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α-Synuclein Expression Levels Do Not Significantly Affect Proteasome Function and Expression in Mice and Stably Transfected PC12 Cell Lines*

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α-Synuclein (α-syn) is a small protein of unknown function that is found aggregated in Lewy bodies, the histopathological hallmark of sporadic Parkinson disease and other synucleinopathies. Mutations in the α-syn gene and a triplication of its gene locus have been identified in early onset familial Parkinson disease. α-Syn turnover can be mediated by the proteasome pathway. A survey of published data may lead to the suggestion that overexpression of α-syn wild type, and/or their variants (A53T and A30P), may produce a decrease in proteasome activity and function, contributing to α-syn aggregation. To investigate the relationship between synuclein expression and proteasome function we have studied proteasome peptidase activities and proteasome subunit expression (α, β-constitutive, and inducible) in mice either lacking α-syn (knockout mice) or transgenic for human α-syn A30P (under control of PrP promoter, at a time when no clear gliosis can be observed). Similar studies are presented in PC12 cells overexpressing enhanced yellow fluorescent protein fusion constructs of human wild type, A30P, and A53T α-syn. In these cell lines we have also analyzed the assembly of 20 S proteasome complex and the degradation rate of a well known substrate of the proteasome pathway, IκBα. Overall the data obtained led us to the conclusion that α-synuclein expression levels by themselves have no significant effect on proteasome peptidase activity, subunit expression, and proteasome complex assembly and function. These results strengthen the suggestion that other mechanisms resulting in synuclein aggregation (not simply expression levels) may be the key to understand the possible effect of aggregated synuclein on proteasome function.

Idiopathic Parkinson disease (PD),[1] dementia with Lewy bodies, a Lewy body variant of Alzheimer's disease, and multiple system atrophy are characterized pathologically by proteinaceous inclusions commonly referred to as Lewy pathology in postmortem brain tissue samples (1, 2). The inclusions occur in the dystrophic (Lewy) neurites that constitute an important part of the pathology of PD and dementia with Lewy bodies. α-Synuclein is the major constituent of Lewy bodies and Lewy neurites (3–5). The initial discovery of two point mutations (A53T and A30P) in the α-syn gene linked to early onset familial PD (6), together with the recently described E46K mutation (7), the ability of the protein to self-aggregate (8), and recent findings *in vivo* in transgenic flies and mice (see for review Refs. 9 and 10) are supporting a central role for α-synuclein in the pathophysiology of diseases with Lewy body pathology.

The proteasome pathway can mediate the degradation of α-syn. The work of several authors (11–13) clearly indicates that α-syn can be directly degraded by the 20 S proteasome without requirement of prior ubiquitylation, even when fused to glutathione *S*-transferase or enhanced green fluorescent protein (13). Accordingly a decrease in proteasome activity may impair α-syn degradation and then facilitate its aggregation. Supporting the above conclusion several reports have described that proteasome inhibitors promote α-syn aggregation in neurons (14–16), neuronal-like cell lines (12, 17–19), and non-neuronal cell lines (20, 21). These results by themselves are not unexpected, as many different pathogenic proteins aggregate when they are overexpressed in cells as a consequence of proteasome inhibition (see for example Ref. 22). The possible relevance of proteasome activity in PD pathology is highlighted by reports that describe a decrease in the proteasome peptidase activity in the substantia nigra of sporadic PD patients (23, 24), accompanied with a decreased expression of proteasomal α-subunits (but not β-type subunits) of the proteasome in the same brain region (25). Nevertheless, other authors have found no change of proteasome peptidase activity in human brain regions with dementia with Lewy bodies pathology (26). Another line of circumstantial evidence is the report by several groups that synuclein overexpression produces a decrease of proteasome peptidase activity when assayed in crude extracts obtained from stably transfected cell lines (17, 19, 27), and *in*

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[1] The abbreviations used are: PD, Parkinson disease; α-syn, α-synuclein; Tg, transgenic; wt, wild type; EYFP, enhanced yellow fluorescent protein; KO, knock-out.

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vitro studies reporting that α-synuclein (and the aggregated form of α-syn at lower concentrations) inhibits the peptidase activity of the proteasome (27, 28). The possible pathological connection between synuclein overexpression and human PD is also suggested by the recent finding of a gene triplication in the so called "Iowa kindred" with an autosomal-dominant familial form of PD (29) and by α-syn overexpression in the substantia nigra of a set of PD patients (30), whereas α-syn overexpression in sporadic PD patients has not been confirmed in other studies (see Ref. 31 and references within)

One emergent suggestion from all these data is that α-synuclein overexpression may directly produce a decrease in proteasome function, initiating a positive reinforcing loop that results in the formation of aggregated synuclein that will promote further proteasome inhibition and synuclein aggregation. As a consequence we decided to explore the relationship between synuclein expression levels and proteasome function. To that end we have studied proteasome peptidase activities and proteasome subunit expression in mice, either lacking α-syn (knock-out mice), or transgenic for human α-syn A30P, and also in PC12 cells overexpressing EYFP fusion constructs of human wild type, A30P, and A53T α-syn. Finally to address the issue of proteasome function *in situ* we have analyzed the assembly of 20 S proteasome and the degradation rate of a well known substrate of the proteasome, Iκbα, in the α-syn overexpressing PC12 cell lines. The data obtained clearly suggest that synuclein expression levels by themselves have no significant effect on proteasome expression and function.

