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Original Article

Experimental Neurobiology

Transmission of Synucleinopathies in the Enteric Nervous System of A53T Alpha-Synuclein Transgenic Mice

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Parkinson's disease (PD) and dementia with Lewy bodies (DLB) are characterized by abnormal deposition of α-synuclein aggregates in many regions of the central and peripheral nervous systems. Accumulating evidence suggests that the α-synuclein pathology initiates in a few discrete regions and spreads to larger areas in the nervous system. Recent pathological studies of PD patients have raised the possibility that the enteric nervous system is one of the initial sites of α-synuclein aggregation and propagation. Here, we evaluated the induction and propagation of α-synuclein aggregates in the enteric nervous system of the A53T α-synuclein transgenic mice after injection of human brain tissue extracts into the gastric walls of the mice. Western analysis of the brain extracts showed that the DLB extract contained detergent-stable α-synuclein aggregates, but the normal brain extract did not. Injection of the DLB extract resulted in an increased deposition of α-synuclein in the myenteric neurons, in which α-synuclein formed punctate aggregates over time up to 4 months. In these mice, inflammatory responses were increased transiently at early time points. None of these changes were observed in the A53T mice injected with saline or the normal brain extract, nor were these found in the wild type mice injected with the DLB extract. These results demonstrate that pathological α-synuclein aggregates present in the brain of DLB patient can induce the aggregation of endogenous α-synuclein in the myenteric neurons in A53T mice, suggesting the transmission of synucleinopathy lesions in the enteric nervous system.

Key words: enteric nervous system, Parkinson's disease, dementia with Lewy bodies, protein aggregation, Lewy body, inflammation

INTRODUCTION

Parkinson's disease (PD) is a progressive, age-related neurodegenerative disorder. Clinically, it is characterized by motor symptoms, such as rigidity, bradykinesia, postural instability, gait disorder, and tremor [1]. However, non-motor symptoms, such as hyposmia, gastrointestinal abnormalities, and autonomic dysfunction are increasingly accepted as integral part of PD clinical manifestations and often precede the classical motor symptoms [2]. Pathologically, selective neurodegeneration and the occurrence of Lewy bodies (LB) in central and peripheral nervous systems are observed in PD at autopsy [3]. The neuronal protein α-synuclein aggregates are major LB component. Mutations

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in α-synuclein gene have been linked to monogenic forms of familial PD [4-8]. Furthermore, genome-wide association studies suggested that sporadic PD is also associated with the genetic variations in α-synuclein gene [9].

Recent neuropathological studies indicate that α-synuclein pathology progresses in a highly specific and predictable pattern in the central nervous system (CNS). Braak and colleagues have divided this progressive pattern into six stages, spreading from the lower brain stem and the olfactory bulb to the limbic system, and finally, to several regions of the neocortex [10, 11]. Although the precise sequence of progression is under debate, it is accepted that α-synuclein pathology spreads from a few discrete regions to larger areas in the brain. An increasing body of evidence suggests that a direct cell-to-cell transfer of α-synuclein aggregates is the underlying mechanism for the pathological spreading [12, 13].

α-synuclein pathology in PD is not limited to the brain but is also found in other nervous systems including sympathetic ganglia, the enteric nervous system (ENS), cardiac and pelvic plexuses, skin, etc. [14]. Gastrointestinal (GI) dysfunction is a common feature in PD, and deposition of α-synuclein in the ENS of PD patients has been reported in the myenteric and submucosal plexuses of GI tracts [15-17]. α-synuclein containing aggregates were also observed in the myenteric plexus of the normal aging rats [18]. α-synuclein aggregates were detected in the ENS prior to the changes in the CNS in a transgenic mouse model [19]. These previous studies have raised the possibility that the α-synuclein pathology could be initiated from the ENS, and propagate to the CNS through the vagus nerve [10].

Here, we determined whether pathogenic α-synuclein aggregates in Lewy body disease could induce α-synuclein pathology in the ENS of a mouse model. Brain extracts from a DLB patient and a normal control subject were injected into the gastric walls of α-synuclein A53T transgenic or C57BL6 control mice, and the α-synuclein pathology and inflammatory responses were assessed.

MATERIALS AND METHODS

Materials

The primary antibodies used are as follows: α-synuclein monoclonal antibody (Syn-1; BD Biosciences, San Diego, CA, USA), human α-synuclein specific monoclonal antibody 274 from our laboratory [20], anti-glial fibrillary acidic protein (GFAP) antibody (Abcam, Cambridge, MA, USA), and anti-MHC-II antibody (eBioscience, San Diego, CA, USA).

Human brain tissues

The human brain tissues were from the South Australia Brain Bank. The normal control brain tissue is from a 72 year old male with 30 hours postmortem delay and had no neurofibrillary tangles, plaques, or Lewy body pathology. DLB patient brain tissue was from a 77 year old male with 19 hours postmortem delay who had diffuse Lewy body pathology. The pathological stains were performed by the Bielschowsky silver and specific immuno-diagnostic stains.

