Abnormal α-Synuclein Interactions with Rab Proteins in α-Synuclein A30P Transgenic Mice

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Abstract. Mutation A30P in the α-synuclein gene is a cause of familial Parkinson disease. Transgenic mice expressing wild mouse and mutant human A30P α-synuclein, Tg5093 mice (Tg), show a progressive motor disorder characterized by tremor, rigidity, and dystonia, accompanied by accumulation of α-synuclein in the soma and neurites and by a conspicuous gliosis beginning in the hippocampal formation at the age of 7 to 8 months and spreading throughout the CNS. Impaired short-term changes in synaptic strength have also been documented in hippocampal slices from Tg mice. α-synuclein aggregates of approximately 34 and 70 kDa, in addition to the band of 17 kDa, corresponding to the molecular weight of α-synuclein, were recovered in the PBS-soluble fraction of brain homogenates from Tg mice but not from brain samples from age-matched wildtype littermates. MPTP-treated Tg and wildtype mice produced α-synuclein aggregates in the PBS-, deoxycholate-, and SDS-soluble fractions. Aggregates of α-synuclein, although with different molecular weights, were also observed in rotenone-treated Tg and wildtype mice. Pull-down studies with members of the Rab protein family have shown that α-synuclein from Tg mice interacts with Rab3a, Rab5, and Rab8. This binding is not due to the amount of α-synuclein (levels of which are higher in Tg mice) and it is not dependent on the amount of Rab protein used in the assay. Rather, α-synuclein interactions with Rab proteins are due to mutant α-synuclein as demonstrated in Rab pull-down assays with recombinant of wildtype and mutant A30P human α-synuclein. Since Rab3a, Rab5, and Rab8 are important proteins involved in synaptic vesicle trafficking and exocytosis at the synapse, vesicle endocytosis, and trans-Golgi transport, respectively, it can be suggested that these functions are impaired in Tg mice. This rationale is consistent with previous data showing that short-term hippocampal synaptic plasticity is altered and that α-synuclein accumulates in the cytoplasm of neurons in Tg mice.

Key Words: α-synuclein; 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP); Parkinson disease; Rab3a; Rab5; Rab8; Rotenone.

INTRODUCTION

α-Synuclein is a highly conserved protein of 140 amino acid residues and is abundant in presynaptic terminals (1–5). α-Synuclein has been linked to the pathogenesis of Parkinson disease (PD) with the discovery of missense mutations (A53T and A30P) in several families affected by early onset familial PD (6–8), a feature that has been demonstrated in sporadic and familial PD (9–12). In addition, α-synuclein is implicated in diffuse Lewy body disease and accumulates in Lewy bodies and aberrant neurites in both familial and sporadic PD and diffuse Lewy body disease (2, 5, 6, 13–17). Although the mechanism of Lewy body formation is not completely known, self-aggregating α-synuclein is the major subunit protein of the filamentous lesions observed in PD (18). Moreover, oxidative dimer formation is the critical ratelimiting step for α-synuclein fibrillogenesis in PD (19).

Transgenic α-synuclein mice manifest variable phenotypes and pathologies that are dependent not only on the type of α-synuclein but also on the characteristics of the transgene promoter (20, 21). Transgenic mice expressing mutant A53T or mutant A30P human α-synuclein show somal and neuritic accumulations of α-synuclein in the brain, accompanied by variable motor deficits (22–24). Expression of nonmutant mice α-synuclein and mutant A30P human α-synuclein in Tg5093 (Tg) mice results in a progressive motor disorder associated with aberrant expression of the protein in the cell soma and with progressive glisosis, but without specific anatomical or biochemical disruption of the nigrostriatal dopaminergic system (25). Finally, Tg mice exhibit altered short-term hippocampal synaptic plasticity (26).