MATERIALS AND METHODS

Plasmid Constructs—Human wild type α-syn cDNA was obtained by PCR with the appropriate oligonucleotides from a human placental cDNA library and cloned into the NdeI and SalI sites of pT7-7 vector. pT7-α-syn A30P and A53T were also obtained by PCR with QuikChange™ site-directed mutagenesis kit from Stratagene. pEYFP-α-syn was obtained by subcloning the amplified α-syn into the XhoI site of pEYFP-C1 vector. The untagged versions of synuclein were obtained by subcloning the amplified α-syn into the NheI/XhoI sites of pcDNA3.1 Zeo. The constructs of variants syn A30P and A53T were obtained by PCR using the same mutagenesis kit from Stratagene. All constructs were fully sequenced by the chain termination method.

Synuclein Purification and Generation of Polyclonal Antibodies—Wild type, A30P, and A53T synucleins were purified essentially as described (32), adding one final step of purification by loading α-synuclein into a DEAE-5PW HPLC column and elution with a linear gradient from 50 mM to 0.6 M NaCl in 50 mM Tris-Cl, pH 7.5. All proteins were dialyzed against ddH_20 or 25 mM Tris-Cl, pH 7.5, and stored frozen at −70 °C until use. Antibodies against α-syn were obtained in rabbits by immunization with the purified protein (100 μg/injection), as described (33).

Mouse Animal Models—Synuclein knock-out mice (−/−) and their corresponding control mice (+/+) have been described previously (34). Transgenic mice for human A30P α-syn under the control of PrP promoter (C57B/6jxSJL F3, Tg5093, and Tg5102, Tg+/−) and the corresponding control mice (Tg −/−) have been described previously (35), the samples analyzed were from 4-month old transgenic mice where no substantial gliosis is apparent (35). Mice were anesthetized and whole brains were removed. Fresh brains were subjected to dissection for obtaining the cerebral cortex, cerebellum, striatum, and ventral midbrain (containing the substantia nigra and ventral tegmental area). All tissue samples were immediately frozen in liquid nitrogen and stored at −80 °C until processed.

Transfected Cell Lines, Metabolic Labeling, and Immunoprecipitations—Rat PC12 cells were transfected with pEYFP-syn plasmids, and stable transfectants were selected with Geneticin and further enriched by fluorescence-activated cell sorting. The expression of EYFP-α-syn (syn wt, A30P, and A53T) fusion proteins was analyzed by Western immunoblotting of total cell extracts prepared by direct lysis of cells in SDS-loading buffer and separation on 14% SDS-PAGE (see below). Protein turnover was studied by the treatment of cells with cycloheximide (50 μg/ml) for the times indicated, and immunoblotting of total cell extracts with anti-Iκbα, as indicated above. Cells were metabolically labeled with 200 μCi/ml of [^{35}S]methionine/cysteine (Amersham Biosciences) for 1 h (pulse) in Dulbecco's modified Eagle's medium without methionine/cysteine and then chased for different periods of time with complete medium. At the times indicated, cells were washed with cold phosphate-buffered saline (2 ×) and lysed in immunoprecipitation buffer (Tris-buffered saline (50 mM Tris-Cl, pH 7.5, 150 mM NaCl) containing 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, 10 μM of leupeptin, and 10 μM MG132 (Calbiochem)). Lysed cells were kept on ice for 5 min and centrifuged at 15,000 × *g* for 30 min at 4 °C to remove any insoluble material. The supernatants were used directly for immunoprecipitation. Immunoprecipitations were performed with rabbit anti-proteasome (36) or anti-Iκbα (Santa Cruz Biotechnology, sc-847) antibodies previously coupled to protein A-Sepharose (Amersham Biosciences) as described previously (36). The immunoprecipitated proteins were eluted with SDS-sample buffer and analyzed by 10% SDS-PAGE and autoradiography.

Proteasome Activity and Immunoblotting—Brain tissue samples were processed for the determination of proteasome peptidase activities and immunoblotting as follows. Samples were homogenized (1:10 w/v) with a Dounce homogenizer in an ice-chilled buffer containing 50 mM Hepes, pH7.4, 150 mM NaCl, 5 mM EDTA, 10 μM leupeptin, 1 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride. Homogenates were centrifuged at 3000 × *g* for 10 min at 4 °C, and the supernatants were used for the determination of proteasome peptidase activity and immunoblotting. Cells were scraped from plates in phosphate-buffered saline, washed two times in phosphate-buffered saline, and the cell pellets were resuspended in a buffer containing: 50 mM Hepes, pH 7.4, 50 mM NaCl, 5 mM EDTA, 10 μM leupeptin, 1 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride. Homogenization was performed either by two cycles of freezing (−70 °C) and thawing at 37 °C or with Dounce homogenizer. Cell homogenates were centrifuged at 3000 × *g* for 10 min at 4 °C, and the supernatants were used for determination of proteasome peptidase activity and immunoblotting. Peptidase activities were determined with synthetic fluorogenic peptides (*N*-Suc-LLVY-MCA, Z-LLE-MCA, and Boc-LRR-MCA) in a Fluorskan microplate reader (excitation, 380 nm; emission, 460 nm) as described (37). The reaction mixture contains in a final volume of 200 μl of 20 mM Hepes, pH 7.4, 50 μM indicated peptide, and different amounts of the corresponding brain or cell extract to be assayed. Assays were kinetically followed for 1 h, and the reaction rates were linear with respect to time and the amount of protein extract (5–20 μg of protein). Parallel reactions containing either 25 μM MG132 or 10 μM Lacatacystin were run as controls. The values reported for proteasome peptidase activities are expressed as relative fluorescence arbitrary units/min/mg of total protein ± S.D. after subtraction for nonspecific peptide hydrolysis (control reactions).

