Human Molecular Genetics, 2012, Vol. 21, No. 3 **485–494**
doi:10.1093/hmg/ddr477
Advance Access published on October 18, 2011

α-Synuclein levels modulate Huntington's disease in mice

Silvia Corrochano[1], Maurizio Renna[2], Sarah Carter[1], Nichola Chrobot[3], Rose Kent[3], Michelle Stewart[3], Jason Cooper[2], Steve D.M. Brown[1], David C. Rubinsztein[2] and Abraham Acevedo-Arozena[1,*]

[1]MRC Mammalian Genetics Unit, Harwell, Oxfordshire OX11 0RD, UK, [2]Department of Medical Genetics, Cambridge Institute for Medical Research, University of Cambridge, CB2 0XY, UK and [3]MRC Mary Lyon Centre, Harwell, Oxfordshire OX11 0RD, UK

Received August 18, 2011; Revised and Accepted October 12, 2011

α-Synuclein and mutant huntingtin are the major constituents of the intracellular aggregates that characterize the pathology of Parkinson's disease (PD) and Huntington's disease (HD), respectively. α-Synuclein is likely to be a major contributor to PD, since overexpression of this protein resulting from genetic triplication is sufficient to cause human forms of PD. We have previously demonstrated that wild-type α-synuclein overexpression impairs macroautophagy in mammalian cells and in transgenic mice. Overexpression of human wild-type α-synuclein in cells and *Drosophila* models of HD worsens the disease phenotype. Here, we examined whether α-synuclein overexpression also worsens the HD phenotype in a mammalian system using two widely used N-terminal HD mouse models (R6/1 and N171-82Q). We also tested the effects of α-synuclein deletion in the same N-terminal HD mouse models, as well as assessed the effects of α-synuclein deletion on macroautophagy in mouse brains. We show that overexpression of wild-type α-synuclein in both mouse models of HD enhances the onset of tremors and has some influence on the rate of weight loss. On the other hand, α-synuclein deletion in both HD models increases autophagosome numbers and this is associated with a delayed onset of tremors and weight loss, two of the most prominent endophenotypes of the HD-like disease in mice. We have therefore established a functional link between these two aggregate-prone proteins in mammals and provide further support for the model that wild-type α-synuclein negatively regulates autophagy even at physiological levels.

INTRODUCTION

Protein conformation disorders (PCDs) or proteinopathies are a growing family of human disorders associated with aggregation of misfolded proteins in specific tissues (1). PCDs include Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and diseases caused by abnormally expanded polyglutamine tracts in mutant proteins, exemplified by Huntington's disease (HD) and spinocerebellar ataxia types 1, 2, 3, 6, 7 and 17. The hallmark of these otherwise unrelated disorders is the presence of aggregates (also known as inclusions) in cells of the target tissues. Huntingtin is the main component of the intraneuronal aggregates seen in HD (2). HD is an autosomal dominant, progressive, neurodegenerative disorder caused by an expanded polyglutamine tract in exon 1 of the HD gene (3). Pathologically expanded exon 1 huntingtin fragments are sufficient to model disease toxicity both *in vivo* and *in vitro*—exon 1 transgenic mice have inclusions and a progressive HD-like phenotype including tremors, weight loss, motor and cognitive impairment and decreased lifespan (4).

α-Synuclein (encoded by the *SNCA* gene) is the main component of Lewy bodies, the intraneuronal aggregates that pathologically characterize PD (5). A causal role for α-synuclein in PD pathology is supported by the findings that rare α-synuclein point mutations as well as duplications or triplications of the wild-type gene are sufficient to cause autosomal-dominant forms of PD (6,7). In some mouse

*To whom correspondence should be addressed at: MRC Mammalian Genetics Unit, Harwell Science and Innovation Campus, Harwell, Oxfordshire OX11 ORD, UK. Tel: +44 1235841130; Fax: +44 1235841200; Email: a.acevedo@har.mrc.ac.uk

models, the overexpression of human wild-type SNCA in neurons is sufficient to cause dopaminergic cell loss (8), although in others, such as the line used here, overexpression of SNCA does not lead to any overt pathology (9). The loss-of-function of this gene is unlikely to cause disease as *Snca* knockout or deleted strains have no reported pathological phenotypes, although they present subtle functional deficits in dopaminergic neurotransmission (10,11).

Macroautophagy (hereafter termed autophagy) is one of the major mechanisms for the clearance of intracytoplasmic aggregate-prone proteins, like α-synuclein and huntingtin. Autophagy initiates when double-membrane structures engulf a portion of cytosol containing the material for degradation in autophagosomes. These ultimately fuse with lysosomes where their contents are degraded. Mutant huntingtin is a well-characterized autophagy substrate and a number of studies have shown that impairment of autophagy increases the number of cells harbouring mutant huntingtin aggregates (12). Conversely, induction of autophagy with drugs such as rapamycin (13) or rilmenidine (14) ameliorates disease phenotypes in HD mouse models. When overexpressed in cell lines, α-synuclein is able to promote the aggregation of mutant huntingtin (15). Recently, we reported that wild-type α-synuclein overexpression impairs autophagy both *in vitro* and *in vivo* through a mechanism involving Rab1 inhibition and mislocalization of the autophagy protein Atg9. Moreover, we also showed *in vitro* that α-synuclein downregulation could promote autophagy (16). Therefore, as α-synuclein impairs autophagy, we hypothesized that its overexpression would worsen the phenotype observed in HD mouse models. Conversely, as α-synuclein depletion enhances autophagy, we hypothesized that the HD phenotype would be partially ameliorated in mice where α-synuclein expression was depleted.

To test these hypotheses in a mammalian system, we crossed two different HD transgenic N-terminal mouse models (R6/1 and N171-82Q) to α-synuclein-deficient mice and to a model overexpressing human wild-type α-synuclein (M7 line). Both R6/1 (4) and N171-82Q (17) are widely used N-terminal models of HD in which the overexpression of exon 1 containing ∼115 glutamines (R6/1) or an N-terminal fragment containing the first 171 residues of mutant huntingtin harbouring 82 glutamines (N171-82Q) results in neurological phenotypes that resemble HD. Both the overexpression of human wild-type α-synuclein in the M7 line (9) and the *Snca* deletion in the C57BL/6OlaHsd (18) strain have been previously reported and do no present any overt neurodegenerative phenotypes.

