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Aggregated α -Synuclein Increases SOD1 Oligomerization in a Mouse Model of Amyotrophic Lateral Sclerosis

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Aggregation of misfolded disease-related proteins is a hallmark of neurodegenerative diseases. Aggregate propagation accompanying disease progression has been demonstrated for different proteins (eg, for α -synuclein). Additional evidence supports aggregate cross-seeding activity for α -synuclein. For mutated superoxide dismutase 1 (SOD1), which causes familial amyotrophic lateral sclerosis (ALS), self-propagation of aggregation and cell-to-cell transmission have been demonstrated *in vitro*. However, there is a prominent lack of *in vivo* data concerning aggregation and cross-aggregation processes of SOD1. We analyzed the effect of α -synuclein and SOD1 seeds in cell culture using protein fragment complementation assay and intracerebral injection of α -synuclein and SOD1 seeds into *SOD1*^{G93A} transgenic ALS mice. Survival of injected mice was determined, and SOD1 aggregates in the facial nuclei were quantified during disease course. We found that α -synuclein preformed fibrils increased the oligomerization rate of SOD1 *in vivo* and *in vitro*, whereas aggregated SOD1 did not exert any effect in both experimental setups. Notably, survival of ALS mice was not changed after inoculation of preformed fibrils. We conclude that misfolded α -synuclein can increase SOD1 aggregation and suppose that α -synuclein seeds are transported from the temporal cortex to the facial nuclei. However, unlike other proteins, the further enhancement of a self-aggregation process by additional SOD1 could not be confirmed in our models. (*Am J Pathol* 2016, 186: 2152–2161; <http://dx.doi.org/10.1016/j.ajpath.2016.04.008>)

Misfolding, accumulation, and deposition of proteins are characteristic for many neurodegenerative disorders [eg, prion diseases, Alzheimer disease (AD), Parkinson disease (PD), Huntington disease, and amyotrophic lateral sclerosis (ALS)].¹ Because aggregated abnormally folded proteins were found to contribute to disease progression, a prion-like seeding property of aggregated proteins has been hypothesized.^{2,3} For prion diseases, Alzheimer disease, and Parkinson disease, the progression of disease pathology along certain neural circuits was suggested many years ago^{4–6} and could currently be confirmed by several independent experimental models.^{7–10}

A similar mechanism was supposed for ALS.¹¹ However, there is a lack of experimental *in vivo* data, although the most widely used animal model of ALS, the *SOD1*^{G93A} model, has existed for two decades.¹²

So far, mutation, oxidation, and posttranslational modification of superoxide dismutase 1 (SOD1) have been found

to favor its aggregation.³ SOD1 aggregation, seeding, and spreading are controversially discussed as potential disease-causing mechanisms.³ It has been demonstrated *in vitro* that aggregated SOD1 and tissue homogenate of mutant *SOD1* transgenic mice are able to seed aggregation of SOD1 and can be transmitted between cells.^{13,14} So far, only one study investigated the self-seeding activity of SOD1 *in vivo*.¹⁵

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A co-presence of α -synuclein—the main component of Lewy bodies in Parkinson disease¹⁶—and SOD1 in the same aggregates has been observed in an ALS patient with *SOD1* mutation,¹⁷ suggesting a cross-seeding activity of α -synuclein or SOD1. Cross-seeding describes a process where a misfolded aggregated protein is able to induce the aggregation of a different protein. However, it has not yet been investigated whether other aggregation-prone proteins are able to induce SOD1 oligomerization *in vivo*.

This could give rise to a different therapeutic strategy: not to target SOD1 aggregates directly but to reduce the trigger for aggregate formation. Because tau and TDP-43 aggregates have been detected after intracerebral injection of α -synuclein, a cross-seeding activity of α -synuclein has been suggested.

Our aim was to investigate whether α -synuclein aggregates are able to induce aggregation and spreading of SOD1. Therefore, we used a SOD1 protein fragment complementation assay (PCA) to study SOD1 aggregation properties in cell culture in the presence of α -synuclein preformed fibrils (PFFs) and brainstem homogenate containing SOD1^{G93A} aggregates. *In vivo*, we investigated SOD1^{G93A} aggregation after injection of α -synuclein PFFs or brainstem homogenate containing SOD1^{G93A} aggregates into the temporal cortex of *SOD1*^{G93A} mice. The mouse model we used expresses a high copy number of the transgene encoding the human ALS causing *SOD1*^{G93A} mutation. It has symptoms and pathologic alterations reminiscent of human ALS cases with *SOD1* mutation.¹⁸ We characterized the mice by determining disease onset, end stage, and disease time course. Furthermore, we investigated SOD1^{G93A} and α -synuclein inclusions in the facial nerve nuclei, which are affected brain regions in ALS and distantly localized from injection site.

