

Original Research

FABP2 is Involved in Intestinal α -Synuclein Pathologies

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Abstract

Background: Recently, the hypothesis that pathological α -Synuclein propagates from the gut to the brain has gained attention. Although results from animal studies support this hypothesis, the specific mechanism remains unclear. This study focused on the intestinal fatty acid-binding protein (FABP2), which is one of the subtypes of fatty acid binding proteins localizing in the gut, with the hypothesis that FABP2 is involved in the gut-to-brain propagation of α -synuclein. The aim of this study was to clarify the pathological significance of FABP2 in the pathogenesis and progression of synucleinopathy. **Methods:** We examined the relationship between FABP2 and α -Synuclein in the uptake of α -Synuclein into enteric neurons using primary cultured neurons derived from mouse small intestinal myenteric plexus. We also quantified disease-related protein concentrations in the plasma of patients with synucleinopathy and related diseases, and analyzed the relationship between plasma FABP2 level and progression of the disease. **Results:** Experiments on α -Synuclein uptake in primary cultured enteric neurons showed that following uptake, α -Synuclein was concentrated in areas where FABP2 was localized. Moreover, analysis of the plasma protein levels of patients with Parkinson's disease revealed that the plasma FABP2 and α -Synuclein levels fluctuate with disease duration. The FABP2/ α -Synuclein ratio fluctuated more markedly than either FABP2 or α -Synuclein alone, depending on the duration of disease, indicating a higher discriminant ability of early Parkinson's disease patients from healthy patients. **Conclusions:** These results suggest that FABP2 potentially contributes to the pathogenesis and progression of α -synucleinopathies. Thus, FABP2 is an important molecule that has the potential to elucidate the consistent mechanisms that lead from the prodromal phase to the onset and subsequent progression of synucleinopathies.

Keywords: FABP2; α -Synuclein; enteric nerves system; synucleinopathy; Parkinson's disease; primary culture; blood biomarkers

1. Introduction

α -Synuclein is a major component of intracellular inclusions called Lewy bodies and Lewy neurites, which are pathological hallmarks of Parkinson's disease and dementia, collectively known as synucleinopathies [1, 2]. α -Synuclein normally exists intracellularly as soluble monomers but aggregates to form β -sheet-rich structures called oligomers and fibrils, which eventually form insoluble Lewy bodies [3,4]. In addition, α -Synuclein exhibits prion-like propagation not only to the cell in which it is expressed but also to surrounding cells [5,6].

One of the proteins that may play an important role in α -synuclein pathologies is fatty acid-binding proteins (FABPs). FABPs act as lipid chaperones and transport lipids into specific compartments in the cell [7]. FABPs have subtypes with different localization sites, such as fatty acid-binding protein 3 (FABP3, H-FABP) in the brain and fatty acid-binding protein 2 (FABP2, I-FABP) in the intestines [8]. Previous studies have shown that FABP3 plays an important role in brain α -Synuclein pathologies. In experiments in which α -Synuclein preformed fibrils (PFF) were injected into substantia nigra pars compacta of mouse midbrain, the motor and cognitive deficits seen in wild-type mice were absent in FABP3 knockout mice

[9]. In our recent research using primary cultured mesencephalic dopaminergic neurons, we found that the uptake of α -Synuclein was not observed in neurons derived from FABP3 knockout mice [10,11]. It has also been shown that α -Synuclein injected into the striatum propagated to the midbrain in wild-type mice but not in FABP3 knockout mice [12]. These results suggest that FABP3 is essential for the intracellular uptake and propagation of α -Synuclein, and is deeply involved in the pathogenesis of synucleinopathies.

Recently, it has become clear that α -Synuclein propagates from the gut via the vagus nerve before it accumulates in the brain [13,14]. Braak *et al.* [15–17], who provided a pathological staging of sporadic Parkinson's disease, showed the dorsal motor nucleus of the vagus nerve as one of the first sites of central nervous system involvement in Parkinson's disease, suggesting propagation of α -Synuclein pathology from the gut to the brain. Kim *et al.* [18] injected α -Synuclein PFF into the pyloric and duodenal muscularis layer of mice and found that pathogenic α -Synuclein was transmitted from the gut to the brain. These results support the hypotheses proposed by Braak *et al.* [15–17]. However, the detailed mechanisms of the events that occur at the cellular level during the initial stages of



α -Synuclein propagation from the gut to the brain, specifically where and how α -Synuclein is taken up by neurons and propagated from the enteric nervous system to the vagus nerve and central nervous system, remain unclear. The relationship between Parkinson's disease and the gut is clinically important. Gastrointestinal dysfunction is common in patients with Parkinson's disease before and early in the disease course [19]. Particularly, constipation is a typical non-motor symptom that occurs in approximately 30% of patients [20,21] and precedes motor symptoms by more than 10 years [22–24].

Among the 12 subtypes of FABPs, FABP2 is localized in the intestine [7]. Therefore, it is possible that FABP2 is involved in α -synuclein pathologies in the intestine, just as FABP3 is involved in α -synuclein propagation and toxicity in the brain. This study aimed to elucidate the pathophysiological significance of FABP2 in the pathogenesis and progression of synucleinopathy. First, to study the mechanism of intracellular uptake of α -Synuclein in enteric neurons, we analyzed the appearance of primary cultured neurons derived from murine small intestine after treatment with α -Synuclein and its relationship with FABP2. In addition, to gain an overview of the pathological conditions caused by synucleinopathies, we analyzed the characteristics and relationships among plasma levels of FABP2 and α -Synuclein using plasma samples of patients with Parkinson's disease and healthy controls.

2. Materials and Methods

2.1 Primary Culture of Murine Enteric Neurons

The primary culture of enteric neurons was performed based on the method described in previous research, specifically that conducted by Smith *et al.* [25] with modifications to suit our experimental environment. The specific experimental conditions and procedures are described below [26].