Mice lacking α-synuclein display functional deficits and abnormal synaptic function in the nigrostriatal dopaminergic system (27). Furthermore, recent studies have demonstrated a small reserve and/or resting pool of vesicles and increased synaptic depression during repetitive electrical stimulation in α-synuclein knock-out mice (28), thus supporting the notion that α-synuclein regulates synaptic vesicle mobilization at nerve terminals, particularly those in the reserve or resting pool (27).

Considering these data, it is feasible that mutant α-synuclein may have several implications in pathology. On one hand, mutated α-synuclein may have properties of solubility and a capacity to form aggregates that differ from those of wild α-synuclein. On the other hand, and
more important in the present context, interactions of \( \alpha \)-synuclein with proteins linked with exocytosis and/or vesicle recycling and with neurotransmission may be impaired by mutant \( \alpha \)-synuclein.

Rab proteins are a large family of GTPases that control membrane transport and cell trafficking by shifting between GTP- and GDP-bound conformations (29). Rab3a is enriched on synaptic vesicles and dissociates from synaptic vesicles during exocytosis (30, 31). Studies in mutants have shown that Rab3a regulates neurotransmitter release during the late steps in exocytosis (32–35). Rab5 participates in ligand sequestration at the plasma membrane and in the motility of endosomes, whereas Rab8 controls the traffic between the trans-Golgi network and the plasma membrane (29).

Exposure to the environmental toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causes selective dopaminergic neuron degeneration in the substantia nigra and loss of dopaminergic terminals in the striatum (36, 37). Chronic exposure to the pesticide rotenone, a highly selective complex I inhibitor, can reproduce anatomical, 

In the present study, we have examined modifications in \( \alpha \)-synuclein solubility and aggregation in transgenic mice expressing wild mouse \( \alpha \)-synuclein and in mutant A30P human \( \alpha \)-synuclein mice (Tg5093 line: Tg) when compared with age-matched wildtype controls. We have also tested the hypothesis that A30P mutant human \( \alpha \)-synuclein may have abnormal interactions with members of the Rab family, including Rab3a, Rab5, and Rab8, thus potentially compromising synaptic vesicle trafficking, endocytosis, and \( \alpha \)-synuclein transport. Tg and wild mice have also been subjected to MPTP or to rotenone treatment to study the effects on A30P \( \alpha \)-synuclein solubility, aggregation, and interaction with Rab target proteins.

**MATERIALS AND METHODS**

**Transgenic Mice**

The generation of Tg mice has been previously described (25). In brief, the cDNA encoding human \( \alpha \)-synuclein containing the A30P mutation was expressed in C57B/6jxSJL F3 hybrid mice using a hamster prion protein (PrP) cosmid vector in which the PrP open reading frame was replaced with the human \( \alpha \)-synuclein open reading frame. This vector has been previously shown to drive transgene product expression throughout neurons in the brain, including the substantia nigra and other vulnerable neural populations in Lewy body disorders. Subsequent generations of mice were produced using C57B/6jxSJL F1 breeders.

The Tg mouse line demonstrates several features of the human synucleinopathies, including a progressive motor disorder characterized by an age-dependent appearance of unilateral and then bilateral tremor, rigidity, and dystonic posturing affecting limbs and axial muscles, accumulation of \( \alpha \)-synuclein in the soma and proximal neurites of neurons, and ubiquitin-immunoreactive astrocytes. However, certain details of these features are distinct from those in humans, most conspicuously the finding that the nigrostriatal dopaminergic system is preserved in these animals and that they do not develop Lewy body-like neuronal inclusions (25). The most prominent neuropathological feature of these mice is an age-related gliosis beginning in the hippocampal formation at the age of 7 to 8 months that spreads to the cerebral cortex, subcortical regions, brainstem, and spinal cord. In addition, impaired short-term changes in synaptic strength have been documented in hippocampal slices from Tg mice (26).

