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BRIEF COMMUNICATION

Lack of Nigral Pathology in Transgenic Mice Expressing Human α-Synuclein Driven by the Tyrosine Hydroxylase Promoter

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α-Synuclein has been identified as a major component of Lewy body inclusions, which are one of the pathologic hallmarks of idiopathic Parkinson's disease. Mutations in α-synuclein have been found to be responsible for rare familial cases of Parkinsonism. To test whether overexpression of human α-synuclein leads to inclusion formation and neuronal loss of dopaminergic cells in the substantia nigra, we made transgenic mice in which the expression of wild-type or mutant (A30P and A53T) human α-synuclein protein was driven by the promoter from the tyrosine hydroxylase gene. Even though high levels of human α-synuclein accumulated in dopaminergic cell bodies, Lewy-type-positive inclusions did not develop in the nigrostriatal system. In addition, the number of nigral neurons and the levels of striatal dopamine were unchanged relative to non-transgenic littermates, in mice up to one year of age. These findings suggest that overexpression of α-synuclein within nigrostriatal dopaminergic neurons is not in itself sufficient to cause aggregation into Lewy body-like inclusions, nor does it trigger overt neurodegenerative changes. © 2001 Academic Press

INTRODUCTION

The potential role of α-synuclein in the pathophysiology of Parkinson's disease has attracted a great deal of attention as mutations in the α-synuclein gene have been found to be responsible for rare, familial cases of Parkinsonism (Polymeropoulos *et al.,* 1997; Kruger *et al.*, 1998). Abnormal α-synuclein has been identified as a major component of Lewy bodies, one of the pathologic hallmarks of Parkinson's disease (Spillantini *et al.,* 1998). Lewy body inclusions are prominent in neurons of the substantia nigra and in addition to α-synuclein fibrils, they also include ubiquitin, neurofilaments, and a range of other proteins (reviewed in Braak and Braak, 2000). In a recent study, mice expressing a wild-type (wt) human α-synuclein transgene driven by the platelet-derived growth factor (PDGF) promoter were reported to develop α-synuclein and ubiquitin immunoreactive inclusions that were argyrophilic and positive for thioflavin S. Although nigral cell body numbers were normal, there was evidence of damage to striatal dopaminergic ter-

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minals (Masliah *et al.,* 2000). A significant difference between the pathologic phenotype of these transgenic mice and that seen in human Parkinsonism was the absence of neurodegeneration in the substantia nigra (Forno *et al.,* 1993). One possible explanation for the lack of nigral cell loss is that the PDGF promoter used by Masliah and colleagues may have failed to induce significant expression of the transgene within dopaminergic neurons of the substantia nigra as suggested by data from Sasahara *et al.* (1991). In mice used in this study, expression of a wt or mutant (A30P and A53T) human α-synuclein transgene was driven by the tyrosine hydroxylase (TH) promoter. Since TH is a key enzyme in the intraneuronal synthesis of catecholamines, the TH promoter would be expected to drive α-synuclein synthesis within catecholaminergic cell groups, including nigral dopaminergic neurons (Banerjee *et al.,* 1992).