2.1.1 Coating of the Cover Glass Chamber

Poly-D-lysine (#168-19041, Fujifilm Wako Pure Chemical, Osaka, Japan) was diluted to 0.1 mg/mL using sterile water, and 120 μ L of this solution was pipetted into each well of an 8-well cover glass chamber (#5232-008, AGC Techno Glass, Shizuoka, Japan). After 10 min of incubation, the poly-D-lysine solution was aspirated, rinsed three times with sterile water, and dried for 2 h. Laminin (#L2020-1MG, Sigma Aldrich, St. Louis, MO, USA) was diluted to 50 μ g/mL using sterile water and added to each well of the poly-D-lysine coated chamber. After incubation at 37 °C for 1 h, the laminin solution was aspirated and rinsed once with sterile water. All the above operations were performed aseptically.

2.1.2 Longitudinal Muscle/Intermuscular Plexus (LMMP) Preparation

Three male 8–10-week-old C57BL/6J mice (SLC, Shizuoka, Japan) were euthanized by overdosing with

isoflurane (#099-06571, Fujifilm Wako Pure Chemical, Osaka, Japan), the abdominal cavity was cut open, and the small intestine was isolated and immersed in ice-cold Hanks' balanced salt solution (HBSS). A 10 mL syringe was used to pass the HBSS through the intestinal tract and remove the fecal matter. The intestinal tract was cut into approximately 2 cm pieces, and the pieces were passed through the tip of an awl to ensure no twisting and fixed with fingers. A portion of the mesentery attached to the intestine was removed using tweezers, and a gap was made in the longitudinal muscles by gently rubbing with the tip of the tweezers along the entire line where the mesentery was attached. Using a cotton swab wetted with HBSS, longitudinal muscle/intermuscular plexus (LMMP) was collected by gently rubbing and placed in a 15 mL tube containing ice-cold HBSS. After all the pieces of LMMPs were collected from all intestinal fragments, the tubes containing LMMP were centrifuged at 1000 \times g for 30 seconds in a centrifuge cooled to 4 °C. To remove debris, isolated LMMPs were rinsed twice as follows, the supernatant was removed using a pipette, resuspended with ice-cold HBSS, and centrifuged at 1000 \times g for 30 seconds in a centrifuge cooled to 4 °C.

2.1.3 Digestion of LMMP

The digestion solution was prepared by adding 13 mg collagenase type II (#LS004174, Worthington, Lakewood, NJ, USA) and 3 mg bovine serum albumin (#A9418-500G, Sigma Aldrich, St. Louis, MO, USA) to 10 mL HBSS. LMMP was washed three times, cut into small pieces with scissors, and digested in a water bath at 37 °C for 60 min. After digestion was complete, cells were collected by centrifugation at 1000 \times g for 5 min in a centrifuge cooled to 4 °C. 0.05% trypsin solution was prepared by adding 1 mL of warm 0.25% trypsin (#201-18841, Fujifilm Wako Pure Chemical, Osaka, Japan) to 4 mL of 37 °C warm HBSS and added to the tube containing the cell pellet after supernatant removal. The cells were digested in a water bath at 37 °C for 7 min with shaking.

2.1.4 Cell Seeding and Culture

Two types of media with different compositions (rinse and neuronal media) were prepared. Rinse medium (D-MEM/Ham's F-12 media with 10% fetal bovine serum [FBS] and antibiotic/antimycotic) was prepared by mixing 500 mL of D-MEM/Ham's F-12 media (#042-30555, Fujifilm Wako Pure Chemical, Osaka, Japan) with 50 mL of FBS (#10437-028, Life Technologies, Grand Island, NY, USA) and 5 mL of 100x antibiotic/antimicrobial solution (#161-23181, Fujifilm Wako Pure Chemical, Osaka, Japan). Neuronal medium (Neurobasal A media with B-27, 2 mM L-glutamine, 1% FBS, 10 ng/mL glial cell line-derived neurotrophic factor [GDNF], and antibiotic/antimycotic) was prepared by mixing 47.5 mL of Neurobasal A medium (#10888022, Life Technologies, Grand Island, NY, USA) with 1 mL of B-27 (50x) (#17504-044,

Life Technologies, Grand Island, NY, USA), 500 μ L of FBS, 500 μ L of 200 mM L-glutamine, 50 ng of GDNF (#079-06111, Fujifilm Wako Pure Chemical, Osaka, Japan), and 500 μ L of 100x antibiotic/antimicrobial solution.

After trypsin digestion, 10 mL of ice-cold rinse medium was added to the tube to neutralize trypsin and the tube was centrifuged at 1000 \times g for 5 min. After centrifugation, the supernatant was removed, and 3 mL of neuronal medium was added. The cell suspension was filtered through a cell strainer, placed in a new 15 mL tube, centrifuged at 1000 \times g for 5 min, and the cells were collected. The cells were resuspended in 1 mL of neuronal medium and counted. Cell suspensions were diluted to the desired density using neuron medium and added to poly-D-lysine/laminin-coated cover glass chambers at 500 μ L per well. The medium was changed by half every 2 days.

2.1.5 α -Synuclein Treatment

ATTO594 labeled human recombinant α -Synuclein PFF (#SPR-322B-A594, StressMarq Biosciences, Victoria, British Columbia, Canada) was used in this experiment. The α -Synuclein PFF was diluted in neuron medium to a concentration of 10 μ M, sonicated for 3 min, and diluted in neuron medium to a final concentration of 1 μ M.