**MPTP and Rotenone Treatment**

Groups of Tg and wildtype mice at ages 3 to 4 months (MPTP or saline) and 6 to 10 months (rotenone or DMSO) received one of the following chronic treatment regimens: group I (n = 4), daily intraperitoneal injection of 16 mg/kg MPTP-HCl in 0.9% saline for 5 consecutive days (total dose = 80 mg/kg); group II (n = 4), daily intraperitoneal injection of 0.9% saline alone for 5 consecutive days; group III (n = 3) intraperitoneal injections of rotenone in DMSO twice a week for 3 consecutive weeks (total dose = 0.250 mg/kg); and group IV (n = 2), intraperitoneal injections of DMSO alone twice a week for 3 consecutive weeks. Animals were killed at 7 and 30 days after the last injection of MPTP or rotenone, respectively. All the animals included in this study were asymptomatic.

**Tissue Preparation**

Mice were killed under \( \text{CO}_2 \) and the entire brain was removed and snap-frozen in dry ice.

**\( \alpha \)-Synuclein Solubility and Aggregation**

\( \alpha \)-Synuclein aggregates were isolated as previously described for glial cytoplasmic inclusions in multiple system atrophy, with slight modifications (39, 40). Brain samples (0.06 g) from treated and untreated Tg and wildtype mice were homogenized in a glass homogenizer in 500 \( \mu \)l of ice-cold PBS\(^+\) (sodium phosphate buffer, pH 7.0, plus protease inhibitors), sonicated, and centrifuged 5,000 rpm at 4°C for 10 min. The pellet was discarded and the resulting supernatant was ultracentrifuged at 37,000 rpm at 4°C for 1 hour. The supernatant (S2) was kept as the PBS-soluble fraction. The resulting pellet was re-suspended in a solution of PBS, pH 7.0, containing 0.5% sodium deoxycholate, 1% Triton, and 0.1% SDS and was ultracentrifuged at 37,000 rpm at 4°C for 1 hour. The resulting supernatant (S3) was kept as the deoxycholate-soluble fraction. The corresponding pellet was re-suspended in a solution of SDS 2% in PBS and maintained at room temperature for 2 hour. Immediately afterwards, the samples were centrifuged at 37,000 rpm at 25°C for 1 hour, and the resulting supernatant (S4) was the SDS-soluble fraction. Finally, the corresponding pellet was re-suspended in a solution of 5% SDS-urea 8 M and incubated by rotation O/N at room temperature. After centrifugation at 37,000 rpm at 25°C for 1 hour, the pellet was discarded and the supernatant (S5) was kept as the urea-soluble fraction.
Equal amounts of each fraction were mixed with reducing sample buffer and processed for 10% SDS-PAGE electrophoresis and Western blot analysis. The membranes were incubated with anti-α-synuclein (Chemicon, Barcelona, Spain) at a dilution of 1:1,000. The protein bands were visualized by the electrogenerated chemiluminescence (ECL) method (Amersham, Barcelona, Spain).

Rab Pull-Down Assay

Brain tissue (0.06 g) from treated and untreated Tg and wildtype mice was homogenized in 600 μl of buffer composed of 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl, and 1% Nonidet P-40, and then sonicated. After centrifugation at 5,000 rpm for 10 min at 4°C, the pellet was discarded and the supernatant S1 was ultracentrifuged at 37,000 rpm for 1 hour at 4°C. The pellet was discarded and the supernatant S2 was pre-cleared with 30 μl of nickel beads. The resulting fraction was divided into 3 equal volumes (200 μl), which were incubated with previously blocked nickel beads and 1 μg of His/Flag-tagged Rab3, Rab5, or Rab8 for 1 hour at 4°C while shaking. Following centrifugation at 12,000 rpm for 30 s, the precipitates were washed with ice-cold PBS and re-suspended with sample buffer.