MATERIALS AND METHODS

Generation of Transgenic Mice

Human α-synuclein cDNA was amplified from total brain cDNA (Clontech, Palo Alto, CA), using *Not*I restriction site tagged primers 5′ forward TCG GAG CGG CCG CTC GAC GAC AGT GTG GTG TAA AGG and 3′ reverse AAT GTG CGG CCG CGG CAC ATT GGA ACT GAG CAC TT. The PCR product was cloned into pcDNA3 vector (Invitrogen, Carlsbad, CA) and mutagenesis was performed to introduce the pathogenic mutation G209A (A53T) and C88G (A30P) using a Clontech kit. The 5′ phosphorylated primers were 5′CTC TGG GGT TGG AAA TGA CCG for selection and 5′ GCA TGG TGT GAC AAC AGT GGC and 5′ GCA GAA GCA CCA GGA AAG ACA. Mutant and wt cDNAs were cloned into the promoter construct (Banerjee *et al.,* 1992) between the 4.8-kb rat TH promoter and SV40 poly(A) signal site. Linearized, purified constructs were microinjected into the pronucleus of single celled embryos derived from Swiss Webster × C57blk6/DBAF1 parents. Transgene-positive derivatives were assessed for transgene integrity by Southern blotting. Transgene expression was assessed by Northern blotting, *in situ* hybridization (using an oligo of sequence 5′ ACA TCC ATG GCT AAT GAA TTC CTT TAC ACC ACA CTG TGC) and Western blotting (833 ng IgG/ml, Transduction Laboratories, San Diego, CA) by standard methods. In total, six lines of TH-A53T mutant mice, two lines of TH-A30P mutant and three lines of wt mice expressing α-synuclein were generated. Transgene protein levels in these lines varied but two mutant lines had equal levels of protein, which was similar to one of the A30P lines and one of the wt lines.

Immunohistochemistry

Sections for immunohistochemistry were prepared as previously reported (Matsuoka *et al.,* 1999). Sections were incubated with human α-synuclein (clone LB509, 0.15 μg IgG/ml, Zymed Laboratories, South San Francisco, CA) (Jakes *et al.,* 1999); endogenous TH (clone 2/40/15*, 0.1 μg IgG/ml, Roche Diagnostics, Indianapolis, IN); and ubiquitin (clone FPM1, 1:100, Novocastra Laboratories, Newcastle upon Tyne, UK). The antibody reaction was visualized using an ABC elite kit (Vector Laboratories, Burlingame, CA). At these dilutions, no immunoreactivity was detected when sections were incubated without primary or secondary antibody. Silver staining (Switzer, 2000) and thioflavin S staining were performed according to standard methods.

Cell Counts and Dopamine Assay

TH-immunopositive cell counts were performed by investigators blind to the genotype of the mouse, in two independent labs on different sets of animals. Stereological equipment and techniques were used for an unbiased determination of the number of dopaminergic neurons in the substantia nigra pars compacta (Chan *et al.,* 1997; Liberatore *et al.,* 1999). Dopamine levels were measured in the striatum by HPLC with electrochemical detection and the results are expressed as ng/mg protein (Kilpatrick *et al.,* 1986). One-way ANOVA failed to show a statistically significant difference between the groups.

RESULTS AND DISCUSSION

In the central nervous system, TH is expressed in the noradrenergic neurons of the brainstem, dopaminergic neurons of the midbrain, periventricular hypothalamic nuclei, and olfactory bulb, and the retinal amacrine cells. A similar and consistent pattern of expression was demonstrated for the α-synuclein transgene in all lines of mice by *in situ* hybridization. Mice with the highest level of expression were further examined by immunocytochemistry (Figs. 1B, 1C, and 1G–1I) and by Western blot analysis (Fig. 1A). Taken together, these findings indicate that we were able to