Materials and Methods

Preparation of Aggregated Proteins

For intracerebral injection of SOD1^{G93A} aggregates, brainstem tissue from transgenic B6SJL-Tg(*SOD1**G93A)1Gur/J mice (Jackson Laboratory, Bar Harbor, ME) at terminal disease stage was sonicated in phosphate-buffered saline (PBS). As control, brainstem tissue from nontransgenic age-matched littermates was used. After centrifugation for 15 minutes at $14,000 \times g$, protein concentration of the supernatant was determined using a bicinchoninic acid assay (Thermo Scientific, Rockford, IL) and adjusted to 5 $\mu\text{g}/\mu\text{L}$ of protein. The PFFs were generated from recombinant human α -synuclein protein with a purity of >95% (a gift from Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach an der Riss, Germany). α -Synuclein was reconstituted in fibril assembly buffer (50 mmol/L Tris, 100 mmol/L NaCl, pH 7.0) at a concentration of 5 $\mu\text{g}/\mu\text{L}$ and 200 μL per tube of the solution and was incubated in an orbital shaker at 37°C and 1000 rpm for 5 days as previously described.¹⁹ Both aggregate-containing preparations were aliquoted and stored at -80°C .

Nonreducing SDS-PAGE

A total of 20 μg of brainstem homogenate or 5 μg of PFFs were separated on 4% to 20% Mini-PROTEAN TGX Gels (Bio-Rad, Hercules, CA) under nonreducing conditions as described previously.²⁰ Proteins were transferred to a polyvinylidene difluoride membrane and probed with antibodies against SOD1 (ab16831, Abcam, Cambridge, UK), α -synuclein (D37A6, Cell Signaling Technology, Cambridge, UK), or hypoxanthine-guanine phosphoribosyl-transferase (ab109021; Abcam). Secondary anti-rabbit antibody labeled with horseradish peroxidase (Dianova, Hamburg, Germany) was used to convert Luminata forte substrate (Merck Millipore, Darmstadt, Germany). Chemiluminescence signal was detected using the ImageQuant LAS 4000 system (GE Healthcare Life Sciences, Chalfont St Giles, UK).

Protein Aggregate Filtration Assay

Five micrograms of brainstem homogenate was passed through a 0.2- μm pore size nitrocellulose membrane using a Dot Blot SF Microfiltration apparatus (Bio-Rad). Hereby only aggregated SOD1 but not the dimeric form is trapped on the membrane and can be immunodetected as described above.^{21,22} Equal protein amounts on the membrane were demonstrated by Ponceau S staining.

Electron Microscopy

Untreated recombinant α -synuclein and PFF solution were diluted 1:20 in PBS, and 5 μL each was incubated for 1 minute on a formvar-coated square 300 mesh copper electron microscopy sample grid (Plano, Wetzlar, Germany), which was dried afterward, incubated for 1 minute with 2% uranyl acetate, and then dried again. Samples were analyzed using an EM10 (Carl Zeiss, Jena, Germany).

Protein Complementation Assay

In the fusion constructs α -synuclein—*Gaussia* luciferase (hGluc1) (S1) and α -synuclein—hGluc2 (S2),²³ α -synuclein was replaced by SOD1 or SOD1^{G93A} cDNA to generate SOD1-hGluc1 (SOD1-L1) and SOD1-hGluc2 (SOD1-L2) or SOD1^{G93A}-hGluc1 (G93ASOD1-L1) and SOD1^{G93A}-hGluc2 (G93ASOD1-L2). As a control, plasmids L1 and L2 that contained hGluc halves only were used. H4 cells in 96-well plates were co-transfected with equimolar ratios of SOD1-L1 + SOD1-L2 or G93ASOD1-L1 + G93ASOD1-L2 or L1 + L2 or in case of mock without plasmid constructs using Fugene6 transfection reagent (Promega, Fitchburg, WI) according to the manufacturer's instructions. Twenty-four hours after transfection, culture medium was replaced with 100 μL of serum- and phenol red-free OptiMEM (Life Technologies, Carlsbad, CA) containing 7 $\mu\text{mol}/\text{L}$ (96 ng/ μL) α -synuclein PFFs, brainstem

