

博士論文（要約）  
Systematic analysis on the seeding activity of  
familial mutant forms of  $\alpha$ -synuclein  
(家族性変異型  $\alpha$ -synuclein のシード活性に関する体系的解析)

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## 【Background】

Parkinson's disease (PD) is one of the most common neurodegenerative diseases characterized by loss of dopaminergic neurons in the midbrain. PD is pathologically characterized by the deposition of aggregated  $\alpha$ -synuclein proteins as Lewy bodies (LB) and Lewy neurites (LN). Six missense mutations in the  $\alpha$ -synuclein gene (*i.e.*, A30P, E46K, H50Q, G51D, A53E, and A53T) have been identified in familial PD cases, further underscoring the pathological importance of  $\alpha$ -synuclein. Recently, interneuronal propagation of aggregated proteins has emerged as a common paradigm explaining how the pathology spreads in the patient brains with neurodegenerative diseases. However, it remains unclear how the pathogenic mutations in  $\alpha$ -synuclein alter the propagation of  $\alpha$ -synuclein aggregates. In this study, I systematically examined the seeding and propagation activities of  $\alpha$ -synuclein mutants in *in vitro* fibrillization assays, primary cultured neurons and wild-type mouse brains.

## 【Results】

### 1. Preparation of $\alpha$ -synuclein seeds

Monomers of full-length human wild-type (h-wt)  $\alpha$ -synuclein and the six mutants were expressed in *E. coli* and purified according to standard procedures. Each monomer was incubated at 37 °C with agitation for 1 week to facilitate the fibrillization. The formed aggregates were collected by ultracentrifugation and then extensively sonicated to prepare fragments. The sonicated aggregates, which I call “seed”, were observed under an electron microscope to confirm the efficient fragmentation of the aggregates. The length of the seeds measured on the electron micrograph was approximately 100 nm, which was not significantly different between wt and the mutants (data not shown).

### 2. Seeding activity: *in vitro* fibril formation

First, the seeding activity of h-wt as well as the mutant forms of  $\alpha$ -synuclein seeds was examined in an *in vitro* fibrilization assay. Each seed was added at a final concentration of 2  $\mu$ M into 100  $\mu$ M monomer of mouse wild-type (m-wt)  $\alpha$ -synuclein. The reaction mixtures were incubated at 37 °C, and the Thioflavin-S (Thio-S) fluorescence was measured every 30 minutes. All mutants but G51D showed a marked increase in the Thio-S fluorescence over time during incubation for 7 h (Figure 1). The aggregation of  $\alpha$ -synuclein was further confirmed by subjecting the reaction mixtures incubated for 8 h to ultracentrifugation and examining the amount of sedimented  $\alpha$ -synuclein by SDS-PAGE followed by Coomassie staining. In this experiment, all mutants including G51D showed a similar amount of sedimentation of  $\alpha$ -synuclein compared to wt (Figure 2). Together, these results suggested that all human  $\alpha$ -synuclein seeds including G51D had an ability to facilitate fibrillization of m-wt  $\alpha$ -synuclein, although

Thio-S fluorescence selectively underestimated the fibril formation with the G51D seed.

### 3. Seeding activity: aggregation in primary neurons

Next, the seeding activity of  $\alpha$ -synuclein seeds were examined in rat cortical primary neurons, which

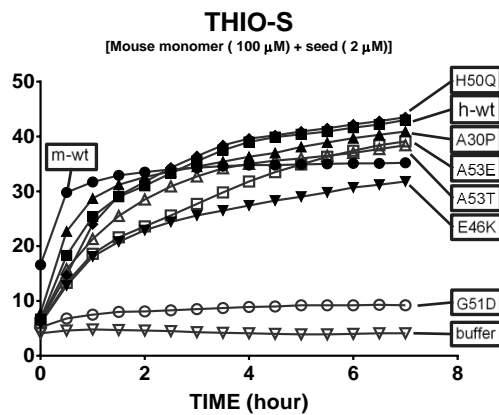


Figure 1: *in vitro* fibril formation of mouse  $\alpha$ -synuclein with different  $\alpha$ -synuclein seeds.

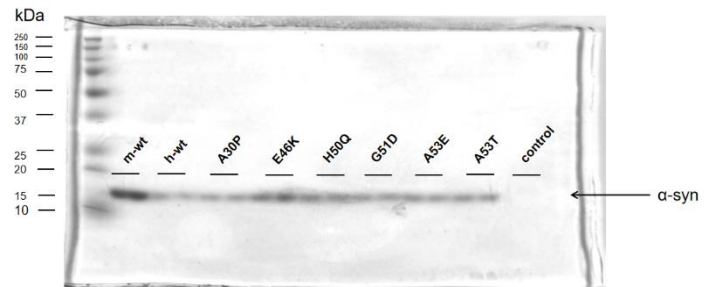


Figure 2: A representative image of Coomassie stained gel showing a similar amount of sedimented  $\alpha$ -synuclein after incubating the mixture of 100  $\mu$ M mouse monomer and 2  $\mu$ M seed at 37  $^{\circ}$ C for 8 hours.