For immunoblot analysis, 20 μg of total protein was loaded onto 12% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with different antibodies. Developing was performed with peroxidase-labeled goat anti-mouse, or anti-rabbit antibodies, at 1/4000 dilution by chemiluminescence method. Anti-EYFP monoclonal antibodies were obtained from BD Biosciences (clone JL-8) and used at 1/2500 dilution. Anti-synuclein was detected with either rabbit polyclonal (1/500) or mouse monoclonal antibodies (1/2000, Transduction Laboratories, clone 42). Anti-Iκbα was used at 1/500 dilution. Anti-C2 (α6, 1/1000), anti-C5 (β6, 1/1000), anti-C8 (α7, 1/1000), and C9 (α3, 1/500) have been described previously (33, 36, 36). Anti-LMP2 (iβ1, 1/500), LMP7 (iβ5, 1/1000), MECL1 (iβ2, 1/1000), PA28α (1/1000), PA28β (1/1000), TBP7, and PSMC4 (1/500) were from Affiniti. Anti-Y (β1, 1/1000) and anti-Z (β2, 1/1000) were from Abcam. Anti-ubiquitin (1/1000) antibodies were from Dako. We also used anti-LMP2 and anti-PA28 rabbit polyclonal antibody made by us in rabbits and affinity-purified against recombinant LMP2 and PA28 as described (33). As control for normalization we used antibodies against α-tubulin (Sigma, clone DM 1a) or anti-protein disulfide isomerase antibodies that have been previously fully characterized (38). Quantification was performed by densitometric scan of the respective immunoblots by use of Quantity One software from Bio-Rad. Values are expressed as arbitrary densitometric units ± S.D.

Statistical Analysis—Results are expressed as the mean ± S.D. They were analyzed statistically by the *t* test between two groups and analysis of variance among multiple groups. Statistical significance was accepted at the $p < 0.05$ level.

RESULTS

Synuclein Expression in Wild Type, Knock-out, and Transgenic Mice—We first analyzed the expression of α-syn in brains obtained from wild type, knock-out, and transgenic mice. Results presented in Fig. 1 show, both in striatum and ventral midbrain (substantia nigra and ventral tegmental area), the

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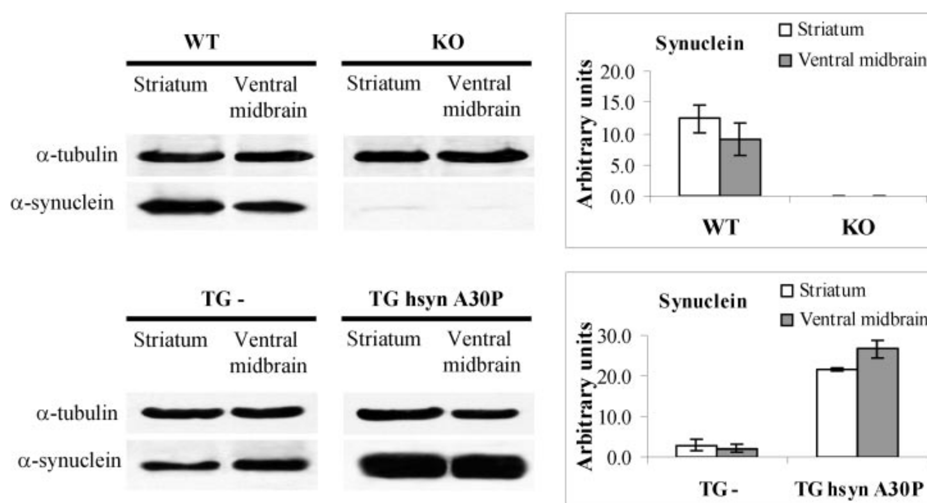

FIG. 1. **α-Syn expression in wild type, KO, and transgenic mice for α-syn.** Brain samples from the striatum and ventral midbrain (substantia nigra and ventral tegmental area) were processed for Western and immunoblot, as described under "Materials and Methods" with polyclonal anti-synuclein antibodies. *Left panels* show representative immunoblots from the different mice under study, KO, hsyn A30P transgenic, and their respective controls labeled wild type (*WT*) and *TG-*. The *right panels* show the quantitation of the immunoblots expressed in arbitrary densitometric units ± S.D. for $n = 3$ different animals, each one assayed by duplicate.

complete absence of synuclein in knock-out mice (as expected) and a 15-fold increase in the amount of α-syn in transgenic mice for human A30P α-syn under the control of PrP promoter (wide expression in all brain areas). Similar results were obtained when an analysis was performed with whole brain, cortex, and cerebellum form the different mice under study and in agreement with previous reports describing those mice (34, 35).