homogenate from transgenic *SOD1*^{G93A} mice, or non-transgenic littermates containing 96 ng/μL protein or the same volume of the corresponding buffer. After 24 hours, 80 μL of the culture medium was transferred to a new 96-well plate (referred to as conditioned medium). Remaining medium was aspirated, and 100 μL of serum- and phenol red-free OptiMEM was added. Luciferase activity of conditioned medium and cells was measured using a luminometer (Victor3 multilabel plate reader; PerkinElmer, Waltham, MA) after addition of cell permeable coelenterazine (20 μmol/L; Nanolight, Pinetop, AZ).

Mouse Breeding and Genotyping

All animal experiments were performed in accordance with national laws and guidelines and were approved by state authorities.

Male mice carrying the human *SOD1*^{G93A} transgene [B6SJL-Tg(*SOD1**G93A)1Gur/J] were obtained from the Jackson Laboratory and cross-bred with female C57BL/6 mice. Offspring was analyzed for the presence of the *SOD1*^{G93A} transgene as previously described.²⁴

To ensure an equal amount of *SOD1*^{G93A} transgene within the experimental groups, the difference in threshold cycle (ΔCT) of *SOD1* and *IL2* amplification of symptomatic mice was analyzed. Therefore, real-time PCR assays were performed using the LightCycler 480 instrument (Roche Diagnostics, Basel, Switzerland) with primers and hydrolysis probes designed by the Roche Universal Probe Library (UPL) system. The following primer sequences and UPLs were used for quantitative real-time PCR: *SOD1*^{G93A}: 5'-AGATGACTTGGGCAAAGGTG-3', 5'-TATTGGGC-GATCCCAATTAS-3', UPL 11, *IL2*: 5'-TCCTGTGAAC-TCTATGTTAATCAGTGT-3', 5'-TTTTCCCACAAGCC-ATCTTT-3', UPL 91. We detected no difference regarding ΔCT values among the differently inoculated mice. The ΔCT levels matched published ΔCT values of the high-copy *SOD1*^{G93A} mouse model.²⁵ Therefore, we can exclude an influence of divergent copy numbers on disease course or survival.

Intracerebral Injection

Forty-day-old *SOD1*^{G93A} transgenic mice were anesthetized with an i.p. injection of 100 mg/kg of ketamine hydrochloride and 16 mg/kg of xylazine and stereotactically

injected with 5 μg of brainstem homogenate containing *SOD1*^{G93A} aggregates (male mice), 5 μg of α-synuclein PFFs (female mice), or 1 μL of 0.9% sodium chloride solution (saline, male and female mice). Mice were injected in the right hemisphere 2 mm posterior and 3.7 mm lateral relative to bregma with a puncture depth of 3.3 mm with an angle of 20° into the insular cortex using a Hamilton syringe at a rate of 0.5 μL per minute. The needle remained at the target site for 2 minutes. After recovery from surgery, mice were monitored regularly and sacrificed 1, 7, 40, or 70 days post injection (dpi) or when they reached disease end stage (Table 1).

Tissue Preparation

Mice were sacrificed using an overdose thiopental and transcardially perfused with 10 mL of PBS followed by 10% neutral buffered formalin solution (Sigma-Aldrich, St. Louis, MO). The brains were removed and postfixed in 10% neutral buffered formalin solution. Tissue was paraffin embedded and cut into 7-μm coronal sections.

Histologic Analysis of *SOD1*^{G93A} Aggregation and α-Synuclein

To test whether B6SJL-Tg(*SOD1**G93A)1Gur/J mice had *SOD1*^{G93A}-positive aggregates in the brainstem, 12-μm cryoconserved sections were fixed for 10 minutes in 10% neutral buffered formalin solution. Afterward, sections were stained in 1% Thioflavin S in PBS for 5 minutes and washed twice in ethanol and twice in PBS. Sections were blocked with 5% goat serum in PBS for 1 hour and incubated with anti-human *SOD1* antibody (ab52950, diluted 1:50; Abcam) at 4°C overnight. A secondary antibody labeled with Alexa 546 dye (Life Technologies) was used for detection of *SOD1*. Sections were mounted in Mowiol containing 25 mg/mL of DABCO [1,4-diazabicyclo(2.2.2)octane] as an antifading reagent and 1 μg/mL of Hoechst 33342 for nuclear staining. Sections were analyzed using a Axiovert 200 fluorescence microscope and AxioVision software version 4.8.2.0 (Carl Zeiss).

