achieved by genetically targeting the individual core components of the Nrf2 pathway provides a useful conceptual framework for future genetic studies: the assessment of negative geotaxis could serve as a screening assay to identify new genetic modifiers of α-synuclein toxicity in vivo, with the PPL1 neuron count serving as a downstream validation assay. Further studies into the mechanisms by which Nrf2 counteracts α-synuclein toxicity are also warranted.

**METHODS**

**Fly stocks**

The upstream activating sequence (UAS)-α-synuclein, tyrosine hydroxylase (TH)-galactosidase 4 (Gal4), UAS-Keap1RNAi/CyO, UAS-CncC/(III) and keap1^{E537}(III) fly stocks have been previously described (Friggi-Grelin et al., 2003; Sykiotis and Bohmann, 2008; Trinh et al., 2008). The UAS-Maf-S fly stock was generated by amplifying the coding sequence of the Maf-S gene by PCR using embryonic cDNA as template and cloning it into the XbaI site of the previously described pUAST-3×HA vector (Sykiotis and Bohmann, 2008) in frame with the C-terminal 3×HA tag. Unless otherwise indicated, flies were reared on standard cornmeal-molasses food at 25°C and transferred onto new vials every 2–3 days. Male flies were used in all experiments.

**Behavioral assays**

Behavioral assays were performed as previously described with minor modifications (Feany and Bender, 2000; Friggi-Grelin et al., 2003).
The locomotor activity was expressed as the percentage of flies that had passed the 2-cm mark after 10 seconds. All behavioral assays were performed at the same time of day to prevent uneven contribution of the circadian cycle to the activity level of the flies.

**Brain dissection, immunofluorescence and neuronal counts**

Dissection of adult fly brains and immunofluorescence of DA neurons were performed according to published protocols (Wu and Luo, 2006; Trinh et al., 2008). Fixed brains were blocked in 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.1% Triton X-100 and 0.5% BSA for 1 hour at room temperature, and incubated overnight with a rabbit anti-TH antibody (1:100; Millipore AB152) and a rat anti-HA antibody (1:100; 3F10 Roche). Primary antibodies were detected with a donkey TRITC-labeled anti-rabbit or a donkey Cy5-labeled anti-rat antibody (1:200; Jackson ImmunoResearch). Whole brains were mounted between two glass coverslips to allow visualization from both sides of the slide. Brain confocal z-series images were acquired with a Leica SP2 confocal microscope equipped with a 40× oil objective (N/A 1.25). The neuronal PPL1 cluster was identified based on position and main neuropil projection (Mao and Davis, 2009). Neuronal counts were performed in a blind manner by inspecting the individual planes of the z-stack.

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**COMPETING INTERESTS**

The authors declare that they do not have any competing or financial interests.

**AUTHOR CONTRIBUTIONS**

M.C.B. designed and performed the experiments and co-wrote the manuscript. G.P.S. and D.B. designed the experiments and co-wrote the manuscript.

**SUPPLEMENTARY MATERIAL**

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