RESULTS

α-Synuclein deficiency increases LC3-II levels in the mouse brain

We previously reported that overexpression of α-synuclein impairs autophagy both *in vitro* and *in vivo* in mouse brains (16). Conversely, we also showed that α-synuclein downregulation promotes autophagy *in vitro*. Here, we tested *in vivo* whether α-synuclein depletion promotes autophagy. We assessed the autophagosome numbers in mouse brains using the autophagosome marker microtubule-associated protein 1 light chain (LC3) (19). This is the standard means of assaying autophagosome numbers directly, as LC3 is processed after translation into LC3-I and then converted to LC3-II. LC3-II specifically decorates autophagosome membranes, and hence LC3-II levels (as a function of actin/tubulin) have been shown to correlate with autophagosome numbers (20). Homozygous ($Snca^{-/-}$) mice had more LC3-II levels (corrected by actin) than the control littermates (Fig. 1A and B; $P = 0.029$). While steady-state LC3-II levels can be increased due to either an increase in autophagosome formation or a decrease in autophagosome degradation (20), one cannot deconvolute these options *in vivo* in the brain. However, these data are consistent with our cell-based studies that showed that α-synuclein knockdown enhanced autophagy and increased both LC3-II levels and LC3-II formation (16).

To test whether LC3-II levels are also modulated by α-synuclein in HD brains, we chose two widely used N-terminal HD transgenic mouse models (R6/1 and N-171-82Q) and crossed them to either a line overexpressing human wild-type α-synuclein (M7 line), or a strain carrying a deletion that encompasses *Snca*, the gene encoding α-synuclein. As in the non-HD context, LC3-II levels were increased in HD brains with no α-synuclein (Fig. 1C and D; $P = 0.041$). Conversely, overexpressing α-synuclein decreased LC3-II levels in HD brains (Fig. 1E and F; $P = 0.037$). Moreover, the levels of p62, a known endogenous autophagic substrate, were significantly increased in brains from HD mice overexpressing α-synuclein (Fig. 1G and H; $P = 0.043$). Taken together, these data show that α-synuclein levels modulate autophagy both in wild-type and HD brains.

α-Synuclein levels affects the age at onset of tremors in two HD mouse models

Since autophagy may protect against HD both by enhancing the removal of the toxic aggregate-prone huntingtin (13) and by reducing the susceptibility to forms of cell death (12), we tested the outcome of disease in the two HD mouse models with increased or reduced α-synuclein. Mice from the four different HD lines generated (see Supplementary Material, Table S1) were phenotyped from 7 weeks of age with tests, including SHIRPA (21), rotarod, grip-strength and weight measurements. In both HD mouse models (R6/1 and N171-82Q), the deletion of *Snca* ($Snca^{-/-}$) significantly delayed the age of tremor onset by at least 2 weeks, when compared with HD littermate controls ($Snca^{+/+}$) (Fig. 2A and B). Also, the loss of one copy of α-synuclein ($Snca^{+/-}$) significantly delayed tremor onset in the HD-R6/1 line when compared with HD littermate controls (Fig. 2B). Conversely, overexpression of human wild-type α-synuclein caused earlier tremor onset in the HD mice in a dose-dependent manner in the N171-82Q line; homozygous α-synuclein overexpressors (N171 HD; M7/M7, Fig. 2C) presented with earlier tremor onset than hemizygotes (N171 HD; M7/0, Fig. 2C), both of which had significantly earlier onset when compared with littermate controls expressing normal levels of α-synuclein (N171 HD; +, Fig. 2C). We fitted a Cox regression model for an age-at-tremor onset for the HD-N171-M7 line adjusted by lineage and stratified by sex on which the