2.1.6 Immunocytochemistry

The cultured cells were fixed on 8 days *in vitro* (DIV), treated with 4% paraformaldehyde for at least 1 h, blocked with 0.1% Triton X-100 and 5% goat serum in phosphate-buffered saline (PBS) for 1 h, and incubated overnight at 4 $^{\circ}$ C with primary antibodies added. The primary antibodies were rabbit anti-beta III Tubulin polyclonal antibody (1:500, #ab18207, Abcam, Cambridge, UK) or chicken anti-beta III Tubulin polyclonal antibody (1:500, #NB100-1612, Novus Biologicals, Centennial, CO, USA) for β III-Tubulin, mouse anti-gial fibrillary acidic protein (GFAP) monoclonal antibody [GA5] (1:300, #3670, Cell Signaling Technology, Danvers, MA, USA) for glial fibrillary acidic protein (GFAP), mouse anti-FABP2 monoclonal antibody [9A9B7B3] (1:500, #GTX83361, GeneTex, Irvine, CA, USA) for FABP2. After washing with PBS, the secondary antibody and nuclear-staining reagent were added and incubated for 1 h. The secondary antibodies used were goat anti-Rabbit IgG H&L (FITC) (1:500, #ab6717, Abcam, Cambridge, UK), goat anti-Mouse IgG H&L (Cy3) (1:300, #ab97035, Abcam, Cambridge, UK), and goat anti-Mouse IgG (H+L) Alexa Fluor 488 (1:500, #A11001, Invitrogen, Waltham, MA, USA) or goat-anti-Chicken IgY (H+L) Alexa Fluor Plus 405 (1:500, #A48260, Invitrogen, Waltham, MA, USA). Hoechst (1:1000, #H341, Dojindo, Kumamoto, Japan) was used for nuclear staining. Stained images were acquired using a confocal laser microscope (TCS SP8, Leica Microsystems, Wetzlar, Germany) and ImageJ (version 1.54f, National Institutes of Health, Bethesda, MD, USA) was used for image quantification.

2.2 Measurement of Plasma Concentrations of FABP2 and α -Synuclein

We used data measured in our previous study [27]. A summary of the measurements are provided below.

2.2.1 Specimens

The specimens used were plasma samples collected from patients and healthy controls at the National Hospital Organization Sendai Nishitaga Hospital. The details of the patients were as follows: Parkinson's disease (89 patients, 73.6 ± 8.4 years of age), all of which had clinically confirmed diagnoses. The healthy controls were elderly people (30 cases, 62.3 ± 5.7 years of age) with no cognitive or motor dysfunctions.

2.2.2 Sample Preparation

Plasma samples stored at -80° C were thawed and centrifuged at 10,000 \times g for 5 min. Samples were diluted in advance with the sample diluent provided with each assay kit or the sample diluent included in the Homebrew Assay Development Kit (#101354, Quanterix, Billerica, MA, USA) and applied to the plate.

2.2.3 Plasma Protein Concentration Measurement

Plasma protein concentrations were measured by enzyme-linked immunosorbent assay (ELISA) using a Simoa HD-X analyzer (#103385, Quanterix, Billerica, MA, USA). Simoa Alpha-Synuclein Discovery Kit (#102233, Quanterix, Billerica, MA, USA) was used to measure α -synuclein, and the instructions for each kit were followed. For FABP2, we constructed a custom assay system using Homebrew Assay. The protocol was performed using the Simoa Homebrew Assay Development Kit (#101354, Quanterix, Billerica, MA, USA), Pierce EDC (#A35391, Thermo Scientific, Waltham, MA, USA), and EZ-Link NHS-PEG4-Biotin (#A39259, Thermo Scientific, Waltham, MA, USA). The captured antibodies were coated onto the magnetic beads at a concentration of 0.3 mg/mL. Biotinylation of the detector antibody was performed in a 1:40 ratio. Mouse monoclonal antibodies against FABP2 were obtained from BioGate (Gifu, Japan). Clone 25E6 was used for capture, and clone 7H8 was used for detector. For calibration curve generation, sequential dilutions of each recombinant protein were used as samples.

2.3 Data Analysis of Plasma Biomarker

As noted above, we used the data measured in our previous studies [27], which were preprocessed using the unified procedure described below for this whole dataset.

2.3.1 Normalization of Data and Removal of Outliers

To facilitate subsequent statistical analysis, markers with a distribution biased toward low values were converted to the logarithm of the normal distribution, which was close to the normal distribution. The Grubbs test was

then repeatedly applied to remove the outliers on the high side. The data were analyzed using R (version 3.6.3, <https://www.r-project.org>).

2.3.2 Age Correction of Data

To exclude the influence of differences in the age of the sample donors among diseases on marker values, age correction was performed for each marker value. First, we tested whether there was any difference in the slope of the relationship between each marker value and age among diseases, and confirmed that there was no significant difference in the slope (FABP2: $p = 0.48$, α -Synuclein: $p = 0.25$). Then, we age-adjusted the data according to the mean age of the sample donors for whom the data for each marker were obtained by analysis of covariance (ANCOVA). The data were analyzed using R (version 3.6.3, <https://www.r-project.org>).

2.3.3 Data Standardization

The data were standardized to compare each marker equally. The mean and unbiased standard deviation of the healthy controls (CN) were used as standardized indices, such that the mean was 5, and the standard deviation was 1 for the healthy controls. The data were analyzed using R (version 3.6.3, <https://www.r-project.org>).

2.3.4 Statistical Hypothesis Testing

As the biomarker values and calculated scores in this study were biasedly distributed, nonparametric tests were used for disease-specific comparisons. Specifically, the Kruskal-Wallis and Dunn's multiple comparison tests were used for multi-group comparisons. Data analysis and plotting were performed using Prism (version 10.1.0, GraphPad Software, Boston, MA) and Python (version 3.9.16, <https://www.python.org>).

3. Results

3.1 α -Synuclein Uptake into Primary Cultured Murine Enteric Neurons

3.1.1 Establishment of a Primary Culture System of Murine Enteric Neurons

As a model for studying uptake of α -Synuclein into enteric neurons, we established an experimental system for the primary culture of murine enteric neurons (Fig. 1A). The staining results showed that many neurons (green) were universally present in the wells, compared to glia (red) and other cells (Fig. 1B). In addition, some neurons had extended projections. These results demonstrate that this method can be used for primary culture of mouse enteric neurons, and we decided to use this method for further experiments.