Proteasome Activity and Expression in Wild Type, Knock-out and Transgenic Mice—If the hypothesis that the levels α-syn expression somehow modify proteasome peptidase activity, we were in good condition to test this hypothesis in the mice under study with zero (knock-out), normal levels (wt), or high levels (15-fold, human synA30P transgenic). As shown in Fig. 2, the three peptidase activities of the proteasome in the striatum and in the ventral midbrain do not significantly change between KO and wt or between transgenic and not transgenic mice. We also found no difference in proteasome peptidase activity in whole brain, cortex, and cerebellum extracts from similar mice (data not shown). The results obtained with brain samples from the human syn A30P transgenic mice are not significantly affected by the gliosis that eventually developed in these animals, as we used heterozygous 4-month-old animals where no clear gliosis can be found (35), and we observed no change in the expression of glial fibrillary acidic protein between control and synA30P transgenic mice by immunoblot analysis (data not shown). These results clearly indicate that there is no correlation between the level of expression of α-syn and proteasome peptidase activity in mice. Nevertheless, we found that the peptidase activity of the proteasome (mainly the chymotrypsin-like activity) in ventral midbrain (substantia nigra and ventral tegmental area) is ~50–70% (analysis of variance, $p < 0.05$) of the activity observed in striatum. This difference is observed for all type of mice used in this study. This reduction of proteasome activity could be attributed to a regional brain difference in proteasome expression (see below), or to the presence of a proteasome "activator" in the striatum, or an "inhibitor" in the ventral midbrain. To test the last possibilities, we performed proteasome peptidase assays with a fixed amount of striatum extract and increasing amounts (up to 4-fold) of the ventral midbrain extract and vice versa. These experiments show additive results; the activity of the mix was equal to the sum of activities of the two extracts assayed independently (data not shown), clearly discarding the presence of an "activator" or "inhibitor" of the proteasome in the striatum and ventral midbrain, respectively.

Then we analyzed the level of expression of proteasome subunits in the same brain regions. We explored the expression of proteasome α, constitutive β, and γ-interferon-inducible subunits. Representative immunoblots of these experiments are presented in Fig. 3. The quantitation of these experiments shows very little change in the level of expression of proteasomal α (C2, α6; C8, α7; C9, α3), β (C5, β6; Y,β1; Z, β2) and β-inducible subunits (LMP2, β1i; LMP7, β5i; MECL-1, β2i) between control, KO, and transgenic animals for the same brain area. We also analyzed the levels of expression of proteasome activator PA28 α in the same brain regions (Fig. 4), PA28β (almost undetectable levels, data not shown), and a component of the 19 S proteasomal regulator TBP7 (PSMC4, data not shown), and found again no differences between control, KO, and transgenic mice. Collectively these data clearly demonstrate the lack of correlation between α-syn expression levels and proteasome subunit expression. When the overall proteasome subunit expression levels were compared between striatum and ventral midbrain (substantia nigra and ventral tegmental area), there is a small (but significant) decrease in the amount of proteasome subunits in the ventral midbrain respect to striatum (20–25%, analysis of variance, $p < 0.05$). This small decrease in level of expression may account for the lower proteasome peptidase activity observed in the midbrain region with respect to the striatum.

Proteasome Activity and Expression in PC12 Cells and Stable PC12 Transfectants with EYFP, EYFPsyn wt, EYFPsyn A30P, and EYFPsyn A53T—Next we investigated proteasome peptidase activities and subunit expression in PC12 cell lines transfected with EYFP-α-synucleins and their respective controls. Fig. 5*A* shows the results of immunoblots of the different cell lines generated with anti-synuclein antibodies. Although endogenous α-syn was expressed in all cell lines, transfected cell lines clearly overexpressed 10–20-fold higher levels of the EYFP-α-syn fusion proteins (Fig. 5*A*, *right panel*). The measurement of proteasome peptidase activities in crude extracts and immunoblots with anti-α, constitutive β, and inducible β subunits are shown in Fig. 4, *B* and *C*, respectively. Proteasome peptidase activities (Fig. 4*B*) are not significantly affected by overexpression of EYFP or the EYFP-α-syn fusion constructs,

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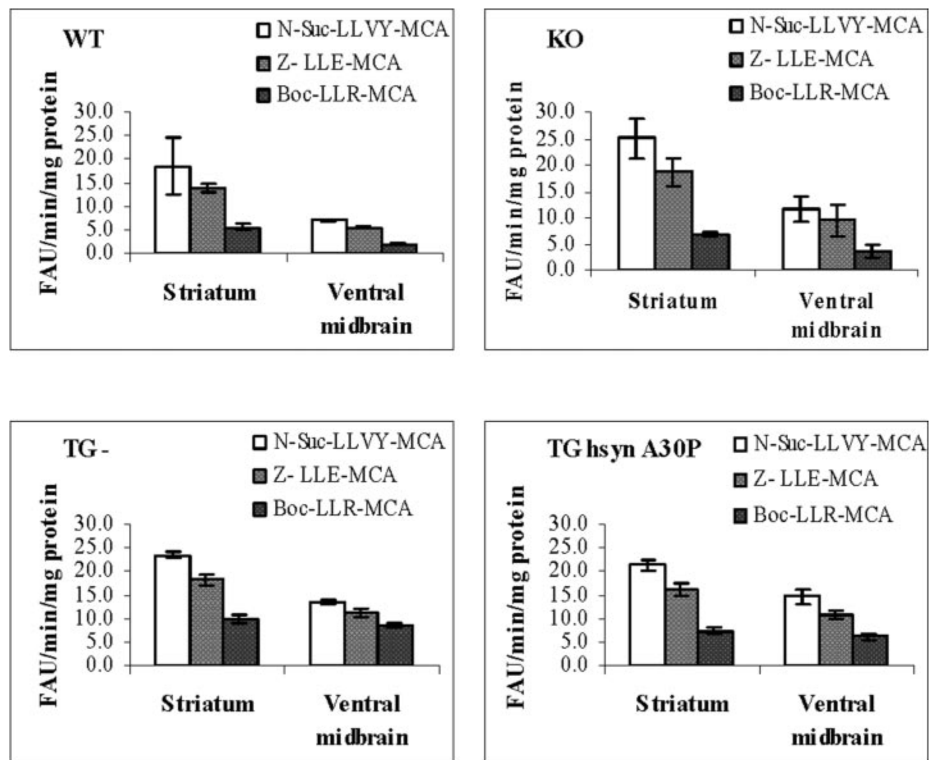

FIG. 2. **Proteasome peptidase activities in wild type, KO (*α-syn*), and transgenic mice (*hsyn A30P*).** Proteasome peptidase activities were assayed in brain samples from striatum and ventral midbrain (substantia nigra and ventral tegmental area) using model fluorogenic peptides *N*-Suc-LLVY-MCA, *N*-Suc-LLE-MCA, and LRR-MCA for assaying the chymotrypsin, postglutamyl-peptidyl-hydrolase, and trypsin-like activities as described under "Material and Methods." Values are expressed as fluorescence (arbitrary units)/min/mg total protein ± S.D. for $n = 3$ different animals, each one assayed by triplicate.