For immunohistochemical detection of *SOD1*^{G93A} aggregates or α-synuclein, paraffin sections were rehydrated and antigen retrieval was achieved by boiling the sections for 5 minutes in water (*SOD1*^{G93A}) or for 20 minutes in 10 mmol/L citrate buffer (pH 6) (α-synuclein) at 800 W in the microwave. Endogenous peroxidases were blocked with 3% hydrogen peroxide in PBS, and the cell membrane

Table 1 Characteristics of Experimental Groups for the Inoculation Study

Inoculum	No. of mice at time of brain dissection					Sex	Age at inoculation, days
	1 dpi	7 dpi	40 dpi	70 dpi	Disease end stage		
Brainstem homogenate containing aggregated <i>SOD1</i>	5	5	5	5	10	Male	40
α-Synuclein PFFs	5	5	5	5	10	Female	40
Saline	5	5	5	5	10	Male and female	40

dpi, days post injection; PFF, preformed fibril.

was permeabilized using 0.2% (SOD1^{G93A}) or 0.5% (α -synuclein) Triton X-100 in PBS. Sections were blocked with 5% goat serum (SOD1^{G93A}) or 2% goat serum/2% bovine serum albumin (α -synuclein) in PBS and incubated at 4°C overnight with anti-human SOD1 antibody (ab52950; Abcam) diluted 1:500 or anti- α -synuclein antibody (D37A6; Cell Signaling Technology) diluted 1:150. After three washes with PBS, sections were incubated with secondary antibody labeled with horseradish peroxidase (Dianova) and developed with DAB (Sigma-Aldrich). Sections were counterstained with 0.1% cresyl violet and mounted with Entellan.

Sections stained for SOD1^{G93A} were digitalized using a Mirax Desk Scanner (Carl Zeiss Microimaging, Göttingen, Germany) and analyzed at 10-fold digital magnification using ImageJ version 1.47v (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>). Cresyl violet–positive, triangle-shaped cells with >15- μ m diameter were classified as motor neurons. SOD1-positive structures with >2- μ m diameter were considered as SOD1^{G93A} aggregates and were counted within the region of the facial nuclei. The numbers of motor neurons or aggregates of every 20th section showing the facial nuclei (five slices per animal) were added together and normalized to the area examined. Calculation was performed for both and single hemispheres individually. Data presented in this article represent the values from analysis of both hemispheres if not indicated differently.

Immunoreactivity and distribution of α -synuclein were analyzed in the facial nuclei using a brightfield microscope with 40-fold magnification (Axioskop 2 plus, Carl Zeiss).

The evaluator (Y.K.) was blinded regarding the age of mice and the injected substance.

Disease Staging

Disease staging was conducted as previously described.²⁴ After surgery, body weight was measured once a week. With the appearance of first disease symptoms, mice were weighed daily to monitor their health and disease progression.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism software version 5.00 (GraphPad Software Inc., La Jolla, CA). The PCA data were analyzed for statistical difference using a two-tailed, unpaired *t*-test. Data from mouse experiments were analyzed using the Kruskal-Wallis test with a Dunn posttest. Survival was analyzed by log rank test. $P \leq 0.05$ was regarded as significant. Data are presented as means $\pm$ SEM.

Results

For seeding and cross-seeding experiments, we proved that our preparations contained defined SOD1^{G93A} aggregates and α -synuclein PFFs. Immunofluorescent analysis of brainstem tissue of end-stage SOD1^{G93A} transgenic mice—which was used for additional experiments later—revealed mostly

Figure 1 SOD1 and α -synuclein preparations contain protein aggregates. **A:** Immunoblot of tissue homogenate from end-stage SOD1^{G93A} transgenic mice and wild-type littermates using SOD1 antibody after nonreducing SDS-PAGE. SOD1 species >100 kDa can be detected only in the transgenic mouse. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) was visualized as loading control. Electron micrograph after negative staining of untreated α -synuclein (**B**) and α -synuclein preformed fibrils (**C**). Scale bars = 600 nm (**B** and **C**).

roundish or oval structures that tested positive for Thioflavin S and human SOD1. Those structures could not be detected in a nontransgenic littermate (Supplemental Figure S1A).