Gels were transferred to nitrocellulose membranes (Bio-Rad, Barcelona, Spain) and blotted with anti-Flag (Sigma, Barcelona, Spain) at a dilution of 1:1,000, anti-α-synuclein (Chemicon) at a dilution of 1:1,000, or anti-α-actin (Sigma) used at a dilution of 1:1,000. Total homogenates and pull-down samples were processed in parallel. A lane containing the S2 fraction and only nickel beads was used as a control for the pull-down assay.

Rab5 Pull-Down with Recombinant α-Synuclein

Recombinant 61–140 wildtype α-synuclein and mutant A30P human α-synuclein (Calbiochem, Barcelona, Spain), both at 1 mg/ml in PBS, were incubated with previously blocked nickel beads and different amounts of His/Flag-tagged Rab3, Rab5, or Rab8 for 1 hour while shaking. Following centrifugation at 12,000 rpm for 30 s, the precipitates were washed with ice-cold PBS and re-suspended with sample buffer.

Gels were transferred to nitrocellulose membranes (Bio-Rad) and blotted with anti-Flag (Sigma) at a dilution of 1:1,000 or anti-α-synuclein (Chemicon) at a dilution of 1:1,000. Twenty μg of nonpulled human recombinant α-synucleins (wildtype and A30P mutant forms) were processed in parallel. A lane containing only the recombinant A30P mutant α-synuclein (20 μg) and nickel beads was used as a control for the pull-down assay.

Rab3a Immunoprecipitation and Immunoblot

Brain tissue (0.02 g) from Tg and wildtype mice was homogenized with 7 volumes of immunoprecipitation buffer (20 mM Tris/Cl pH: 8.0, 1 mM EDTA, 150 mM NaCl, and 1% Nonidet p-40) and then sonicated. After centrifugation at 5,000 rpm for 10 min at 4°C, the pellet was discarded and the supernatant S2 was pre-cleared with 40 μl protein G-sepharose for 30 min at 4°C. The protein concentration was determined by the Bradford method, and 2.5 mg of the pre-cleared sample was incubated at 4°C overnight with anti-Rab3 antibody (Santa Cruz Biotechnology, Madrid, Spain) immobilized with G-sepharose beads (60 μl). After washing, bound proteins were eluted by boiling the beads in SDS sample buffer without mercaptoethanol. The sample buffer consisted of 3.8 ml of de-ionized water, 0.5 M Tris-HCl, pH: 6.8, 1.0 ml 0.8 ml glycerol, 1.6 ml SDS 10% (w/v) and 0.4 ml bromophenol blue 1% (w/v). Then the samples were subjected to 12% SDS-PAGE electrophoresis followed by Western blot analysis with anti-Rab3a (monoclonal, Dr. J. Blasi), anti-α-synuclein (Neomarkers, Bionova, Barcelona, Spain), anti-rabphilin (Transduction Lab, Lexington, KY) and anti-β actin (Sigma). The anti-α-synuclein antibody recognizes human α-synuclein but not mouse α-synuclein. The antibodies were used at dilutions of 1:1,000, 1:2,000, 1:100, and 1:1,000, respectively. The protein bands were visualized with the ECL method (Amersham). Total homogenates were processed in parallel with a lane containing the antibody used for immunoprecipitation and protein G-sepharose with no sample.

In addition, Rab3a immunoprecipitation assays were carried out with normal human cerebral cortex obtained at postmortem following generous donation for research to the Institute of Neuropathology and Brain Bank of Bellvitge, and according to the recommendations of the local ethics committee. Rab3a immunoprecipitation was carried out with Rab3a antibody (Calbiochem) 2 μg/ml. Western blot analysis was conducted with Rab3a monoclonal antibody (Dr. J. Blasi) used at a dilution of 1:100 and with the monoclonal anti-α-synuclein antibody (Neomarkers) used at a dilution of 1:100.