3.1.2 α -Synuclein PFF Uptake into Enteric Neurons and FABP2

To observe α -Synuclein uptake into enteric neurons, primary cultured murine enteric neurons at 6 DIV were

treated with α -Synuclein PFF at a concentration of 1 μ M and fixed after 48 hours. Immunocytochemistry was performed, and the neurons were observed with confocal laser microscopy.

Intracellular uptake of α -Synuclein was observed in cells treated with α -Synuclein PFF (Fig. 2A). The areas of α -Synuclein accumulation and FABP2 localization were observed to overlap; therefore, we conducted quantitative analysis. In the β III-Tubulin positive cells, we defined the FABP2 localized areas as "FABP2 positive areas" and the other areas as "FABP2 negative areas" and quantified the average fluorescence intensity of fluorescently labeled α -Synuclein PFF in each area. As a result, the mean fluorescence intensity of fluorescently labeled α -Synuclein per unit area was significantly higher ($p < 0.0001$) in FABP2 positive areas than in FABP2 negative areas (Fig. 2B).

In addition, the correlation between the fluorescence intensity per unit area of fluorescently labeled α -Synuclein PFF and that of the FABP2 antibody was analyzed for micrographs such as Fig. 2A. The results revealed a significant positive correlation between the fluorescence intensity of α -Synuclein and FABP2 ($r_s = 0.4237$, $*p = 0.0276$) (Fig. 2C).

3.2 Analysis of FABP2 Levels in Plasma of Patients with Parkinson's Disease

3.2.1 Relationship between Plasma FABP2 Level and Disease Progression

In our previous study, we measured plasma FABP2 levels using plasma samples from patients with Parkinson's disease and other related disorders, as well as healthy controls [27]. In this study, we used these data to further analyze the changes in plasma FABP2 levels in patients with Parkinson's disease.

As previously reported, FABP2 levels tend to be elevated in Parkinson's disease [27]. To clarify the relationship between plasma FABP2 levels and the progression of Parkinson's disease, we analyzed its relationship with the Hoehn and Yahr Scale, a representative index of disease severity, and duration. To simplify the comparison with healthy controls and statistical processing, plasma FABP2 levels in healthy controls were standardized to a mean of 5 and a standard deviation of 1. FABP2 levels in patients with Parkinson's disease were calculated based on the mean and standard deviation of the healthy controls.

First, in terms of the relationship between disease severity and FABP2 levels, there was no significant difference between stages, although there was an increasing trend in Stages 2 and 4 compared to healthy controls (Fig. 3A). Next, we analyzed the relationship between disease duration and FABP2 levels. The FABP2 levels tended to decrease along with duration (Fig. 3B). Therefore, we further analyzed the relationship between disease duration and FABP2 levels by stratifying patients according to disease duration. Patients were divided into five groups: 2 years or less (≤ 2 years), 2–5 years (≤ 5 years), 5–10 years (≤ 10

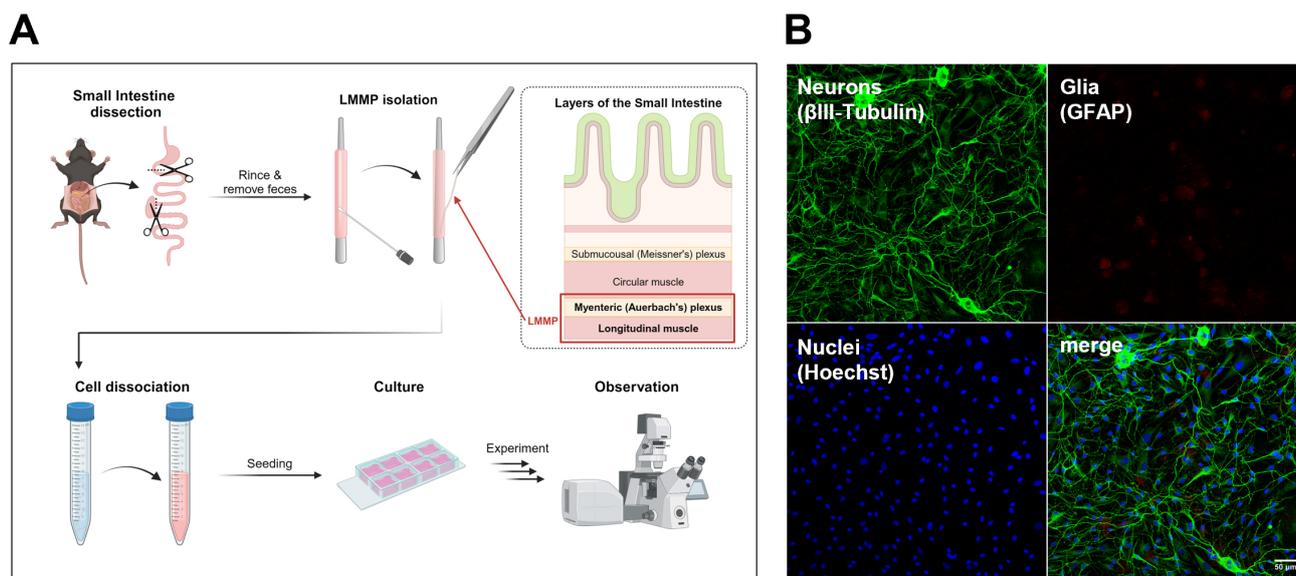


Fig. 1. Establishment of murine enteric neuron primary culture system. (A) Summary of experimental procedures (Created with BioRender.com). (B) Representative images of primary cultured cells derived from longitudinal muscle/myenteric plexus (LMMP) obtained from the small intestine of 8-week-old C57BL/6J mice (male), fixed at 8 DIV. The cells were immunostained with antibodies specific for β III-Tubulin (green), a marker of neurons; GFAP (red), a marker of glia; and Hoechst (blue) for cell nuclei staining (Scale bar: 50 μ m). DIV, days *in vitro*. GFAP, glial fibrillary acidic protein.

years), 10–15 years (≤ 15 years), and over 15 years (> 15 years). Although no significant differences were observed on each pair of groups, the results suggested that FABP2 levels increased with increasing disease duration, peaked at 5–10 years, and then decreased, returning to the same level as that of healthy controls after 15 years (Fig. 3C).