To investigate whether SOD1^{G93A} aggregates were still present after homogenization of the brainstem, nonreducing SDS-PAGE (Figure 1A) and protein aggregate filtration assay (Supplemental Figure S1B) were conducted. Using anti-SOD1 antibody, high-molecular SOD1 species were detected in SOD1^{G93A} transgenic but not in wild-type mice, indicating the presence of aggregated SOD1^{G93A}.

To control α -synuclein PFF preparation, negative stain electron microscopy was performed. Untreated recombinant protein exhibited a dot-like roundish morphology (Figure 1B), whereas PFFs had a fibrillar morphology. Fibrils had a length between 50 and 500 μ m and assembled to a net-like structure (Figure 1C). In addition, the presence of high-molecular α -synuclein species within PFF preparation was confirmed by nonreducing SDS-PAGE (Supplemental Figure S1C).

These preparations were used to determine their influence on SOD1 aggregation *in vitro*. For this, we used the hGluc PCA which has already been used to analyze α -synuclein oligomerization.^{23,26} Human H4 neuroglioma cells were co-transfected with plasmid constructs encoding for SOD1-L1 and SOD1-L2. Because of SOD1 interaction and oligomerization, the two hGluc fragments are brought together and hGluc enzymatic activity is reconstituted. Cells were incubated with α -synuclein PFFs, transgenic or wild-type mouse brainstem homogenate or corresponding buffers for 24 hours, followed by luciferase activity measurement.

Incubation with α -synuclein PFFs increased luciferase activity 1.6-fold in living cells transfected with SOD1-L1 and SOD1-L2 ($P = 0.0001$) (Figure 2A) and their conditioned medium ($P < 0.0001$) (Figure 2D). In the medium of cells

transfected with G93ASOD1-L1 + G93ASOD1-L2, a twofold increase in luciferase activity was also observed ($P < 0.0001$) (Figure 2D). In contrast, α -synuclein PFFs did not influence the luciferase activity in mock or L1 and L2 transfected cells (Figure 2A) or their conditioned medium (Figure 2D), suggesting that α -synuclein PFFs increase the oligomerization of SOD1 and SOD1^{G93A}.

Incubation with mouse brainstem homogenate from SOD1^{G93A} transgenic mice did not influence luciferase activity in cells (Figure 2B) and their conditioned medium (Figure 2E) except in cells transfected with SOD1-L1 + SOD1-L2. SOD1^{G93A} mouse brain homogenate led to a decrease of approximately 20% ($P = 0.0258$) in luciferase activity of cells transfected with SOD1-L1 + SOD1-L2 compared with cells treated with the corresponding buffer (Figure 2B). In the conditioned medium of mock transfected cells, a 20% increase in luciferase activity was detected after incubation with brainstem homogenate of transgenic mice (Figure 2E). Incubation with brainstem homogenate of a wild-type littermate did not result in any changes in luciferase activity in living cells (Figure 2C). In conditioned medium of cells transfected with SOD1-L1 + SOD1-L2, wild-type brainstem homogenate led to a 15% decrease of luciferase activity ($P = 0.02$) (Figure 2F). These data indicate that α -synuclein but not SOD1 is able to increase SOD1 oligomerization.

To determine whether α -synuclein PFFs or brainstem homogenate containing SOD1^{G93A} aggregates influence SOD1 aggregation *in vivo*, we inoculated SOD1^{G93A} transgenic mice with α -synuclein PFFs or brainstem homogenate containing SOD1^{G93A} aggregates into the temporal cortex and investigated the number of SOD1^{G93A}-positive aggregates in the facial nerve nuclei at different time points by SOD1^{G93A} immunohistochemistry. Representative sections of the facial nerve nuclei used for quantification of motor neurons and aggregates are shown in Figure 3.