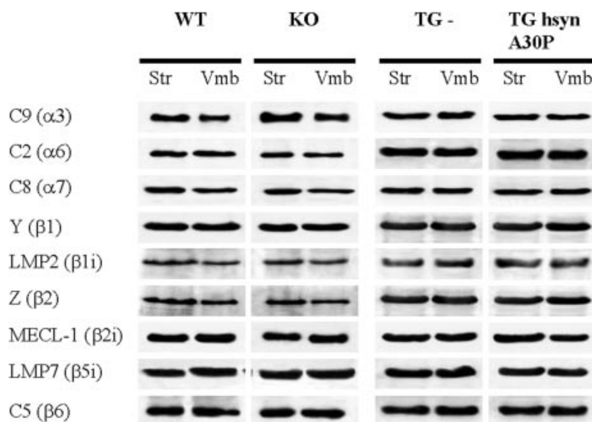

FIG. 3. **Proteasome subunit expression levels in wild type, KO (*α-syn*), and transgenic mice (*hsyn A30P*).** Samples from striatum and ventral midbrain (substantia nigra and ventral tegmental area) from different animals (representative blots are presented) were analyzed by Western immunoblotting with different proteasomal subunits, as indicated. Quantitation was done by densitometric scanning, as described under "Materials and Methods," using anti-tubulin or anti-protein-disulfide isomerase antibodies as controls for protein loading (not shown).

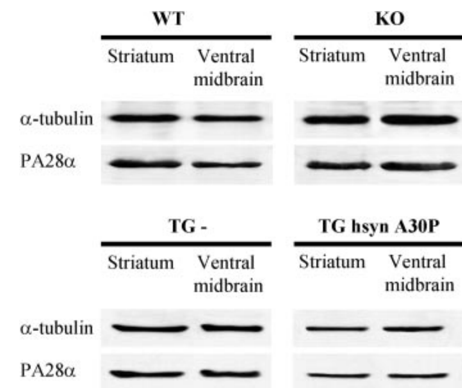

FIG. 4. **PA28 α expression levels in wild type, KO (*α-syn*), and transgenic mice (*hsyn A30P*).** Samples from the striatum and ventral midbrain (substantia nigra and ventral tegmental area) from different animals (representative blots are presented) were analyzed by Western immunoblotting with antibodies against PA28 α. Quantitation was done by densitometric scanning, as described under "Materials and Methods," using anti-tubulin antibodies as controls for protein loading.

similarly, the quantification of the immunoblots (Fig. 5*C*) shows no significant changes in the expression of proteasomal subunits. Furthermore proteasome activator PA28 α, PA28 β (expressed at very low levels in PC12) cells, and TBP7, PSMC4, also did not significantly change (data not shown).

Proteasome Assembly in PC12-transfected Cell Lines—As mentioned in the Introduction, it has been reported that in the substantia nigra of PD patients there is a decrease of proteasomal α-subunits expression with little change of β subunits (25). One possibility to explain these results is that α-syn may alter the assembly line of the proteasome. To test this hypothesis we analyzed the proteasome complex in brains from the different mice strains (wild type, KO, and transgenic) and from the different PC12-transfected cell lines by immunoprecipitation with anti-whole proteasome complex antibodies (36). The results obtained demonstrate that the proteasome complex contains both α- and β-subunits, and that no free α- or β-subunits remained in the supernatants after immunoprecipitation (data not shown). Furthermore a gel filtration experiment of crude soluble extracts from mouse brains or stably transfected PC12 cells failed to detect any free α- or β-proteasomal subunit (data not shown). To study more deeply the possible effect of α-syn overexpression in proteasome assembly, we performed pulse-chase experiments in the transfected PC12 cell lines. As shown in Fig. 6, anti-proteasome antibodies immunoprecipitated proteasome precursors after the pulse, clearly identified by the presence of pre-Z (pre-β2) in the immunoprecipitated complex and the association with the immature complex of p17, the homologue of Ump1p (36). After 8 h of chase, both in PC12 cells overexpressing EYFP and the fusion EYFP-α-syn, the mature proteasome is fully assembled with similar kinetics, as denoted by the absence of the β-subunit precursor and the p17 chaperon