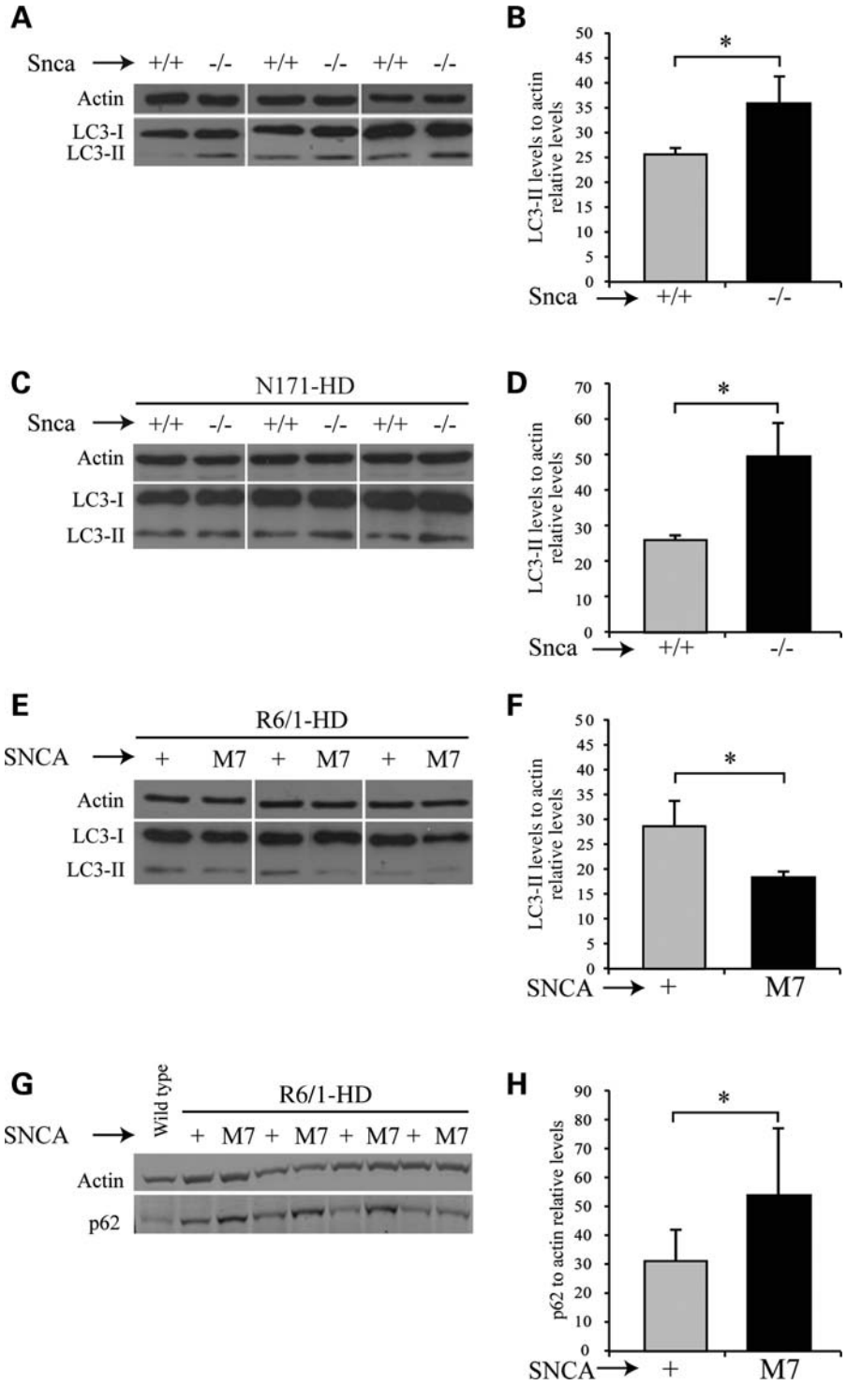

Figure 1. α-Synuclein levels modulate autophagy *in vivo*. (**A** and **F**) LC3-II levels assessed by SDS–PAGE in brain lysates from mice expressing different levels of α-synuclein. Error bars represent standard error of the mean ($^*P < 0.05$). (A and **B**) Representative samples from non-HD wild-type ($Snca^{+/+}$) and α-synuclein-deleted mice ($Snca^{-/-}$) are shown. (B) Quantification of LC3-II levels from (A) relative to actin by densitometry (Student's *t*-test; $n = 3$ ($P = 0.029$). (**C** and **D**) LC3-II levels from wild-type (HD-N171; $Snca^{+/+}$) or α-synuclein-depleted (HD-N171; $Snca^{-/-}$) HD mice. (D) Quantification of LC3-II levels from (C) relative to actin (Student's *t*-test; $n = 3$ ($P = 0.041$). (**E** and F) LC3-II levels from wild-type (HD-R6/1; +) and α-synuclein overexpressing HD mice (HD-R6/1; M7/0). (F) Quantification from (E) relative to actin (Student's *t*-test; $n = 3$ ($P = 0.037$). (**G** and **H**) p62 levels in brain lysates from HD mice overexpressing α-synuclein and controls. (H) Quantification of p62 levels from (G) relative to actin (Student's paired *t*-test; $n = 4$ ($P = 0.043$).