RESULTS

α-Synuclein Aggregates

α-Synuclein (19 kDa) was mainly recovered in the PBS-soluble fraction, whereas small amounts were recovered in the deoxycholate- (DC-) and SDS-soluble fractions in untreated Tg and wildtype mice (Fig. 1). The amount of α-synuclein was higher in Tg, as previously described in these mice (25). In addition, α-synuclein-immunoreactive bands of approximately 34 and 70 kDa were observed only in the PBS-soluble fractions of Tg mice. No similar bands were recognized in untreated wildtype mice (Fig. 1). Interestingly, MPTP-treated Tg and wildtype mice produced α-synuclein aggregates distributed in the PBS-, DC-, and SDS-soluble fractions. Aggregates were also observed in rotenone-treated Tg and wildtype mice. Yet the molecular weight of the bands differed in the 2 experimental models. Several bands of variable molecular weight were found in the PBS-soluble fraction in rotenone-treated mice, whereas bands of 45 kDa were recovered in MPTP-treated mice. Bands of about 66 kDa in the PBS-, DC-, and SDS-soluble fractions were equally observed in MPTP-treated and rotenone-treated Tg and wildtype mice (Fig. 1). No bands were found in the urea-soluble fraction in untreated and treated Tg and wildtype mice (Fig. 1). The same results were observed in several consistent blots.
**α-synuclein aggregates**

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**Fig. 1.** α-Synuclein solubility and aggregation in brain homogenates of untreated, MPTP-treated, and rotenone-treated mice, and blotted for α-synuclein. Samples from 2 groups of mice were processed in parallel, with Tg5093 mice expressing wildtype mouse α-synuclein and mutated human A30P α-synuclein (Tg), and wildtype littermates of transgenic mice expressing only wildtype mouse α-synuclein (W). Several fractions were recovered in the different lanes: PBS-, deoxycholate- (DC-), SDS-, and urea (U)-soluble fractions. In addition to the larger amount of PBS-, DC-, and SDS-soluble α-synuclein of 19 kDa in Tg compared with W mice, bands of high molecular weight of 34 kDa and about 70 kDa are detected in the PBS-soluble fraction of Tg mice. Treatment with MPTP results in the formation of high molecular weight α-synuclein aggregates (45 and 66 kDa) and modifications in α-synuclein solubility in both W and Tg mice. Treatment with rotenone results in the formation of different synuclein aggregates that are multiple in the PBS-soluble fraction and of approximately 50 kDa in the PBS-, DC-, and SDS-soluble fractions in both W and Tg mice. In no case was α-synuclein recovered in the urea-soluble fraction.
Rab Pull-Down Assays

Pull-down assays were carried out by using His/Flag-tagged Rab3a, Rab5, and Rab8 in brain samples of untreated, MPTP-treated, and rotenone-treated α-synuclein Tg and wildtype mice.

α-Synuclein, which manifested as a band of approximately 19 kDa with specific antibodies that recognize human and mouse α-synuclein, was recovered in total brain homogenates of untreated wildtype and Tg mice. Pull-down assays showed α-synuclein-specific bands of 19 kDa in pulled samples of untreated Tg mice. No such bands were observed in pulled samples of untreated wildtype mice. No signal was obtained in the lane corresponding to the S2 fraction incubated with nickel beads but without His/Flag-tagged Rab (Fig. 2). The same membranes were immunostained for Flag that recognizes common sequences of Rab proteins. Flag immunoreactivity in Rab3a, Rab5, and Rab8 pull-down assays was similar in wildtype and Tg mice (Fig. 2), thus showing that differences in α-synuclein immunoreactivity were not due to different Rab protein loading in pull-down assays. Control of protein loading was tested by incubating the membranes with anti-β-actin. In spite of similar protein loading in the lanes corresponding to wildtype and Tg total brain homogenates, as revealed by the β-actin signal, the amount of α-synuclein was higher in Tg than in wildtype mice (Fig. 2).