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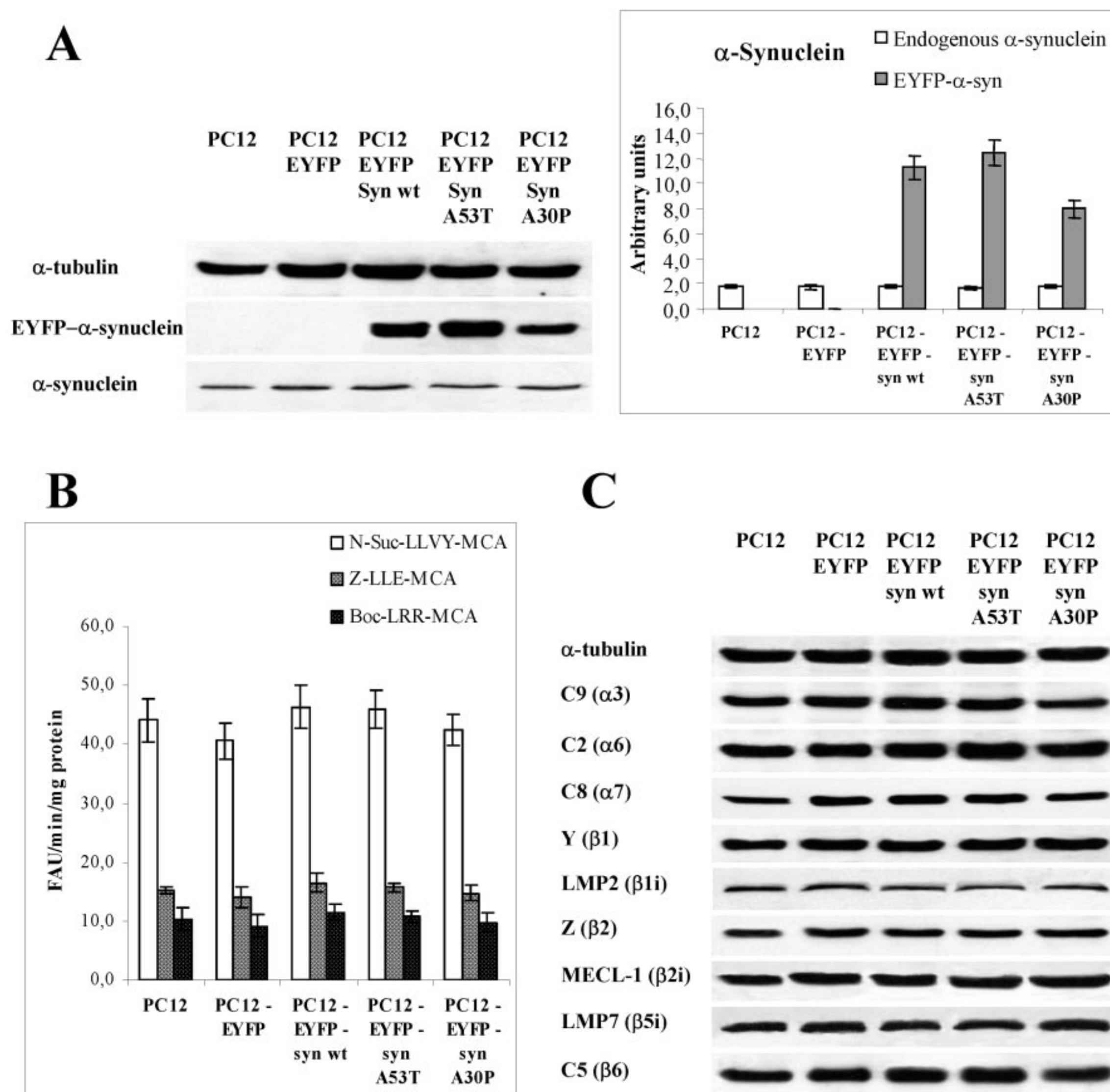

FIG. 5. **Proteasome peptidase activities and subunit expression in control and stably transfected PC12 cells.** *A*, immunoblot analysis with anti-synuclein antibodies of PC12 untransfected and stably transfected with EYFP, EYFP-syn wt, EYFP-syn A30P, and EYFP-syn A53T. The *graph* shows the quantitation in arbitrary densitometric units ± S.D. ($n = 3$). *B*, representation of the proteasome peptidase activities of the different cells lines assayed with synthetic fluorogenic peptides as indicated, results are the average ± S.D. ($n = 3$) run in triplicate. *C*, representative immunoblots of total cell extracts obtained from the different PC12 cells lines analyzed with antibodies to the indicated proteasomal subunits. For detailed description see "Materials and Methods." Quantitation was done by densitometric scanning using anti-tubulin antibodies as controls for protein loading.

(note also the incorporation of processed LMP2 subunit). These results and similar results obtained with PC12 cell lines transfected with EYFP-α-syn A30P and A53T, clearly indicated that overexpression of α-syn has no significant impact on proteasome complex assembly.

Iκbα Turnover in PC12 α-Syn-transfected Cell Lines—To study the impact of synuclein overexpression on proteasome function in a cellular context, we investigated protein ubiquitylation in the stably transfected PC12 cell lines. As judged by immunoblots of total cell proteins with anti-ubiquitin antibodies, we found no increase of ubiquitylated proteins in cells overexpressing the different EYFP-syn constructs, and all of the cell lines responded with an increase in the polyubiquitylated proteins in response to the addition of proteasomal inhibitors (data not shown). To gain further insight on the possible functional impairment of the proteasome, we decided to study the turnover of a classical short life protein, Iκbα, in PC12 cells untransfected, transfected with EYFP, or the fusion constructs EYFP-α-syn. Fig. 7*A* shows immunoblot analysis with anti-Iκbα antibodies of total cell extracts from the different cell lines under study after treatment of the cells with cycloheximide for the times indicated. The results obtained demonstrate that EYFP or EYFP-α-syn (wt, A30P, and A53T) overexpression did not significantly altered the degradation rate of Iκbα. Furthermore, overexpression of α-syn also did not affect the rapid turnover of newly synthesized IκBα as judged by pulse-chase experiments and shown in Fig. 7*B*. In both sets of experiments the inclusion of 10 μM lacatacystin completely prevents Iκbα degradation.