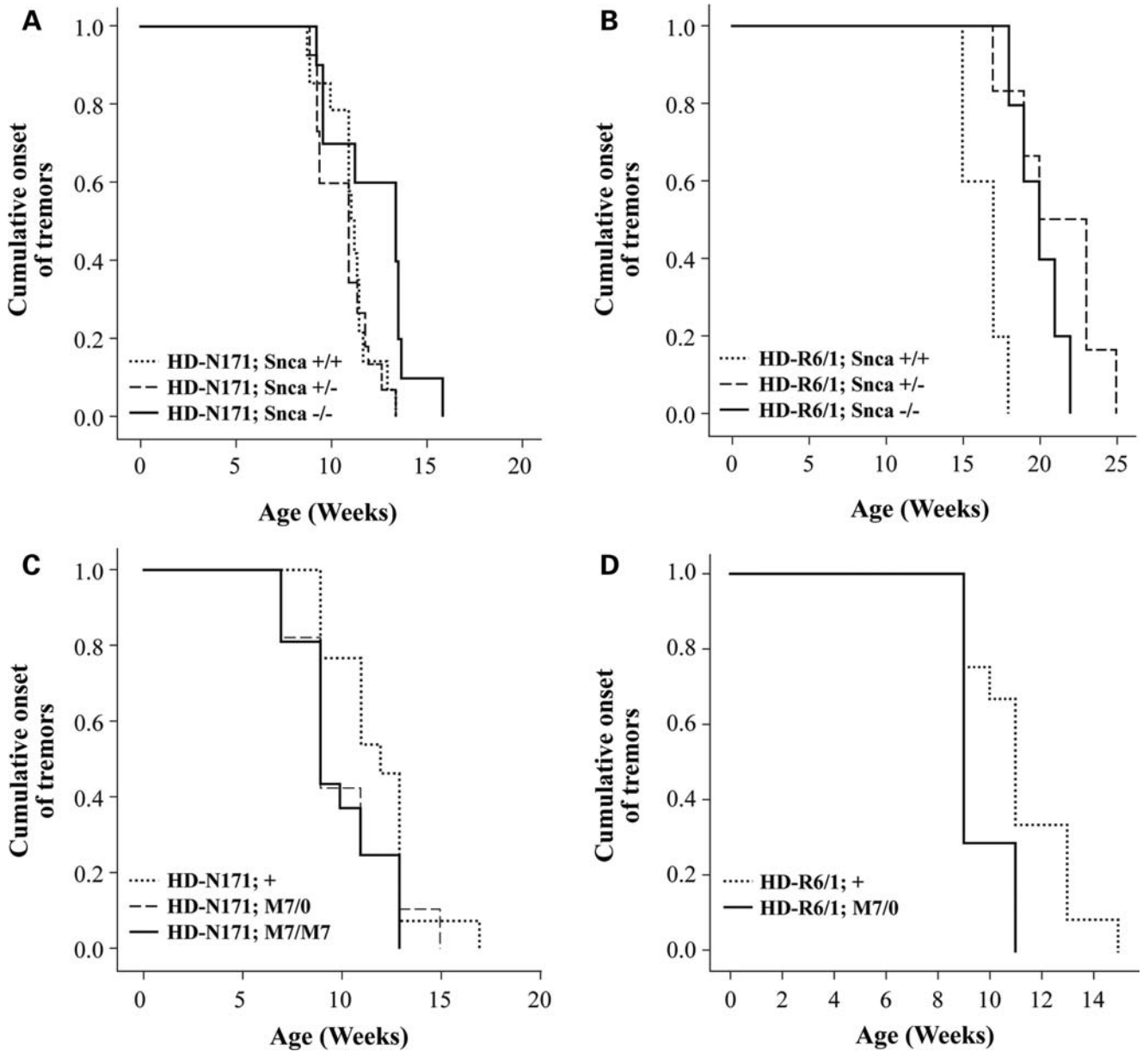

Figure 2. α-Synuclein modifies the age-at-onset of tremors in two HD mouse models. Onset curves showing the age at appearance of tremors measured qualitatively via the SHIRPA test for the four HD lines. All plots represent the proportion of mice presenting tremors (cumulative tremor onset) versus the age at which tremors first appeared. (**A**) Age-at-onset of tremors of HD mice by *Snca* genotype in the HD-N171-Snca-F2 line. HD-N171; $Snca^{-/-}$ mice showed a delayed tremor onset when compared with the HD-N171; $Snca^{+/+}$ littermates ($P = 0.017$). No differences between HD-N171; $Snca^{+/-}$ and the control littermates HD-N171; $Snca^{+/+}$ ($P = 0.9$). HD-N171; $Snca^{+/+}$ ($n = 14$), HD-N171; $Snca^{+/-}$ ($n = 15$), HD-N171;$Snca^{-/-}$ ($n = 10$). (**B**) Age at tremor appearance in males of the HD-R6/1-Snca-F2 line. Both the HD-R6/1; $Snca^{-/-}$ and the HD-R6/1; $Snca^{+/-}$ had later onset of tremors when compared with the HD-R6/1; $Snca^{+/+}$ littermates ($P = 0.007$ and $P = 0.009$, respectively, when comparing with HD-R6/1; $Snca^{+/+}$ controls). Only males are represented in this graph, but the result was similar for females (data not shown). [HD-R6/1; $Snca^{+/+}$ ($n = 5$), HD-R6/1; $Snca^{+/-}$ ($n = 6$), HD-R6/1; $Snca^{-/-}$ ($n = 6$).] (**C**) Age-at-onset of tremors for HD-N171-82Q mice overexpressing human wild-type α-synuclein: HD-N171-M7-F2 line. Homozygotes for the α-synuclein transgene (HD-N171; M7/M7, solid black line) had tremors earlier than their HD-N171; + littermates ($P = 0.02$). Hemizygous mice for M7 transgene (HD-N171; M7/0) also showed earlier onset of tremors than the HD-N171; + littermates ($P = 0.043$) HD-N171; + ($n = 13$); HD-N171; M7/0 ($n = 28$), HD-N171; M7/M7 ($n = 16$). (**D**) Age at tremor appearance in males of the HD-R6/1 line overexpressing SNCA: HD-R6/1-M7-F1 line. HD mice overexpressing human α-synuclein in hemizygosity (HD-R6/1; M7/0, $n = 7$) had an earlier age of tremor onset than their control littermates (HD-R6/1; +, $n = 12$) ($P = 0.038$).

SNCA genotype was significantly associated with onset of tremors ($P = 0.03$). The estimated hazards for presenting an earlier tremor onset were obtained for all α-synuclein genotypes: when the hazard of developing an early tremor onset was referred to that of homozygous α-synuclein transgenic carriers (HD-N171; M7/M7, their hazard for developing early tremor onset is 1), hemizygous male α-synuclein transgenic carriers (HD-N171; M7/0) presented a hazard of