During the disease course, we found an increasing number of SOD1^{G93A} aggregates per square millimeter until 40 dpi, when aggregate counts reached their maximum in all groups. From 40 dpi until disease end stage, SOD1^{G93A} aggregate counts decreased but a more intense surrounding staining for SOD1^{G93A} could be observed (Figure 3 and

Figure 2 α -Synuclein but not SOD1 aggregates increase luciferase activity in protein fragment complementation assay. Luciferase activity measurement of mock/L1 + L2/SOD1-Luc1 + SOD1-Luc2/G93ASOD1-Luc1 + G93ASOD1-Luc2 transfected H4 cells and conditioned medium after incubation with 7 μ mol/L α -synuclein preformed fibrils (PFFs) (A and D), brainstem homogenate from SOD1^{G93A} transgenic (B and E), or wild-type mice and corresponding buffer for 24 hours (C and F). Incubation with PFFs increases luciferase activity significantly in SOD1-L1 + SOD1-L2 transfected cells (A) and conditioned medium from SOD1-L1 + SOD1-L2/G93ASOD1-L1 + G93ASOD1-L2 transfected cells (D). Data are from three to six independent experiments. Data are expressed as means $\pm$ SEM. $n = 9$ to 18. * $P < 0.05$, *** $P < 0.001$ (two-tailed, unpaired *t*-test).

Figure 3 Representative images of sections stained for SOD1 in the area of the facial nucleus 1 (A–C), 7 (D–F), 40 (G–I), and 70 (J–L) days post injection (dpi) and at disease end stage (M–O). Scale bars = 50 μ m (A–O).

Figure 4A). When we compared the different groups at certain time points, we found a significant difference in the number of aggregates per square millimeter between α -synuclein- and saline-injected mice at 70 dpi (509.2 ± 86.11 versus 308.3 ± 91.24 , $P = 0.03$) (Figure 4A). However, a trend toward higher numbers of SOD1^{G93A} aggregates per square millimeter in α -synuclein- compared with NaCl-injected mice could also be detected at 40 dpi (729.5 ± 65.88 versus 526.9 ± 156.2 , $P = 0.06$) and disease end stage (400.4 ± 68.5 versus 279.2 ± 39.22 , $P = 0.11$). At 1 and 7 dpi, no differences between the groups could be detected. For SOD1^{G93A} mice injected with SOD1^{G93A} aggregate containing brainstem homogenate, we could not find any differences in SOD1^{G93A} aggregate numbers, suggesting that SOD1^{G93A} aggregates do not influence SOD1^{G93A} aggregation under these *in vivo* conditions (Figure 4A).

In addition, we compared the diameter of SOD1^{G93A} aggregates of the groups at different time points. During the disease course, the aggregate diameter significantly ($P = 0.03$) increased in α -synuclein-injected mice

between 40 dpi and disease end stage (Figure 4B). The aggregate diameter tended to increase in all experimental groups. The different groups had similarly sized aggregates at certain time points, indicating that higher SOD1^{G93A} aggregate counts in α -synuclein-injected mice are not attributable to the differences in the size of the aggregates.

Because the number of motor neurons in SOD1^{G93A} mice correlates with severity of disease symptoms, we evaluated the number of motor neurons in the facial motor nuclei, one of the affected brain regions in ALS. We found a significant decrease in the number of motor neurons per square millimeter in all experimental groups between 1 dpi and disease end stage, but no significant difference between the groups at any time point was detected (Figure 4C).

We compared aggregate counts, motor neuron counts, and α -synuclein staining in the facial nuclei from the left and right hemisphere to evaluate possible changes after unilateral injection of aggregated SOD1^{G93A} or α -synuclein PFFs. No differences between the hemispheres were detected (Figure 4D).

Figure 4 Elevated numbers of SOD1 aggregates after injection of α -synuclein preformed fibrils into the temporal cortex of *SOD1^{G93A}* mice, whereas aggregate size and motor neuron count do not differ. **A:** A significantly higher number of SOD1^{G93A} aggregates can be detected in the facial nucleus 70 days post infection (dpi) between α -synuclein- and saline-injected mice. A trend toward higher aggregate numbers in α -synuclein injected mice can be seen 40 dpi and at disease end stage. **B:** A significant increase in SOD1^{G93A} aggregate diameter can be detected in α -synuclein-injected mice over time. A trend toward bigger aggregate diameter can be observed in all experimental groups. No differences between the groups could be detected at any time point investigated. **C:** The number of motor neurons detected by cresyl violet staining decreases during disease course but does not differ between experimental groups. **D:** In α -synuclein-injected mice, no difference between the left and right hemispheres regarding SOD1^{G93A} aggregate counts can be detected. $n = 4$ to 5. * $P < 0.05$ (Kruskal-Wallis test and Dunn posttest).