DISCUSSION

In the present work we have evaluated the hypothesis that α-synuclein expression levels may alter proteasome activity and function. If such a relationship exists, one would expect it to pop up clearly in the experimental conditions that have been analyzed. First we analyzed mice brains, either with no α-synuclein expression (knock-out mice), normal levels of α-synuclein (wt mice), or transgenic for human α-synuclein A30P (with 10–15-fold overexpression of α-syn A30P in many brain areas). We found no significant difference in proteasome

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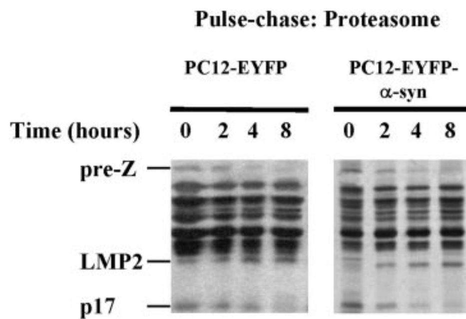

FIG. 6. **Proteasome assembly in transfected PC12 cell lines.** The figure shows an autoradiogram of immunoprecipitated proteasome complex from pulse (*0 h*)-chase (hour indicated) experiments of PC12 transfected with EYFP or EYFP-α-syn wt. *Lines indicate* the precursor of Z subunit (pre-β2) and the p17 chaperone, which disappear during the chase, a clear indication of the formation of mature proteasome complex, and LMP2 (β1i) that is progressively incorporated upon processing during maturation of the proteasome complex.

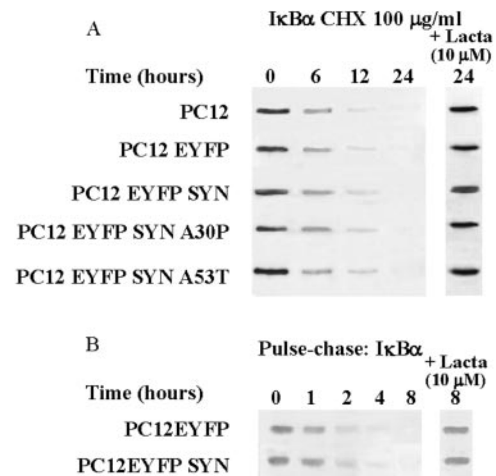

FIG. 7. **Iκbα turnover in untransfected and stably transfected PC12 cell lines.** *A*, immunoblot experiments of total cellular extracts from the different PC12 cells (untransfected or transfected with EYFP, EYFP-α-syn wild type, A30P, and A53T) that have been treated with cycloheximide for the times indicated. *B*, an autoradiogram of immunoprecipitated Iκbα from pulse (*0 h*)-chase (hour indicated) experiments of PC12 transfected with EYFP or stably transfected with EYFP-α-syn. Controls under the same conditions, but treated with lactacystin 10 μM, are also shown.

peptidase activities, α- and β-subunit (constitutive and inducible) and PA28 α expression, in the striatum and ventral midbrain (substantia nigra and ventral tegmental area) brain areas, regions considered highly relevant to PD pathology. We also did not find changes in other brain areas examined, like the cerebral cortex and cerebellum, even in brains from A30P transgenic animals that show some motor disturbances (35).

Similar to what we found in transgenic mice, the overexpression of EYFP fusion constructs of human α-syn wild type or their point variants (A30P and A53T) in PC12 cells has no significant effect in proteasome peptidase activity or expression of proteasome subunits and PA28 α. In addition, similar results were obtained with cells transfected with untagged α-syn wt and A30P variant (data not shown).

The results on proteasome peptidase activities seem in contrast with results published by other groups as briefly mentioned in the Introduction. Snyder *et al.* (27) reported a 50% decrease in the chymotrypsin-like activity in stably (but not in transiently) transfected BME-17 cells with human α-syn wt. Tanaka *et al.* (17) report a small reduction of proteasome peptidase activity in PC12 cells stably transfected with human α-syn A30P, (22.1, 17, and 24% reduction in the chymotrypsin-like, trypsin-like, and post-glutamyl hydrolase activities, respectively) but no change in peptidase activity when they overexpressed α-syn wt. Similarly, Stefanis *et al.* (19) report a small reduction of the chymotrypsin-like activity (20–35%, the only peptidase activity measured in this work) in PC12 cells overexpressing α-syn A53T, but no difference was observed in cell lines overexpressing α-syn wt. Except for the report of Snyder *et al.* (27), the results of the other two groups agree with the results presented here that overexpression of α-syn wt does not produce any significant changes in the peptidase activities of the proteasome. The reported changes in proteasome peptidase activities in crude extracts from cells overexpressing the two α-syn variants (A30P and A53T) are small, 17–35%, and no explanation was given to these findings. The amount of α-synuclein present in the crude cell extracts from overexpressing cell lines could, if measurements of proteasome peptidase activity are not done with careful control of the assay conditions, cause variable inhibition of proteasomal peptidase activities. In this context, it is worth recalling that synuclein and EYFP-synuclein fusion protein (and their synuclein variants) are direct substrates of the 20 and 26 S proteasome and that the synthetic fluorogenic substrates used for the peptidase assay of the proteasome inhibit the degradation of synuclein, and vice versa, as expected (13).[2] As the method of assay of proteasome peptidase activity varies somewhat between the different groups, we have also performed the assay of the peptidase activities in the presence of glycerol and ATP to preserve the 26 S proteasome complex and homogenizing cells by Dounce (19) and assaying the peptidase activities in the presence or in the absence of 5 mM magnesium-ATP. Once again the results showed no significant difference in the peptidase activities of the proteasome. All the data presented clearly allow us to conclude that α-syn overexpression has no clear significant effect on proteasome peptidase activity or expression. Even in the case that a small reduction of peptidase activity of the proteasome is observed in cell lines overexpressing α-syn and their variants, we have shown here that α-synuclein (wt, A30P, and A53T) overexpression does not affect proteasome assembly or the turnover rate of short half-life proteins, like Iκbα. These results clearly indicate that proteasome function is not significantly impaired by α-syn overexpression in a cellular context.