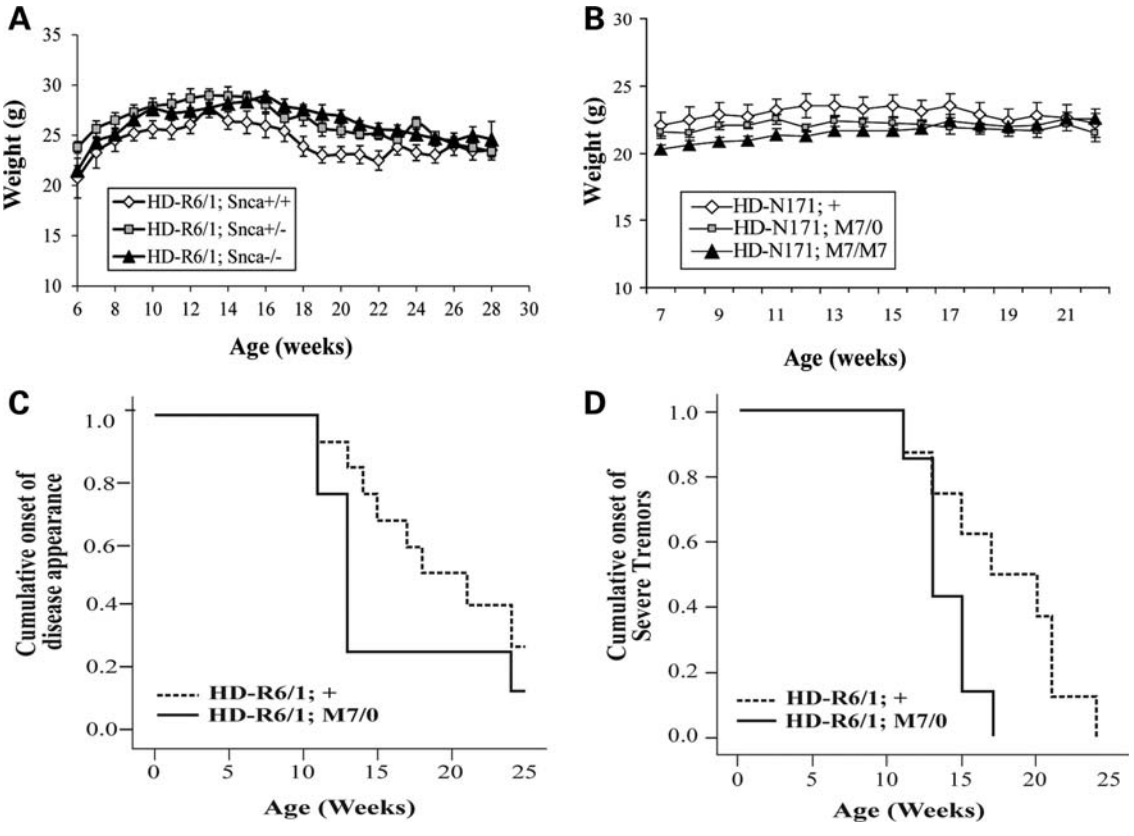

Figure 3. α-Synuclein levels modify different aspects of HD mice phenotype. (**A** and **B**) Weight curves for selected HD lines. For all time points, statistical analysis was performed using ANOVA for all the groups followed by paired comparison between the groups by LSD *post hoc* tests. (A) Weight curves of males from the HD-R6/1-Snca-F2 line. HD-R6/1; $Snca^{-/-}$ (solid black triangle) had a slower rate weight loss when compared with HD-R6/1; $Snca^{+/+}$ littermates (white diamonds), showing significant differences between both groups from 15 to 22 weeks of age. Heterozygous HD-R6/1; $Snca^{+/-}$ mice also maintained weight better than control littermates HD-R6/1; $Snca^{+/+}$ (significant differences found at most time points between 9 and 25 weeks of age. *P*-value comparisons in Supplementary Material, Table S2). HD-R6/1; $Snca^{+/+}$ ($n = 5$), HD-R6/1; $Snca^{+/-}$ ($n = 5$) HD-R6/1; $Snca^{-/-}$ ($n = 6$). (B) Weight curves for females of the N171-82Q HD line overexpressing the human SNCA gene (M7 transgene): HD-N171-M7-F2 line. Significant differences were observed from 9 to 15 weeks of age when compared with HD-N171; M7/M7 to HD control littermates (HD-N171;+). *P*-value comparison in Supplementary Material, Table S3. HD-N171; + ($n = 12$); HD-N171; M7/0 ($n = 15$), HD-N171; M7/M7 ($n = 11$). (**C** and **D**) SHIRPA analysis of the HD-R6/1; M7 line. (C) Onset curves showing the age at onset at which mice first presented 'disease appearance' ($P = 0.06$ between HD-R6/1; M7/0 ($n = 8$) and littermate controls HD-R6/1; + ($n = 12$)). (D) Onset curve showing the onset of severe tremors occurrence. HD-R6/1; M7/0 ($n = 8$) mice (solid black line) developed severe tremors earlier than the controls HD-R6/1; + ($n = 12$) ($P = 0.031$).

developing an early tremor onset of 0.65 whereas HD littermates not carrying an α-synuclein transgene (HD-N171; +) presented a hazard of 0.43. Consistent with these data, hemizygous overexpression of human wild-type α-synuclein in the other HD line used on this study (R6/1 HD; M7/0) also significantly worsened the tremor age-at-onset, when compared with HD littermate controls (R6/1 HD; +, Fig. 2D). Thus, overexpression of α-synuclein accelerates, whereas deletion delays tremor onset on two HD mouse models.

HD-related weight loss is affected by α-synuclein levels

Both HD mouse models used in this study tend to gain weight normally until around 12 weeks of age. After that, these mice do not gain weight normally, and end up losing weight as a consequence of the disease progression. If they lose >30% of their maximum body weight, we humanely terminate them in accordance to our local animal welfare recommendations. Modifications in the rate at which an HD mouse lose weight are indicative that the gene of study is having an effect on disease progression. For the four lines of study, we weighed HD and non-HD littermate controls weekly from 7 weeks of age until they reached humane end point. No differences on weights between any of the non-HD genotypes were found (data not shown). We found sex-specific differences on weights depending on α-synuclein expression levels. In HD males, weight loss is delayed by α-synuclein deficiency in the HD-R6/1-*Snca* line. Statistically significant differences between male control HD mice (HD-R6/1; $Snca^{+/+}$) and mice carrying one copy (HD-R6/1; $Snca^{+/-}$) or no copies of *Snca* (HD-R6/1; $Snca^{-/-}$) are evident at most time points between 9 and 25 weeks of age (Fig. 3A, *P*-values in Supplementary Material, Table S2). However, the weights of females from the HD-R6/1-*Snca* line or HD-N171-*Snca* mice were not statistically different between any of *Snca* genotypes (Supplementary Material, Fig. S1B).

As previously described for tremor onset, we observed that weight loss is accelerated at least in females from the HD mice

overexpressing α-synuclein in homozygosity (HD-N171; M7/M7, Fig. 3B), showing significant differences between 8 and 15 weeks of age when compared with HD controls (HD-N171; +, *P*-value comparisons in Supplementary Material, Table S3). However, although HD-N171; M7/M7 males weighed consistently less than littermate controls, no significant differences were observed between males at any time point (Supplementary Material, Fig. S1C). Hemizygous α-synuclein over expressing lines (HD-N171; M7/0 and HD-R6/1; M7/0) are indistinguishable from HD littermate controls (Supplementary Material, Fig. S1C and D), inferring a dose effect of α-synuclein levels affecting HD weights.