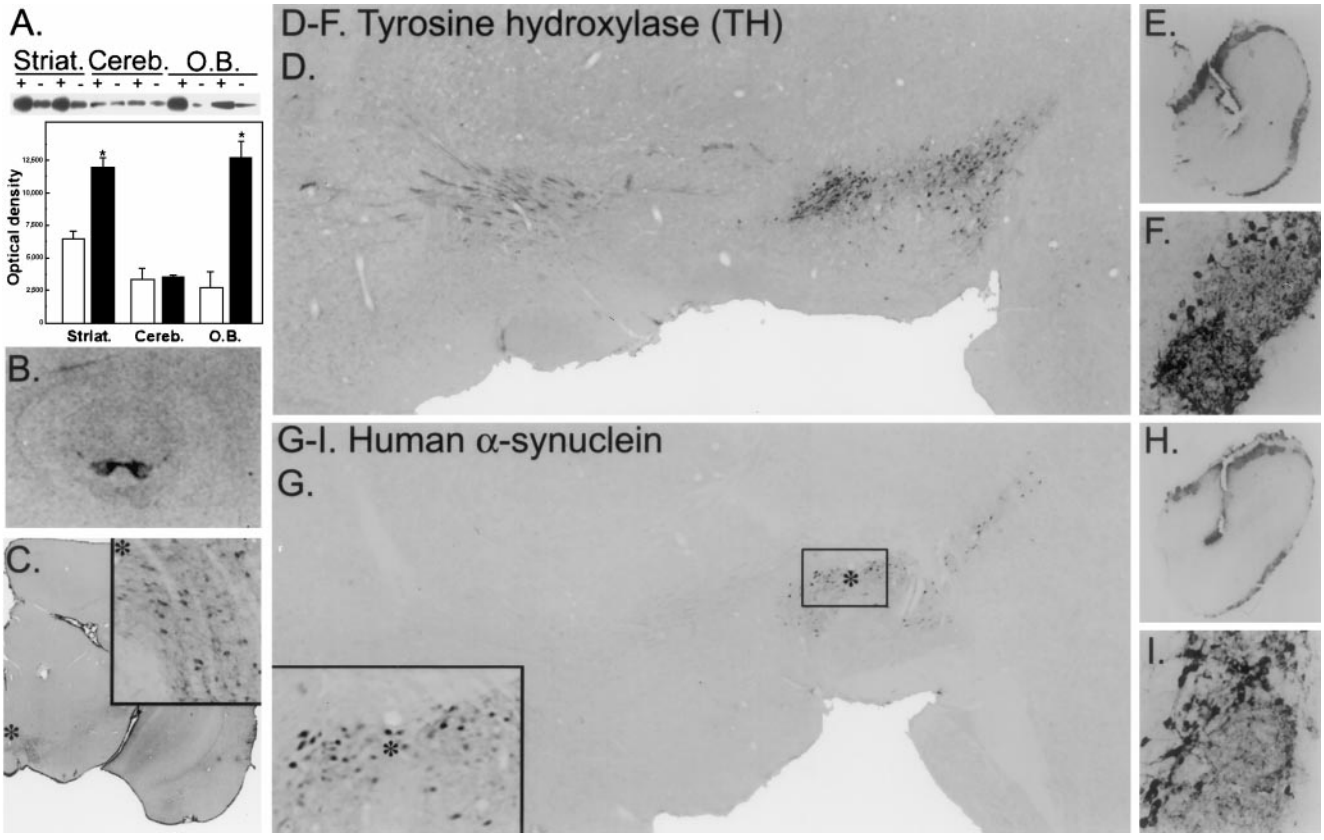

FIG. 1. Mice over-expressing human mutant (A53T) α-synuclein (+) and nontransgenic controls (−) show the 19-kDa α-synuclein band (A, Striat, Striatum; Cereb, Cerebellum; O. B., Olfactory bulb). Alpha-synuclein is statistically increased in the striatum and the olfactory bulb of transgenic mice (closed column) compared to nontransgenic mice (open column) ($P < 0.01$ by two-tailed Student's t test). Human α-synuclein mRNA and protein were expressed in the substantia nigra (B and C, respectively). Expression of endogenous TH and human α-synuclein were compared (D–F vs G–I). In the striatum (D vs G) and the olfactory bulb (E–F vs H–I) the pattern of expression of human α-synuclein was very similar to the distribution of TH-immunoreactivity. Human α-synuclein was predominantly observed in cell bodies and was also present in terminals in the substantia nigra and (insets in C and G) striatum. Asterisks indicate the same point in the panel and its inset. Original magnification is ×4 in C–E, G, and H (montaged); ×40 in F and I.