Another question is the meaning of the results presented here to the understanding of PD pathology in human brain. No extrapolation can be made directly, as factors modifying synuclein or proteasome function may occur in PD patients (aging, oxidative stress) that could alter the cell homeostasis of synuclein synthesis and disposal. In a preliminary approach we have analyzed brain samples from three PD patients with Lewy body pathology in many brain areas including substantia nigra, as it is usual observation at post-mortem studies because of the marked heterogeneity in the clinical and pathological phenotype of PD patients (39). These preliminary results confirmed our data from mice, namely that proteasome peptidase activity is also higher in human brain striatum than in the substantia nigra pars compacta. Furthermore, with respect to age-matched controls, we observed no significant changes in proteasomal subunit expression levels (either α or β, using tubulin or protein disulfide isomerase as controls of protein loading). These preliminary results, obtained with the only human samples made available to us until now, are in sharp

[2] B. Alvarez-Castelao, I. Mayo, and J. G. Castaño, unpublished results.

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contrast with the reported decrease in proteasomal peptidase activities and the unexplained reduction of expression of α-subunits (but not β-subunits) of the proteasome in the substantia nigra of patients with sporadic PD (23, 24). Our preliminary data are in better agreement with those reported by other authors that found no changes in proteasome peptidase activities in brain regions affected with dementia with Lewy body pathology (26). We believe, in agreement with the comments in a recent review (40), that the study of proteasome activity and subunit expression in the human brain from PD patients and other synucleinopathies require further detailed examination before reaching the conclusion that proteasome function is impaired in substantia nigra of PD patients.

Finally, we want to stress that the results presented in this work are not in contradiction with the fact, shown by several laboratories, that proteasome inhibition causes aggregation of α-syn and their point variants when overexpressed (we also obtained similar results with the EYFP fusion and untagged synuclein constructs). Further studies are required to fully understand the degradation pathway of the long half-life α-syn protein (11) and whether aggregation or fibrillation of α-syn actually inhibits proteasome activity in the cell, maybe through binding to the S6′ subunit of the 19 S proteasome complex (27).

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α-Synuclein Expression Levels Do Not Significantly Affect Proteasome Function and Expression in Mice and Stably Transfected PC12 Cell Lines

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ADDITIONS AND CORRECTIONS

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PAGES 52986 AND 52987:

The data shown in Figs. 1 and 3 were not correct. The lower α-tubulin blot images in Fig. 1 were reused in Fig. 3 and were incorrectly labeled MECL-1 (β2i). The last four bands in the Y(β1) panel in Fig. 3 were reused in the Z(β2) panel. The corrected figures represent results from replicate experiments performed at the same time as the original experiments. These corrections do not change the interpretation of the results or the conclusions of this work.

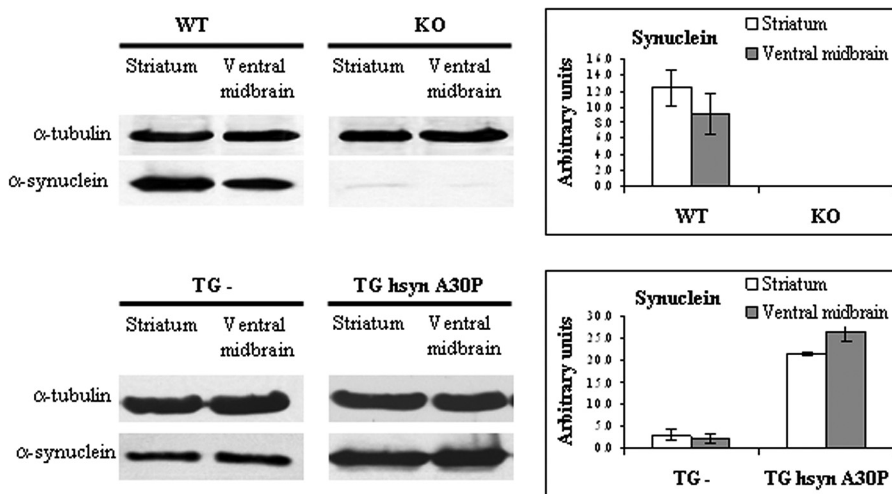

Fig. 1

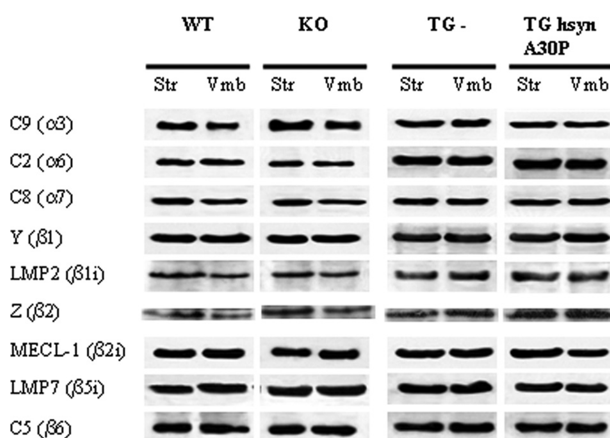

Fig. 3

Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.