Disease progression on R6/1 HD mice over expressing α-synuclein

We analysed the general disease progression of HD mice longitudinally from 7 weeks of age, fortnightly, until they reached the humane end point. We assessed the general appearance of HD mice as part of the SHIRPA battery of test and examined factors including grooming and activity in the home cage and SHIRPA arena. Once an animal stopped grooming normally and lacked activity in either home cage or SHIRPA arena, it was defined as having a 'diseased appearance'. HD mice manifested this 'disease appearance' after the onset of other endophenotypes, such as tremor onset. The onset of this 'diseased appearance' trait was accelerated in HD-R6/1 mice overexpressing α-synuclein (HD-R6/1; M7/0) when compared with HD littermate controls (HD-R6/1; +) (Fig. 3C), although the differences did not reach statistical significance ($P = 0.06$). Following the trend found in the age at onset of tremors, male HD-R6/1 mice overexpressing α-synuclein (HD-R6/1; M7/0) also developed severe tremors earlier than their HD control littermates (Fig. 3D, $P = 0.03$).

Motor functions, measured by rotarod and grip strength every 2 weeks from 8 weeks of age until they reached humane end point, were not altered by α-synuclein levels on any of the four lines tested (Supplementary Material, Fig. S2A–F). Hind-limb clasping is a prominent feature of the phenotype of the R6/1 HD strain, appearing earlier in the HD-R6/1; M7/0 mice when compared with their HD-R6/1; + littermates, although the differences did not reach significance (Supplementary Material, Fig. S2G, $P = 0.1$). Time until they reached the humane end point (lifespan) was also not significantly altered by either the depletion (Supplementary Material, Fig. S2H) or overexpression (Supplementary Material, Fig. S2I) of α-synuclein on any of the four HD lines tested in this study.

α-Synuclein status effects on inclusion numbers and soluble huntingtin levels

To test the effect of the α-synuclein status on soluble mutant huntingtin levels, we examined brain lysates from HD mice via western blotting at an early disease time point (12 weeks of age). Although we generally found more soluble huntingtin in α-synuclein overexpressors and less in *Snca*-deficient brains, these differences were not statistically significant (Supplementary Material, Fig. S3). We also counted intranuclear inclusions (INI) detected with an antibody against mutant huntingtin in the HD-R6/1 mice brains. We found no differences in the total number of inclusions between mice that have depleted (HD-R6/1; $Snca^{-/-}$) or overexpressed α-synuclein (HD-R6/1; M7/+) when compared with littermate controls at early disease time points (data not shown). It should be noted that both the HD-N171 and the α-synuclein overexpressor line (M7) are under the control of the same mouse prion promoter, so they would be expected to have similar expression patterns apart from insertion events. As both are under the control of the same promoter, we tested whether the expression of either transgene at the RNA level affected the expression of the other and found a non-significant decrease in the HD transgene expression on the HD-N171; M7/M7 homozygous line, whereas hemizygous HD-N171; M7/0 were indistinguishable from littermate controls HD-N171; + (data not shown). Hence, to avoid any confounding issues regarding the coexpression of both transgenes, we used the HD-R6/1; M7/0 line for the analysis of huntingtin soluble levels and inclusion formation as above.

We explored the possibility that an impairment on autophagy or any other toxic effect due to α-synuclein overexpression in the HD context may have had an impact in the survival of brain cells. In order to detect cell death in the brains of HD mice overexpressing α-synuclein, we performed terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labelling (TUNEL) staining on brain slices at an early and late stage of disease on HD mice brains. TUNEL staining did not reveal any active cell death (data not shown). We also stained brain slices for a general marker of neurodegeneration (Fluoro-Jade) and did not find any differences between HD-R6/1; M7/0 and their littermate controls (HD-R6/1; +, data not shown).

There is at least one study reporting α-synuclein as a component of HD inclusions (22). We could not find obvious widespread α-synuclein co-localization with huntingtin INI on both HD strains at early and late time points. However, minimal co-localization occurs between huntingtin and α-synuclein on both R6/1 and R6/1; M7/0 brains (Supplementary Material, Fig. S4). The same pattern was obtained when using HD-N171; M7/0 brains at early and late stage (data not shown). The staining found for α-synuclein was intense and widely spread and obviously more intense in the M7 overexpressing mice. We also stained for p62 and, as expected (23), found evident co-localization between p62 and huntingtin INI that was not affected by α-synuclein levels (Supplementary Material, Fig. S4).

DISCUSSION

We recently reported that overexpression of human wild-type α-synuclein in cell models, *Drosophila* and mouse brains impaired autophagy. Conversely, α-synuclein downregulation induced autophagy in cell models (16). Here, we show that *Snca* deletion increases the levels of LC3-II (relative to actin), an autophagy marker, in wild-type (Fig. 1A and B) and HD mouse brains (Fig. 1C and D). Conversely, LC3-II levels are diminished in HD brains when α-synuclein is over expressed (Fig. 1E and F). These complementary findings suggest that even physiological levels of α-synuclein impact

on autophagy in mouse brains. We then decided to test *in vivo* the effects of α-synuclein levels in HD pathology.

Our initial hypothesis was that HD mice overexpressing α-synuclein would show an earlier disease onset and more rapid disease progression. Conversely, HD mice deficient in α-synuclein would have delayed disease onset. We found that at least two prominent endophenotypes present in HD mouse models conformed to our initial hypothesis. Both tremor onset (Fig. 2) and weight loss (Fig. 3A and B) were dependent on α-synuclein levels. Reassuringly, we found the same opposite effects on both endophenotypes in the four independent HD mouse lines tested: *Snca* deficiency resulted in a protective effect on both HD mouse models tested, whereas α-synuclein overexpression was deleterious again in both HD models. Resting tremors are one of the first endophenotypes arising in both the N171-82Q and R6/1 HD lines and we and others have previously shown that its onset can be modulated by HD modifier genes (24) or by autophagy modulators (14). Thus, α-synuclein may modulate the onset of disease in the HD models.