abundantly express endogenous  $\alpha$ -synuclein. The aggregation of endogenous  $\alpha$ -synuclein can be induced by exogenously added  $\alpha$ -synuclein seeds to the culture media. In this experimental paradigm, immunocytochemical detection of phosphorylation at Ser129 of  $\alpha$ -synuclein (p-syn) is utilized as a surrogate for monitoring the aggregation of  $\alpha$ -synuclein in cells. Neurons isolated from rat cerebral cortices were treated with  $\alpha$ -synuclein seeds at DIV (days *in vitro*) 10 and kept treated for 7 days. All mutants induced the formation of p-syn positive aggregates in primary neurons (Figure 3). Quantification of p-syn (+) area on the micrographs revealed that A53T significantly increased p-syn (+) aggregation, whereas A30P significantly decreased (Figure 4).

### 4. Seeding activity: *in vivo* propagation

Finally, effects of the mutations on the propagation of  $\alpha$ -synuclein pathology were examined in wild-type mouse. The  $\alpha$ -synuclein seeds were unilaterally administered into the striatum of 2-month-old male

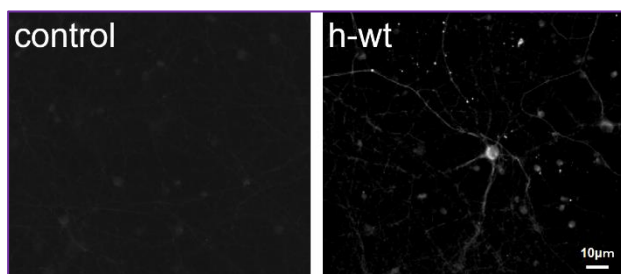


Figure 3: A representative image of p-syn positive aggregates in primary neurons. Scale bar, 10  $\mu$ m.

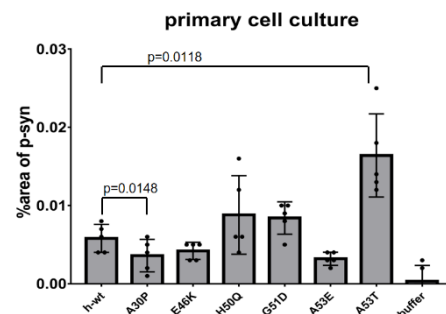


Figure 4: Quantification of p-syn (+) area on the micrographs. N=3, Dunnett' test.

mice by stereotaxic injection (n = 5). 3 months after administration, the h-wt seed induced the p-syn (+) staining primarily in the injected side of the brains. The p-syn (+) staining was observed not only in the neuronal perikarya (LB-like) but also in neurites (LN-like) (Figure 5). The p-syn staining was also observed in the brain regions contralateral to the injection side, suggesting the intraneuronal propagation of  $\alpha$ -synuclein aggregates. The number of LB-like pathology in the amygdala as well as in the cerebral cortex in the contralateral side was manually counted, which demonstrated that A53T significantly increased the LB-like pathology in the amygdala, whereas the rest of mutant seeds decreased it (Figure 6).

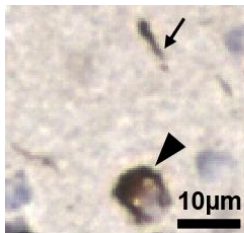


Figure 5: A representative image of the contralateral amygdala injected with h-wt seed. Arrowhead point to LB-like inclusions and arrow point to LN-like pathology.

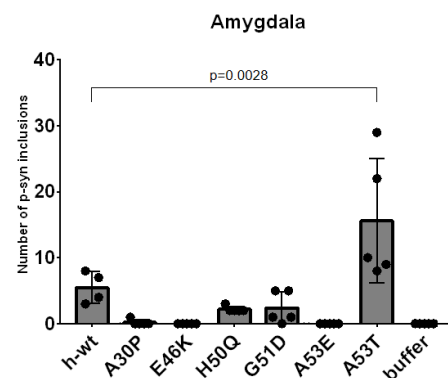


Figure 6: The number of LB-like p-syn (+) inclusions in the contralateral amygdala. N=5, Dunnett' test.

## 【Conclusion】

In conclusion, although all seeds showed a similar seeding activity in test tubes, they induced different pathologies in the primary neurons and mouse brains. As all mutations except A53T failed to show an increase in the formation of p-syn (+) aggregates in primary neurons as well as in mouse brains, it would be reasonable to speculate that the pathogenicity of  $\alpha$ -synuclein mutations is not attributed to their effect on the seeding activity. Of note, we found that some mutations (e.g. A30P, E46K and A53E) preferentially induced LN-like pathology over LB-like inclusions, while quantitative analyses of LN-like pathology should be performed in future. Nevertheless, the distinct aggregate formation at neurite and cell body might reflect the differences in intracellular milieu and/or factors that affect the aggregation process of  $\alpha$ -synuclein mutants. Further molecular and biochemical studies would clarify these issues.