Taken together, using immunohistochemistry, we found that α -synuclein PFFs are able to form SOD1^{G93A} aggregates *in vivo*. Despite increased numbers of SOD1^{G93A} aggregates in both hemispheres of the brain, disease-related pathologic changes (ie, motor neuron counts) were not influenced in *SOD1^{G93A}* mice.

Because aggregated α -synuclein induces its own aggregation in wild-type mice in a prion-like manner and spreads by so far unknown mechanisms along neural circuits after intracerebral inoculation,^{7,27} we also investigated α -synuclein levels in the facial nuclei of the mice. α -Synuclein immunoreactivity was similar in the differently injected mice except for 70 dpi. At this time point, α -synuclein staining was much higher in α -synuclein PFF-injected animals compared with aggregated SOD1- or NaCl-injected mice (Figure 5).

To evaluate the effect of increased SOD1^{G93A} aggregation in α -synuclein-injected mice, we analyzed the time course of symptoms and survival of the mice. Only

sex-specific differences were found. Weight development (Figure 6A), age when first symptoms appeared (Figure 6B), first pareses of the hind limbs appeared (Figure 6C), or overall survival of the mice (Figure 6, D and E) was not altered after injection of SOD1 aggregates or α -synuclein PFFs. In addition, no significant differences in the duration of symptomatic phase (early and late disease phase) was observed (data not shown).

Discussion

Since Braak and Braak⁵ reported a constant spreading pattern of pathologic changes in Alzheimer disease, the idea of aggregate self-propagation arose over time not only for prion diseases but also for other neurodegenerative diseases. Recently, the principle of aggregate cross-seeding has also been suggested for aggregation-prone proteins.^{7,28,29}

Figure 5 Representative images of sections stained for α -synuclein showing the facial nucleus of mice 70 days post injection of brainstem homogenate from SOD1^{G93A} transgenic mice (A), α -synuclein PFFs (B), or saline (C). Scale bars = 50 μ m (A–C).

In our cell culture model, we found that SOD1 has a higher oligomerization rate in the presence of α -synuclein PFFs. *In vivo* we were able to confirm this effect because a higher amount of SOD1^{G93A} accumulation after α -synuclein PFF inoculation was observed.

Using PCA, we found that α -synuclein PFFs increase oligomerization of SOD1. Enhanced oligomerization of wild-type SOD1 was detected in living cells and conditioned medium, whereas mutant SOD1 oligomerization was increased in conditioned medium only. PFF treatment might result in a fast secretion of mutant SOD1 because an additional secretion mechanism for mutant SOD1 is known.³⁰ Furthermore, a higher level of dimer formation in untreated SOD1^{G93A} transfected cells could conceal the effect elicited by α -synuclein on SOD1^{G93A} oligomerization inside the cells. A similar increase in SOD1 oligomerization has been found in the presence of untreated recombinant α -synuclein in a recent publication, which also reports the specificity of the SOD1– α -synuclein interaction.³¹ However, aggregated SOD1^{G93A} was not able to increase SOD1 aggregation in our experiments. This finding contradicts results of other groups—even if they used a noncellular environment, different mutations, or fluorescent tags—who demonstrated the ability of misfolded SOD1 to seed its aggregation.^{13,14,32} This finding might be explained by the use of PCA detecting interaction of SOD1 and not its morphology as used by previous studies.^{13,14,32} Regarding our result, additional SOD1 possibly acts as competing counterpart for SOD1 protein complementation because the added SOD1 might form oligomers with the SOD1 labeled

with the first half of the luciferase, thereby preventing it from joining the second half of the luciferase. In this way, the decreased luciferase activity in the presence of SOD1^{G93A} aggregates can be explained. Because a direct interaction of α -synuclein and SOD1 has been reported,³¹ α -synuclein and SOD1-luciferase constructs potentially compete for the same binding side on the other SOD1-luciferase half, which could lead to an underestimation of oligomer induction caused by α -synuclein.