We did not observe any improvement in motor function measured by rotarod and grip-strength performance for any of the HD lines studied. In both HD mouse models used here, rotarod and grip-strength deficits appear once disease is already underway, weeks after the onset of tremors. It is tempting to speculate that the consequences of modulating autophagy through α-synuclein levels may impact more on the disease onset. However, it is entirely possible that the effect of α-synuclein levels on HD endophenotypes could also be due to other reasons apart from the impairment or induction of autophagy. While we did not observe a significant improvement in lifespan, in our experiments the assessment of lifespan was confounded by our local animal welfare rules requiring us to euthanase mice when their disease exceeds the defined humane end points for moderate severity. Since the great majority of the animals were euthanized, we could not obtain survival data with the same precision as would have occurred if all mice had been allowed to die from their polyglutamine disease.

We measured mutant soluble huntingtin levels in the brains of HD mice expressing different amounts of α-synuclein. Although we generally found more soluble huntingtin in α-synuclein overexpressors and less in *Snca*-deficient brains, these differences were not statistically significant (Supplementary Material, Fig. S3). We measured huntingtin levels at a relatively early disease time point (12 weeks), and therefore it may be possible that changes may be evident at earlier or later disease stages. A failure to see differences at this early disease time point may be due to mouse-to-mouse variation necessitating much larger numbers of mice, and the possibility that changes in huntingtin levels below the sensitivity of western blotting in mice (e.g. 20% changes) may be sufficient to enable modification of the phenotype in these HD models. We also looked at huntingtin inclusion numbers in different brain areas including striatum, cortex and cerebellum but did not observe any significant differences on inclusion numbers on any of the HD lines regardless of the α-synuclein status at early disease stages. However, in an accompanying report, a reduction in inclusion numbers was seen only at late disease stages in R6/1-HD; $Snca^{-/-}$ brains when compared with littermate controls (25). Another reason that could explain why we did not observe an increase in inclusions in the mice that overexpress α-synuclein is that this overexpression may be toxic in an HD context, leading to neuronal cell death in the cells with highest expression. We assessed this possibility on brains from HD mice overexpressing α-synuclein, but did not find any sign of active cell death (as measured by TUNEL) or neuronal degeneration (measured via fluoro-jade staining). Previous reports have shown co-localization of α-synuclein in HD huntingtin inclusions (22). Although we did not find a widespread α-synuclein co-localization with HD inclusions in the mouse models used here, we did find some rare co-localization events in both R6/1 and R6/1; M7/0 brains (Supplementary Material, Fig. S4).

Taken together, these results are consistent with previous data, suggesting that α-synuclein may be toxic in certain conditions. For example, α-synuclein levels rise after administration of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (26). In this context, *Snca* null mice are resistant (27) whereas human wild-type α-synuclein overexpressors are susceptible to MPTP neurotoxicity (28). Moreover, rapamycin treatment (a known autophagy inducer) has been shown to ameliorate the dopaminergic neuronal loss after MPTP treatment (29). However, other studies have suggested that wild-type α-synuclein may also be neuroprotective in certain contexts (30–33).

In summary, we have tested the effects of modulating α-synuclein levels in HD mouse models. Our data suggest that increasing levels of this protein enhances the phenotype in two different models, while decreasing α-synuclein levels has beneficial effects. This suggests that even physiological levels of α-synuclein modulate the HD phenotype and that it is even possible that modest reductions in α-synuclein may constitute a therapeutic target for HD (and possibly other proteinopathies). While our data suggest that the ability of α-synuclein to modulate autophagy *in vivo* may constitute one mechanism for its effects on the HD phenotype, it is likely that α-synuclein acts via multiple pathways.

MATERIALS AND METHODS

Mice

All procedures were performed with the appropriate UK Home Office and local animal welfare and ethical committee approval. Both HD mice, R6/1 mice [B6CBATgN (HDexon1)61Gpb] (4) and N171-82Q mice [B6C3F1/TgN(HD82Gln)81Dbo] (17) were obtained from The Jackson Laboratory (Bar Harbour, ME, USA) and subsequently backcrossed for at least 10 generations onto a C57BL/6J background. The R6/1 line expresses the first exon of human huntingtin carrying ∼120 glutamines under the control of the ubiquitously expressed human huntingtin promoter. The N171-82Q expresses the N-terminal 171 residues of human huntingtin carrying 82 glutamines under the mouse prion promoter, which is expressed at high levels in neurons. Mice were genotyped by polymerase chain reaction (PCR) with DNA extracted from ear-clips at 3 weeks of age (primer sequences available on request). α-Synuclein-deficient animals $Snca^{-/-}$ (C57BL/6JOlaHsd)

obtained from Harlan Olac (Bicester, UK) were genotyped also by PCR (18). Mice overexpressing human wild-type α-synuclein (M7 line: B6;C3-Tg(Prnp-SNCA)7Vle) were a gift from V.M.-Y. Lee (University of Pennsylvania, Philadelphia, PA, USA) and were genotyped by real-time PCR from ear-clip DNA (9). The expression of the M7 transgene is also under the control of the mouse prion promoter. All genotypes were checked again after death by a second extraction of DNA from the tail and M7 expression levels were verified by western blotting when possible. Both HD lines (R6/1 and N171-82Q) were maintained in hemizygosity on a C57BL/6J background, whereas both the *Snca*$^{-/-}$ and the overexpressing M7 mice were maintained in homozygosity. To generate the double mutants with the different combinations of lines, we crossed males from both HD lines (R6/1 and N171-82Q) to *Snca*$^{-/-}$ or M7 females. Selected HD males were then intercrossed with non-HD females from the first generation to generate all possible genotypes in the F2. We produced all but one of the four lines of double mutants in the F2. We used F1 mice for the HD-R6/1/M7 cross (HD-R6/1-M7-F1), as we did not produce homozygous α-synuclein transgenics for that line (see Supplementary Material, Table S1).

Behavioural analysis

Animals were housed together in groups of mixed genotypes, and experienced observers were blind to the genetic status during testing. Mice were tested on a longitudinally designed battery of test from week 7 until they reached the humane end point. Mice were carefully monitored daily, and weighed every week. A minimum of five females and five males per genotype and line were analysed for specific tests. They were housed under conventional conditions with food and water *ad libitum*.