3.2.2 Relationship between Plasma FABP2 Level and α -Synuclein Level

To determine the relationship between plasma levels of FABP2 and α -Synuclein in patients with Parkinson's disease, we analyzed the correlation between FABP2 and α -Synuclein levels (Fig. 4A). The results showed that plasma FABP2 levels were negatively correlated with α -Synuclein levels in patients with Parkinson's disease.

As with FABP2, we stratified patients with Parkinson's disease according to the duration of disease and checked changes in α -Synuclein level. The results showed that α -Synuclein levels decreased as the duration of disease prolonged, peaked around 5 years, and then increased (Fig. 4B). Interestingly, this contrasted with the FABP2 level, which increased until the disease duration reached 5–10 years, peaked, and then declined.

Therefore, we defined a new index, the FABP2/ α -Synuclein ratio, as the FABP2 level divided by the α -Synuclein level for each plasma sample and checked its change with disease duration. The FABP2/ α -Synuclein ratio fluctuated more significantly (Fig. 5A). The abilities to differentiate were evaluated in two groups: patients 2–5 years after onset of the disease, when the FABP2/ α -Synuclein ratio is highest, and healthy controls. The

FABP2/ α -Synuclein ratio was shown to differentiate disease groups from the healthy controls with higher accuracy than FABP2 or α -Synuclein alone (Fig. 5B).

4. Discussion

In this study, we used murine primary cultured enteric neurons to study α -Synuclein uptake into neurons in the intestine. In α -Synuclein treated primary cultured enteric neurons, α -Synuclein was concentrated in the FABP2 localizing area, suggesting that FABP2 is involved in the intracellular uptake and aggregation of α -Synuclein. The cells used in this study were derived from the Auerbach's plexus (myenteric plexus) of the small intestine. It has long been known that Lewy bodies are found in the Auerbach's plexus, as demonstrated by autopsy studies of patients with Parkinson's disease [28]. Thus, the findings of α -Synuclein uptake in primary cultured neurons derived from mouse Auerbach's plexus in this study are consistent with the results of previous studies. Therefore, the primary culture system of mouse enteric neurons that was used in this study is a suitable model for studying α -Synuclein pathology in the enteric nervous system.

We have previously developed a selective ligand for FABP3, which plays an important role in brain α -Synuclein pathology and reported that this ligand inhibits α -Synuclein oligomerization and spreading in the brain [9,29]. By developing such a ligand for FABP2, which is the focus of this study, we hope to achieve a specific mechanistic understanding of the contribution of FABP2 to α -Synuclein pathology and apply it to develop a treatment for synucleinopathies targeting FABP2.