Consistent with the findings from PCA, we could also demonstrate an elevation of SOD1^{G93A} aggregate counts after intracerebral injection of α -synuclein *in vivo*. Similar to the reported aggregation speed of α -synuclein in wild-type mice,⁷ a higher accumulation rate of SOD1^{G93A} was observed 70 dpi and not at earlier stages. We did not detect a significant difference at end stage of disease, which might derive from the clinical definition of end stage (ie, each individual has a maximal general pathologic degeneration covering the differences regarding higher aggregation rates observed in α -synuclein PFF inoculated mice). Consistent with the proposed aggregation speed of α -synuclein in wild-type mice,⁷ α -synuclein is elevated in α -synuclein-injected animals at 70 dpi as well. We could not demonstrate changes of survival, motor neuron counts. However, this might be caused by a fast disease progression of SOD1^{G93A} mice. Possibly in a slower progressing mouse model with longer survival, motor neuron counts might also be influenced. In addition, additional studies should include methods that are able to detect functional differences between the

Figure 6 No changes in disease course of aggregate injected SOD1^{G93A} mice. **A:** No difference in body weight between the experimental groups is seen. **B:** There is no difference with age of mice at disease onset. **C:** There is no difference regarding the age when mice had first pareses. Kruskal-Wallis test and Dunns post test were performed (B and C). **D** and **E:** A sex-specific difference but no difference between aggregate injected and corresponding controls is observed in the survival time. Data are expressed as means $\pm$ SEM. *n* = 5 NaCl-injected mice (male and female; A, D, and E); *n* = 10 SOD1^{G93A}- and α -synuclein-injected mice (A, D, and E); *n* = 5 NaCl-injected mice (male and female; C); *n* = 7 NaCl-injected mice (female; B); *n* = 8 NaCl-injected mice (male; B); *n* = 10 SOD1^{G93A}-injected mice (male) and α -synuclein-injected mice (female; C); *n* = 13 α -synuclein-injected mice (female; B); *n* = 15 SOD1^{G93A}-injected mice (male; B).

differently inoculated mice (eg, motor unit number estimation).

Recently, another group found sparse SOD1 aggregation within the spinal cord 2.8 months after injection of mutated SOD1 and a general induction of motor neuron symptoms in heterozygous *SOD*^{G85R-YFP} mice.¹⁵ This model is normally asymptomatic and reveals no general pathologic accumulation. In contrast, the *SOD1*^{G93A} transgenic mice used here have an increasing load of SOD1^{G93A} accumulation in brainstem during the disease course.^{33,34} Therefore, it might not be surprising that no effect of SOD1^{G93A} aggregate inoculation on the total aggregate load in *SOD1*^{G93A} transgenic mice could be observed because pathologic degeneration might cover the presence of aggregate formation processes. Consistent with our data, Ayers et al¹⁵ did not detect an accelerated disease progression in *SOD1*^{G93A} mice after SOD1^{G93A} inoculation.

In addition, evidence is emerging that different strains of SOD1 aggregates exist whose abundance correlates with disease progression in mice.³⁵ Notably, only the less potent SOD1 strain could be isolated from *SOD1*^{G93A} mice.³⁵

Because the detailed mechanism of α -synuclein and SOD1 interaction has not been characterized yet, we can only speculate on the influence of α -synuclein on SOD1^{G93A} aggregation. On the one hand, α -synuclein might act as a trigger for SOD1^{G93A} to form seeds that are further transported through neural networks and form aggregates only in susceptible areas. On the other hand, α -synuclein itself could be transported and influence SOD1^{G93A} that is already in the aggregation process. In addition, the importance of a special cellular environment or regional susceptibility in the process of SOD1^{G93A} aggregation should be addressed in the future. However, we observed that the amount of α -synuclein found in the facial nuclei is higher 70 dpi after injection of α -synuclein PFFs compared with the injection of SOD1^{G93A} and NaCl. This finding indicates that α -synuclein levels influence the number of SOD1 aggregates directly because this measure is elevated in the same animals.

Regarding the transport of seeds, we hypothesize that they spread from insular cortex to the motor cortex. Both are interconnected according to tracing experiments.^{36,37} From the motor cortex, which is interconnected between both hemispheres through the corpus callosum, the corticospinal tract arises, projecting ipsilaterally and contralaterally to the motoric areas of the brainstem.³⁸ In addition, projections exist from the motor cortex to relay neurons located in the brainstem.³⁹ Therefore, we can state that the injection site is connected to the facial nucleus by polysynaptic connections, suggesting a route of transport. However, this route of seed transportation should be validated using tracers in further experiments.

Taken together, this study indicates for the first time that α -synuclein PFFs increase SOD1 aggregation *in vitro* and *in vivo*. The underlying mechanism seems to enhance self-aggregation of wild-type and mutant SOD1. Suppressing such an influence might be an additional therapeutic target

in ALS. Therefore, in future studies, cofactors in the aggregation process should be investigated.

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2016.04.008>.

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