Rab pull-down assays in MPTP- and rotenone-treated rats showed α-synuclein-specific bands of 19 kDa in pulled samples of MPTP-treated and rotenone-treated Tg mice, but not in pulled samples of MPTP-treated and rotenone-treated wildtype mice. No signal was obtained in the lane corresponding to the S2 fraction incubated with nickel beads but without His/Flag-tagged Rab (Fig. 3).

To demonstrate that the bindings observed in these pull-down experiments were specifically due to mutated α-synuclein and to rule out the possibility that the amount of protein was not responsible for the differences between wildtype and Tg mice, Rab5 pull-down assays were carried out using recombinant wildtype and recombinant A30P mutant α-synuclein. Pull-down assays incubated with the anti-α-synuclein antibody revealed a specific band of 19 kDa in nonpulled total homogenates of wildtype and Tg mice and in Rab5-pulled samples with recombinant mutant A30P α-synuclein. However, no bands were observed in Rab5-pulled samples with recombinant wildtype α-synuclein (Fig. 4). Yet Flag-immunoreactive bands were recovered equally in His/Flag-tagged Rab5 pull-down assays with recombinant wildtype and A30P mutant α-synucleins (Fig. 4).

Rab3 Immunoprecipitation Assays

To control and corroborate the results of pull-down assays, Rab3a immunoprecipitation studies were carried out in Tg and wildtype mice. Samples were run in parallel with total brain homogenates of Tg and wildtype mice. Rab3a-pulled samples immunoblotted for anti-Rab3a showed similar bands at the appropriate molecular weight in Tg and wildtype samples; yet α-synuclein was recovered only in pulled Tg samples and total Tg. Since this antibody does not recognize mouse synuclein, no signals were recovered in wildtype samples (Fig. 5). Membranes immunoblotted for rabphilin disclosed similar immunoreactive bands in Tg and wildtype samples, thus indicating Rab3a/rabphilin binding.

To test that Rab3a/α-synuclein interactions in Tg mice were due to mutant/α-synuclein and not to wildtype human synuclein, immunoprecipitation studies with Rab3a were carried out in control human cortex. Rab-3a was recovered in immunoprecipitated samples but α-synuclein was not recovered in Rab3a-immunoprecipitated samples, although it was present in total brain homogenates (Fig. 6).

DISCUSSION

The present study has shown α-synuclein aggregates in brain homogenates of Tg mice expressing wild mouse α-synuclein and mutant A30P human α-synuclein (Tg), but not in brain homogenates of age-matched nontransgenic mice (wildtype). Modifications of α-synuclein solubility have been previously observed in human Lewy body diseases and these are recapitulated in transgenic mouse models (42–46). Changes in α-synuclein solubility and α-synuclein aggregation occur in brain homogenates in wildtype and Tg mice treated with MPTP or with rotenone. These results complement previous observations showing α-synuclein upregulation and increased capacity of α-synuclein aggregation in target neurons following MPTP treatment in mice and baboons (47, 48). Similarly, the present results further support α-synuclein aggregation following rotenone exposure (49).

Rab3a is exclusively associated with synaptic vesicles and dissociates from membranes during exocytosis (29, 30, 50–52). Rab3a null-mutant Caenorhabditis elegans (33) and Rab3a-null mice (32) are viable, suggesting non-essential functions for Rab3. Yet Ca2+-triggered fusion of vesicles is altered in the absence of Rab3 in mice (32), whereas Rab3-null worm mutants have fewer synaptic vesicles, especially near the active zone, but more synaptic vesicles at ectopic sites (33), suggesting that the transport of synaptic vesicles at the nerve terminal is impaired in the absence of Rab3a. Recent data indicate that Rab3a regulates a late step in synaptic vesicle fusion (34).