Humane end points. Animals were humanely terminated once they reached one of the following moderate severity end points: (i) hunched posture coupled with a lack of movement and (ii) 30% weight loss from maximum body weight over the animal lifetime or 20% weight loss over a single week.

SHIRPA. The modified *SHIRPA* (19) battery of behavioural tests was performed on the mice every 2 weeks from 7 weeks of age onwards. To measure the tremors, mice were placed on a grid in a clear perspex cylinder for 5 min. Tremors were scored as follows: 0, none; 1, present; 2, severe; 3, outrageous.

Rotarod. Rotarod (Accelerating model, Ugo Basile, Italy) was performed every 2 weeks, on alternate weeks to the SHIRPA testing, from 8 to 29 weeks of age. Mice were given two trials per day, three times per week. The rotarod was set to increase speed from 4 to 40 rpm in 250 s. Latency to fall was recorded, 300 s being the maximum time allowed for a mouse. On the first week of testing, animals were trained to acquire the task; they were given three training sessions per day on three different days. An average performance for each animal and week was used to group animals by genotype.

Grip strength. Mice were held by the tail and placed on the apparatus (BIOSEB, France) according to manufacturer's instructions. The strength of all four paws was measured. Each session consisted of two trials for which the average force was used per animal and week.

Immunocytochemistry

HD mice from the deletion (HD-R6/1-Snca-F2) and the overexpressing (HD-R6/1-M7-F2) lines were perfused transcardially with 4% (w/v) paraformaldehyde (Sigma-Aldrich) in phosphate saline buffer (PBS), pH 7.4. Coronal 30 μm cryosections of brains were free floating stained to detect INI. We used the MAB5374 anti-huntingtin antibody (Millipore) (or the previous EM-48 from Chemicon), using a secondary AlexaFluor488-conjugated antibody (Invitrogen) and mounted on Vectashield with nuclear counterstaining DAPI (Vector labs). Confocal projections (typically the sum of 12 images per projection) were used to count the total of inclusions per ×60 field, using a Leica SP5 microscope (Leica Microsystems). Striatum and forebrain cortex aggregates were counted in coronal sections, ±1 mm from the bregma, on at least three sections per animal. Usually, at least three fields were counted per section, between three and six slices per mouse, and at least three animals per genotype were tested on each line with a matching age and gender littermate controls. After manually counting the inclusions, we used the Volocitys software 5.4.1 (Perkin Elmer, USA) on the Z-stack projections to verify the counting obtained and corrected by nuclei's DAPI+ per image. TUNEL was performed on those brain sections using an *in situ* cell death fluorescent detection kit (Promega) according to the manufacturer's instructions. Secondary AlexaFluor488-conjugated, AlexaFluor594-conjugated and AlexaFluor647-conjugated antibodies (Invitrogen) were used and confocal images were taking using a Zeiss 710 microscope.

For co-localization studies, brains from mice of different ages [typically an early (8–12 weeks old) and late (20–24 weeks old) time points] were selected from both HD models and followed the above procedure to co-stain for mutant huntingtin and α-synuclein (Millipore) and mutant huntingtin and C-terminal p62 (Progen, Germany). For the α-synuclein–huntingtin co-localization, prior staining, 30 μm cryosections were boiled in SSC buffer and allowed to cool down in dH_2O. Six to ten fields in the piriform cortex were imaged in 30 μm z-stacks. A mean of five INI per image were taken per mouse. Two mice per genotype were used. Images were analysed by Volocity software (version 5.4.1). Pearson's coefficients were used for reporting co-localization.

Immunoblot from brain homogenates

Brains were frozen immediately after removal and stored at −80°C for all possible genotypes. We used at least four animals on each group, grouped by gender, at 12 weeks of age. Brains were mechanically homogenized in 2.5 volumes of buffer B with protease inhibitors (Roche Diagnostic) in lysing matrix tubes D (MP Biomedicals, Germany) and a Fast-Prep-24 homogenizer at 4°C. Homogenates were then centrifuge at 13 000 rpm for 15 min and the supernatant was

retained. Protein concentration was determined (Bradford reagent, Sigma) and equal amount of proteins from each sample was resolved by SDS–PAGE (NUPAGE system, Invitrogen) and transferred on low fluorescent PVDF (LF-PVDF) membranes (Millipore). Primary antibodies include MAB1574 (also known as 1C2, Millipore) for the detection of the mutant huntingtin, anti-α-synuclein (Millipore), anti-β-actin (Sigma) and anti-LC3 (Novus). Blots were probed with anti-mouse or anti-rabbit IgG–HRP and visualized with ECL-plus detection kit (GE Healthcare). When quantifying was necessary, we used infrared secondary antibodies IRDye® (Li-Cor Biosciences) and quantified using the Odyssey imaging system (Li-Cor Biosciences).

Statistics

Significance levels were determined for comparisons between groups using SPSS software (version 17.0, SPSS Inc.). Parametric data were analysed by ANOVA followed by *post hoc* test (LSD) and Student's *t*-test. Non-parametric mouse data (age-at-tremors, survival, limb grasping onset) were visualized using Kaplan–Meier survival curves and tested using the Wilcoxon test as we hypothesized a stronger effect of α-synuclein genotype early on. We used Cox's regression to model the effect of α-synuclein overexpression on age-at-tremor onset. On the model, we adjusted the data for lineage (grouping F2 mice by their HD F1 parent to control for possible differences on HD transgene expression) and stratified by sex.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

ACKNOWLEDGEMENTS

We are grateful to Professor Virginia Lee (University of Pennsylvania) for α-synuclein overexpressor mice. We are indebted to Jim Humphreys and the imaging service (MRC Harwell) for help with perfusions and figures, respectively.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by a UK Medical Research Council programme Grant (to D.C.R. and S.D.B.) and by a Wellcome Trust/Medical Research Council strategic award in neurodegeneration (to D.C.R.). D.C.R. is a Wellcome Trust Senior Clinical Research Fellow.

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