achieve high levels of expression of both wt and mutant human α-synuclein within nigrostriatal dopaminergic neurons. Although human α-synuclein accumulated to high levels in the cell body and terminals of dopaminergic cells (Fig. 1), the same cells were negative for ubiquitin immunoreactivity and for thioflavin S and silver staining (data not shown). By these criteria, we therefore believe that evidence of Lewy-type pathology was not seen. In addition, α-synuclein accumulation did not impact the number of nigral neurons, nor the levels of striatal dopamine in animals up to one year of age (Table 1). These findings suggest that overexpression of either wt or mutant human α-synuclein within nigrostriatal dopaminergic neurons is not sufficient to cause pathogenic aggregation,

TABLE 1

	Nigral cell counts at 12 months	Dopamine level at 12 months
Nontransgenic	10694 ± 1597 (9)	114.2 ± 9.8 (5)
Wild-type mice	9586 ± 2001 (7)	122.5 ± 12.4 (5)
A53T	11903 ± 1104 (9)	125.1 ± 10.8 (5)
A30P	10633 ± 1717 (3)	ND

Note. The table shows mean ± SD of the number of TH immunopositive cell bodies in the nigra and the mean ± SD level of striatal dopamine (ng/mg protein). The number of animals studied is shown in parentheses. Statistical analysis by one-way ANOVA showed there was no significant difference between the groups. In addition, overall neuron numbers in the pars compacta was not significantly different between mouse groups as shown by Nissl-staining (data not shown).

nor does it trigger overt neurodegenerative changes in these animals.

Phenotype development in other transgenic mice overexpressing human α-synuclein is variable, and none of the mice reproduce the features of human Lewy body pathology completely. One line of mice overexpressing wt human α-synuclein under the control of the PDGF promoter develops inclusions and neuritic pathology in different areas of the brain (including "occasional" inclusions in the substantia nigra) and shows evidence of damage to striatal dopaminergic terminals (Masliah *et al.,* 2000) but with no overt loss of nigral cell bodies. Although the inclusions are α-synuclein, ubiquitin and silver-stain positive, they are not composed of fibrillar synuclein as seen in human Lewy bodies. Conversely, a second set of PDGF-driven α-synuclein mice (wt and mutant A53T) demonstrated accumulated α-synuclein, but did not develop the same type of inclusions as the mice described in Masliah *et al.* despite high-level expression of the transgene (Goldberg *et al.,* 2000). Mice have also been generated expressing either mutant or wt α-synuclein under the control of the thy-1 promoter. Nonfibrillar synuclein accumulation was again seen in cell bodies and neurites in several brain regions (Kahle *et al.,* 2000; van der Putten *et al.,* 2000) but in one of the models, α-synuclein accumulation was most prominent in brainstem and motor neurons. Some, but not all of these motor neurons were argyrophilic and ubiquitin immunoreactive (van der Putten *et al.,* 2000).

As the mouse models are well matched in terms of protein level, it is difficult to explain why the pathology differs between them. It is particularly puzzling that we could not observe inclusions using the TH promoter which expresses well in the nigra, whereas they were found (albeit rarely) in mice using the PDGF promoter (Masliah *et al.,* 2000) that is relatively ineffective for transgene expression in this region. While it is possible that strain differences may account for the discrepancy, it seems unlikely as the strains of mice used in both studies were similar (C57B6/DBA2 × Swiss Webster for the TH-synuclein mice, as compared to C57B6 × DBA2 for the PDGF-synuclein mice).

Surprisingly, the most convincing Lewy-type pathology has been re-created not in mice, but in transgenic *Drosophila.* In this animal model, the inclusions were composed of fibrillar α-synuclein and their formation resulted in neuronal loss. Furthermore, mutant synuclein was more pathogenic than wild type, supporting the idea that α-synuclein does play a role in Parkinson's disease pathogenesis as predicted by the genetic data. What is clear from the mouse studies is that synuclein accumulation does not lead directly to fibrillarization of the protein and overt cell loss as it does in the fly models, and it is probable that a "second hit" approach may be necessary to enhance the phenotype and provide us with a more accurate mouse model for the synucleinopathies.

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