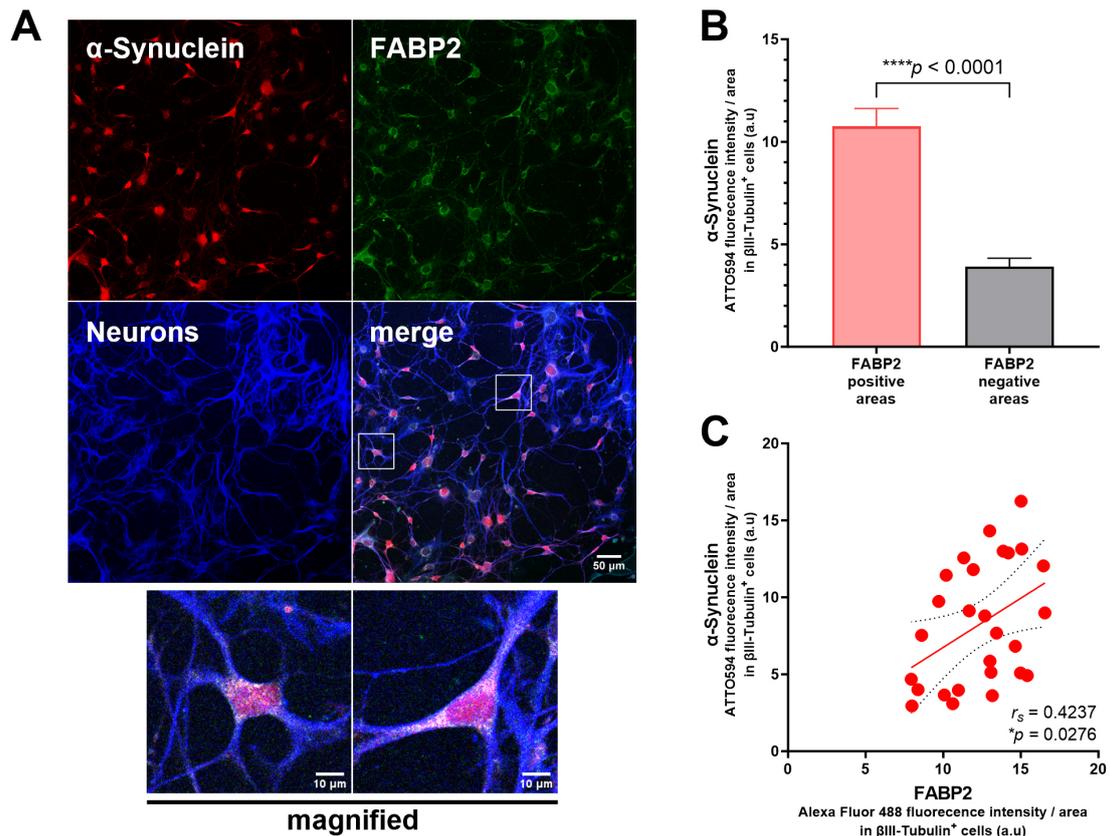


Fig. 2. Taken up α -Synuclein concentrated in FABP2 positive area in enteric neurons. (A) Representative images of cells treated with fluorescently labeled α -Synuclein PFF (red) at a concentration of 1 μ M and cultured for 48 hours. Immunostaining was performed using antibodies specific for the neuronal marker β III-Tubulin (blue) and FABP2 (green). The bottom images are magnified images of the white frame areas of the merge image. (B) In β III-Tubulin-positive cells, FABP2 positive areas were defined as areas where FABP2 was localized, and FABP2 negative areas were defined as areas where FABP2 was not localized. The average fluorescence intensity of ATTO594 within each area was quantified. Quantitative analysis was performed using 27 microscopic images, as shown in (A). FABP2 positive and FABP2 negative areas in the same images were paired and tested using a paired t -test, and the obtained p -values are shown in the figure (Error bars: Mean with SEM). (C) The correlation between the fluorescence intensity per unit area of fluorescently labeled α -Synuclein taken up into the β III-Tubulin-positive cells and FABP2 antibody was analyzed (r_s : Spearman's rank correlation coefficient). FABP2, fatty acid-binding protein; SEM, standard error of the mean.

In the analysis of FABP2 and α -Synuclein levels in patient plasma, focusing on the disease duration, there was a tendency for the plasma FABP2 levels to increase until approximately 5–10 years after the onset of the disease, and then to decrease. It is interesting to note that FABP2 levels do not change uniformly over the duration of the disease but rather increased once before decreasing. In patients with Parkinson's disease, plasma α -Synuclein levels also tended to fluctuate over the duration of disease. The fluctuation was similar to that of FABP2, which peaked approximately 5 years after the onset of the disease, decrease once, and then increased again. We defined a new index, the FABP2/ α -Synuclein ratio, which was the FABP2 level divided by the α -Synuclein level and examined its relationship to the duration of disease. The changes in the FABP2/ α -Synuclein ratio were more clearly observed than FABP2 or α -Synuclein alone. These results suggest that

FABP2 and α -Synuclein are involved in the progression of Parkinson's disease and their effects are apparent in plasma levels.

In the central nervous system, the accumulation of α -Synuclein is known to increase with the progression of Parkinson's disease [30]. On the other hand, the plasma levels of disease-related proteins such as FABP2 and α -Synuclein do not uniformly increase or decrease with the progression of the disease but vary from period to period, possibly because the sites and tissues from which these proteins are released or accumulated also vary with the duration of the disease as follows: (1) If the tissue in which proteins such as α -Synuclein have accumulated is a site where proteins that have diffused extracellularly due to cell death or other causes are likely to leak into the blood, then plasma levels increase; (2) If the site diffuses outside the cell but is mostly taken up by surrounding cells and is unlikely to leak