The present study has also shown that mutant A30P human α-synuclein, but not wildtype α-synuclein, interacts with Rab proteins Rab3a, Rab5, and Rab8, as seen in Rab pull-down assays. This binding is not dependent on the amount of Rab protein used in the assay, as Rab5 pull-down with recombinant wildtype and with mutant...
Rab pull-down in non-treated mice

Fig. 2. Pull-down studies using His/Flag-tagged Rab3, Rab5, and Rab8 in untreated wildtype littermates (W) and Tg5093 (Tg) mice processed for α-synuclein immunoreactivity show characteristic bands of 19 kDa in Tg mice, while no bands are present in W mice. Membranes were processed in parallel with total brain homogenates (tW and tTg) showing specific bands of 19 kDa in W and Tg mice. These homogenates were also processed with nickel beads alone as a control for the pull-down experiment and no specific bands were observed. The same membranes were incubated with anti-Flag antibodies that showed similar bands in pull-down samples obtained from W and Tg mice, indicating similar Rab protein content in pulled W and Tg samples. Finally, control of protein loading in the lanes corresponding to the total brain homogenates was tested by incubating the membranes with antibodies to β-actin.

A30P α-synuclein shows α-synuclein immunoreactivity not only with the mutant form, but also Flag immunoreactivity (which recognizes Rabs) in the lanes corresponding to wildtype and mutant α-synuclein. Nor is binding of mutant α-synuclein with Rab dependent on the amount of α-synuclein, as Rab5 pull-down assays with increasing doses of recombinant wildtype and mutant α-synucleins demonstrates Rab5 binding only with the mutant form even in conditions in which the system is saturated for mutant α-synuclein.
Rab pull-down in MPTP- and rotenone-treated mice

Pull-down studies have been corroborated by Rab3a immunoprecipitation assays in which α-synuclein from Tg mice, but not α-synuclein from wildtype mice, interacts with Rab3a. Yet Rab3a interacts with rabphilin, as is well documented (53, 54). Finally, Rab3a/α-synuclein interactions are not simply related to the presence of human α-synuclein because no Rab3a/α-synuclein binding has been previously reported in normal conditions, and no similar interactions have been here observed in Rab3a immunoprecipitation assays with control human cortical samples. Therefore, combined pull-down and immunoprecipitation methods demonstrate that mutant synuclein interacts with Rabs in Tg mice.

These data, together with observations derived from α-synuclein and Rab3a mutants, permit us to delineate a scenario in which α-synuclein and Rab3a play complementary and independent functions in the regulation of synaptic vesicle trafficking. Lack of α-synuclein or Rab3a is associated with impaired synaptic vesicle transport, whereas mutated α-synuclein results in abnormal binding with Rab3a, thus presumably contributing to abnormal synaptic function. Consistent with this, it is worth stressing that short-term hippocampal synaptic plasticity is altered in the transgenic mice used in the present study (26).

Defective sequestration of dopamine into vesicles, leading to the generation of reactive oxygen species in...
Pull-down studies using His/Flag-tagged Rab5 and recombinant wildtype and A30P mutant human α-synuclein processed with α-synuclein antibodies show bands of 19 kDa in lanes with A30P mutant α-synuclein. No bands were observed in the pull-down assay with wildtype α-synuclein. The same membranes processed for anti-Flag show similar bands at the appropriate molecular weights in wildtype and Tg mice. Increasing amounts (10, 20, and 50 μg) of α-synuclein (wildtype and A30P-mutant) were processed with His/Flag-tagged Rab5. Twenty μg of wildtype and A30P mutant α-synuclein without Rab5 were used as a control for the pull-down assay. Early saturation explains similar signals for the different doses of α-synuclein in this particular assay.

the cytoplasm, has been suggested as playing a role in the degeneration of dopaminergic neurons (55). Furthermore, cells expressing mutant α-synuclein are more sensitive to a variety of toxic insults (56) and may cause increased susceptibility to dopaminergic toxicity (11, 12, 57). Recent studies have shown that Rab5 participates in the endocytosis of α-synuclein and dopamine (58). Binding of mutant α-synuclein to Rab5, as observed in the present study, may account for abnormal endocytosis of selected neurotransmitters and may increase dopamine toxicity in substantia nigra pars compacta neurons.