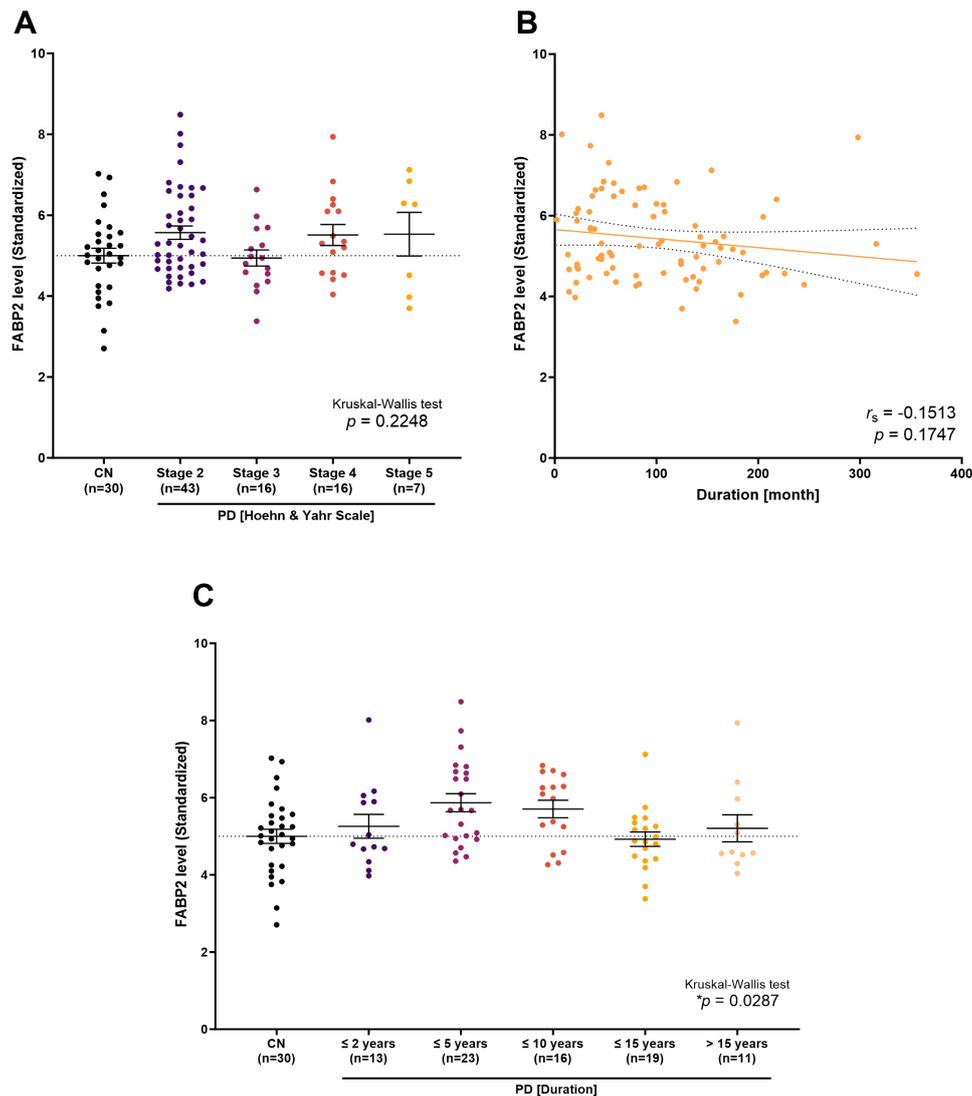


Fig. 3. Relationship between plasma FABP2 levels and Parkinson's disease progression. (A) FABP2 levels were compared between patients with Parkinson's disease stratified by the Hoehn & Yahr scale and healthy controls (CN). When $p < 0.05$, by applying the Kruskal-Wallis test, the difference between groups was tested using Dunn's multiple comparison test (n: number of samples, Error bar: Mean with SEM). (B) The duration of disease (Duration) was used as the abscissa, and the FABP2 level was used as the ordinate to compare the relationship between the two (r_s : Spearman's rank correlation coefficient). (C) The duration of Parkinson's disease was classified into five groups, and FABP2 levels were compared with those of healthy controls (CN). When $p < 0.05$, by applying the Kruskal-Wallis test, the difference between the groups was tested using Dunn's multiple comparison test (n: number of samples, Error bar: Mean with SEM). PD, Parkinson's disease.

into the bloodstream, no change in plasma levels occurs; (3) Rather, plasma levels are reduced in tissues that are more likely to take up proteins in the blood. As mentioned in the introduction, the vagus nerve is suggested as one of the pathways by which α -Synuclein is transmitted from the intestine to the brain. However, it is important to note that the vagus nerve not only leads from the gut to the brain but also extends from the brain to other tissues [14]. Considering this, the plasma levels of each protein may vary depending on the stage of accumulation and release of disease-related proteins, not only in the gut and brain, but also in other tissues.

Previous studies have shown that FABP2 is associated with intestinal inflammation and that intestinal injury releases FABP2 into the circulation [31]. Also, intestinal inflammation has been suggested to induce α -Synuclein pathology in the intestine [14]. Based on these previous findings and the results of this study, it is possible to infer mechanisms of pathogenesis and progression of synucleinopathy as follows: First, early inflammation caused by stress response or drug exposure leads to accumulation of intestinal α -Synuclein, which propagates to and accumulates in the brain as the disease progresses. Then α -Synuclein and other disease-related proteins accumulating

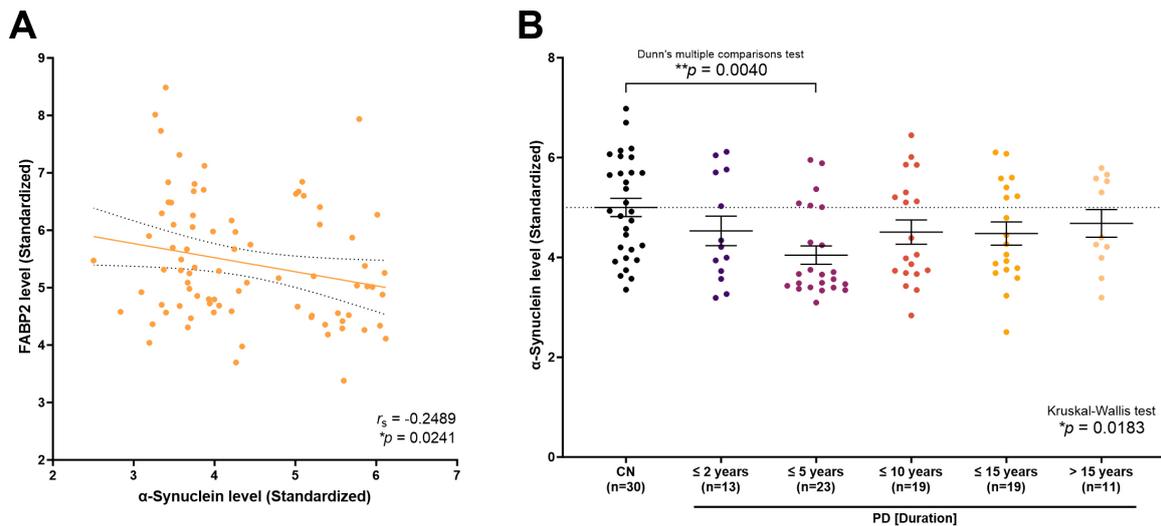


Fig. 4. Correlation between FABP2 and α -Synuclein and change of α -Synuclein level according to disease duration. (A) The correlation between α -Synuclein level and FABP2 level was analyzed using α -Synuclein level as the horizontal axis and FABP2 level as the vertical axis (r_s : Spearman's rank correlation coefficient). (B) The duration of Parkinson's disease was classified into five groups, and their α -Synuclein levels were compared with those of healthy controls (CN). When $p < 0.05$, by applying the Kruskal-Wallis test, the difference between the groups was tested using Dunn's multiple comparison test (n: number of samples, Error bar: Mean with SEM).

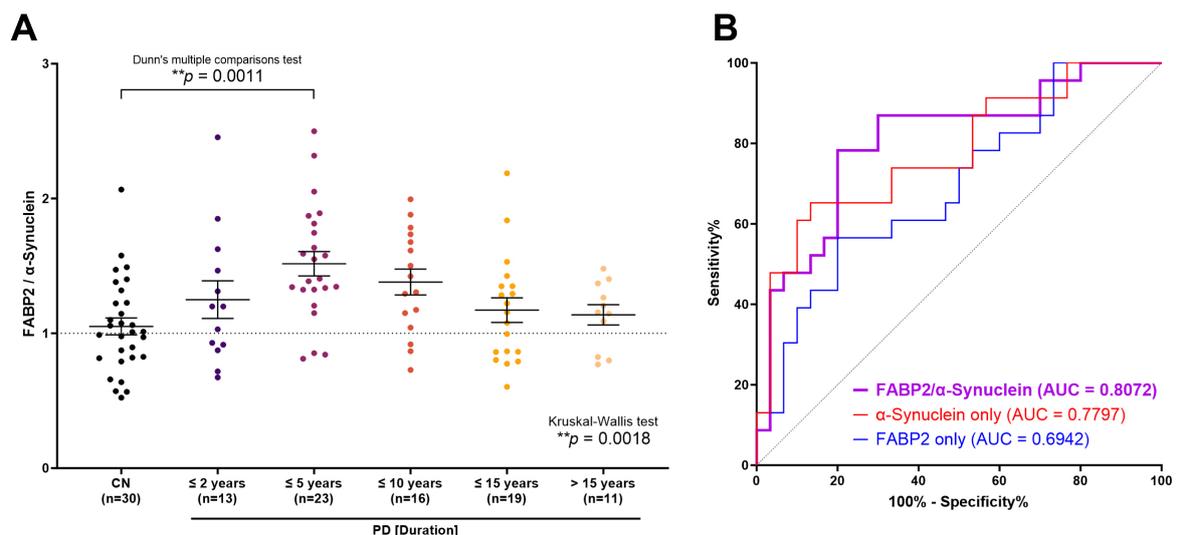


Fig. 5. FABP2/ α -Synuclein ratio shows higher discriminant ability than FABP2 or α -Synuclein alone. (A) The duration of Parkinson's disease was classified into five groups, and the FABP2/ α -Synuclein ratio was compared with that of healthy controls (CN). When $p < 0.05$, by applying the Kruskal-Wallis test, the difference between the groups was tested using Dunn's multiple comparison test (n: number of samples, Error bar: Mean with SEM). (B) ROC curve showing the discriminative ability of the FABP2/ α -Synuclein ratio, as assessed by discriminating between controls and patients with PD 2–5 years post-onset (AUC: area under the curve). ROC, receiver operating characteristic; PD, Parkinson's disease.

in the cells leak into the blood, and the plasma concentrations of these proteins change. Eventually, most enteric neurons die, and the plasma concentration of these proteins reaches a steady state when no proteins leak from the intestine into the plasma.

Although this study suggests that FABP2 is potentially involved in the pathogenesis and progression of synucleinopathy, several questions remain unanswered. In the

experiment of α -Synuclein uptake in enteric neurons, the taken up α -Synuclein was concentrated at the FABP2 localization site, but the details of the mechanism of how FABP2 is specifically involved in the formation of α -Synuclein pathology were not clarified in this study. It is necessary to clarify the specific role of FABP2 in the process of α -Synuclein uptake, aggregation, and accumulation in enteric neurons. We are currently investigating the pharmacolog-

ical effects of FABP2 knockdown and FABP ligand treatment on α -Synuclein uptake to elucidate the pathogenesis of FABP2-targeted synucleinopathy and its therapeutic application in the future. However, these experiments showed that the primary cultured neurons used in this experiment were inefficient for siRNA lipofection and the introduction of viral vectors containing shRNA, making it difficult to obtain knockdown effects at the protein level. This issue should be addressed in future studies. Additionally, because we used mouse-derived primary cultured cells in this study, what we observed is the interaction between mouse endogenous FABP2 and human α -Synuclein PFF. In accordance with previous studies that investigated the properties of human α -Synuclein in rodents [32], we used human α -Synuclein in this study. Future studies should analyze the relationship between human FABP2 and human α -Synuclein using human-derived tissues and cells. In the analysis of plasma FABP2 levels, we were unable to examine how FABP2 levels changed from pre-onset to disease onset because we used samples collected from patients who had already been diagnosed with Parkinson's disease. Although it was found that FABP2 levels fluctuate with the duration of Parkinson's disease, it is necessary to clarify in detail what kinds of fluctuations are observed before the onset of the disease, how they are related to the onset and progression of the disease, and why plasma FABP2 levels fluctuate in the first place.

5. Conclusions

This study suggests that FABP2 is potentially involved in two aspects of α -Synuclein pathology. One is the possibility that it plays some role in the uptake and accumulation of α -Synuclein in enteric neurons, and the other is its potential as a biomarker for changes occurring in the patient's body during the progression of Parkinson's disease. Focusing on FABP2 may lead to the elucidation of a consistent mechanism from the prodromal stage to the onset and subsequent progression of synucleinopathy. Our findings may contribute to the development of novel diagnostic biomarkers and fundamental therapeutics.

Abbreviations

FABP, fatty acid-binding protein; CN, healthy controls; PD, Parkinson's disease; DLB, dementia with Lewy bodies; LMMP, longitudinal muscle/myenteric plexus; PFF, preformed fibrils.

Availability of Data and Materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

Conceptualization: TSeK, KF and IK; methodology: TSeK and IK; software: TSeK; validation: TSeK, KF, HO,

TB, TT, AT and IK; formal analysis: TSeK, KF, HO, TB, TT, AT and IK; investigation: TSeK and IK; resources: HO, TB, TT, and AT; data curation: TSeK, KF, HO, TB, TT, AT, TSas and IK; writing—original draft preparation: TSeK; writing—review and editing: KF and IK; supervision: KF, AT, TSas and IK; project administration: KF, AT and IK; funding acquisition: KF and IK. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All the participants or their legal representatives provided written informed consent. The human experiments conducted in this study adhered to the principles outlined in the Declaration of Helsinki. The research protocols were approved by the Ethics Committees of Tohoku University (approval number PH19-5) and the National Hospital Organization Sendai Nishitaga Hospital (approval numbers 29-3 and 29-10).

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Conflict of Interest

The authors declare no conflict of interest. Kohji Fukunaga and Ichiro Kawahata are serving as Guest editors of this journal. We declare that Kohji Fukunaga and Ichiro Kawahata had no involvement in the peer review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to François Ichas.

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