Rab8 participates in the trans-Golgi network plasma membrane traffic (29). Therefore, it can be suggested that mutant α-synuclein binding to Rab8 may have consequences on α-synuclein traffic in the cytosol. An interesting aspect in several transgenic models, including the present transgenic mice (25), is the translocation of mutant α-synuclein to the cytosol. Although several factors may be involved in this process, abnormal interactions of mutant α-synuclein with Rab8 may account for α-synuclein deposition in the cytoplasm.

Finally, in contrast to their effects on α-synuclein solubility and aggregation, MPTP and rotenone do not have any effect on the interaction of α-synuclein with Rab proteins. It is not the effect of the toxicity but rather the nature of α-synuclein (mutant α-synuclein in the present paradigm) that gears the fate of α-synuclein binding with Rab3a, Rab5, and Rab8.
Fig. 5. Rab3a immunoprecipitation assay and Western blot (ipRab3a) from Tg and wildtype (W) mice processed in parallel with total brain homogenates (tTg and tW) show specific Rab3a bands at the appropriate molecular weight. No signal is observed in the lane corresponding to anti-Rab3a and protein G sepharose (Rab3a + protG). Yet, α-synuclein is found only in Tg mice, thus indicating Rab3a/α-synuclein binding in Tg but not in W mice. In contrast, rabphilin immunoreactivity is recovered in total homogenates and immunoprecipitated samples from both Tg and W mice. Finally, protein loading in total homogenates was tested with β-actin.

Yet, we have recently shown that Tg mice are more vulnerable than littermate controls to MPTP in a dose-dependent manner. Tg mice exhibit higher mortality rates, more accentuated loss of TH-positive neurons in the substantia nigra pars compacta, and more pronounced decrease in striatal dopamine and TH levels when compared to age-matched littermate controls. These findings support the idea that over-expression of mutant A30P α-synuclein in Tg mice increases the vulnerability of their dopaminergic nigrostriatal system to MPTP (unpublished observations). The mechanism by which over-expression of A30P α-synuclein in Tg mice enhances their sensitivity to MPTP remains to be elucidated. It has been previously shown that the selective toxicity of MPP+ for DA neurons derives, at least in part, from its high affinity for the DA transporter (DAT). Intraneuronally, MPP+
Rab3a immunoprecipitation in human cortex

Fig. 6. Rab3a immunoprecipitation assay and Western blot in human cortex (ipC) discloses specific Rab3a bands at the appropriate molecular weight in total homogenates (tC) and Rab3a-immunoprecipitate sample (ipC). No signal is observed in the lane corresponding to anti-Rab3a and protein G sepharose (Rab3a + prot G). α-Synuclein is found only in total homogenates but not in ipC samples.

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can be sequestered into synaptic vesicles by the vesicular monoamine transporter (VMAT) and sequestration into vesicles decreases MPP⁺ toxicity by preventing its interaction with the mitochondria. According to this model, cellular vulnerability to MPTP in Tg mice might occur if level and activity of DAT or VMAT were altered in these mice. We have previously demonstrated that VMAT-2 binding is unaffected in the striatum of Tg mice (25). It is possible, though, that a compromise at other steps of membrane trafficking pathways could account for the increased dopaminergic neuron vulnerability seen in these mice. In this sense, the abnormal interaction between α-synuclein and Rab proteins in Tg mice described here could potentially interfere with sequestration of MPP⁺ into synaptosomal vesicles, enhancing its direct interaction with mitochondria and thus its toxicity. In addition, the finding that these abnormal interactions occur at presymptomatic clinical stages preceding the motor dysfunction that characterizes the phenotype of Tg mice suggests that they could potentially underlie the clinical and electrophysiological abnormalities found in this transgenic line.
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