Mouse strains

C57BL6 and A53T tg [B6.Cg-Tg(Thy1-SNCA*A53T)1Sud/J] mice were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). Housing and breeding of animals were performed at the animal care facility of Konkuk University. The experiments were approved by the Institutional Animal Care and Use Committee of Konkuk University (IACUC #KU11023).

Preparation of brain extracts

The brain homogenates from normal and DLB patient brains were obtained by sonicating unfrozen temporal cortices in PBS with protease inhibitor cocktail (Sigma, St. Louis, MO, USA). The protein concentration was determined by BCA assay (Pierce, Rockford, IL, USA).

Western blotting

The procedure for western blotting is described elsewhere [21]. Chemiluminescence detection was performed using the FUJIFILM Luminescent Image Analyzer LAS-3000 and Multi Gauge (v3.0) software (FUJIFILM, Tokyo, Japan).

Stomach injection experiments

Ten to twelve weeks old male and female mice were anesthetized by intraperitoneal (*i.p.*) injection of Zoletil50 (75 mg/kg; Virbac, Carros cedex, France), rompun (5 mg/kg; Bayer AG, Leverkusen, Germany), and saline mixture (1 : 1 : 18). Using the clean forceps and scissors, the stomach cavity was opened and 100 μg brain extract proteins was injected using the syringe into the several places of body area in the stomach wall, being careful not to puncture the stomach wall. After suturing, mice were maintained as usual. The number of animals used are described below: saline 1month (3), normal brain extract 1month (4), DLB extract 1month (5), saline 2 months (4), normal brain extract 2 months (5), DLB extract 2 months (5), saline 3months (2), normal brain extract 3 months (2), DLB extract 3 months (4), saline 4 months (4), normal brain extract 4 months (2), and DLB extract 4 months (6).

Tissue preparation

Mice were anesthetized by *i.p.* injection of Zoletil50 (75 mg/kg), rompun (5 mg/kg), and saline mixture (1 : 1 : 18). The mice

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were transcardially perfused with 0.9% saline, followed by 4% paraformaldehyde (PFA). Stomach, brain, and spinal cord were removed and postfixed and stored for further analyses. Stomach was cut and laid flat on the sponge before post-fixing with 4% paraformaldehyde overnight. Fixed stomach tissue was then rinsed in DMSO three times 5 minute each to ease the separation of stomach wall layers. It was rinsed in PBS several times to remove DMSO. Using the thin forceps, mucosa, submucosa, muscularis externa layers were carefully removed to expose myenteric plexus.

Whole-mount immunohistochemistry

The prepared stomach tissue was immunostained for further analysis. After washing 3 times in PBST (PBS/0.2% Triton X-100), the tissue was blocked using Vector M.O.M. kit (Vector Labs, Burlingame, CA, USA) for 1 hour at RT. The primary antibodies were diluted in blocking buffer and incubated for 48 hours at 4°C with slow rotation. After washing 3 times in PBST, fluorescence dye (Cy2-, Rhodamine Red X-) conjugated secondary antibodies were added and incubated for 1 hour at RT. After washing 3 times in PBST, the nuclei were stained with TOPRO-3 (Invitrogen, Carlsbad, CA) for 10 min at RT and washed again in PBST. The stained tissue was mounted on slide using Antifade reagent from Invitrogen.

Statistical analysis

All experiments were blind-coded and repeated three-to-four times. The values in the figures are expressed as the mean ± SEM. Differences were considered significant if p values were < 0.05. The graphs were drawn with Prism 5 software (Graphpad Software Inc., La Jolla, CA, USA). For determination of statistical significance, values were compared by one-way ANOVA with Bonferroni's post-test using InStat (version 3.05) software (Graphpad Software Inc.).

RESULTS

α-synuclein aggregates in the brain extract of a DLB patient

α-synuclein in the brain extracts from the normal subject and a DLB patient were compared by Western blotting (Fig. 1). The amount of monomer (arrow) was not different between the normal and DLB extracts. However, higher levels of α-synuclein aggregates were detected in the DLB brain extract than in the normal extract. Immunohistochemical analyses of temporal cortex of opposite hemisphere revealed comparable background α-synuclein staining in both cases, but only the DLB patient contained LBs (data not shown). We have used these extracts in the following experiments to determine possible interactions of exogenous α-synuclein with the endogenous protein and transmission of pathogenic protein aggregates.

Deposition of α-synuclein in the myenteric plexus of injected A53T mice

Brain extracts were injected into the stomach walls of the A53T heterozygous tg mice, and the α-synuclein deposition was examined in the enteric nervous system within the stomach tissues1 to 4 months after the injection (Fig. 2). After 30 days of injection, mostly diffused α-synuclein immunoreactivity was more prominent in the myenteric plexus neurons of the DLB extract-injected mice than in those of saline-injected or the normal extract-injected mice (Fig. 2A; enlarged). At 60 days, α-synuclein staining in the DLB extract-injected mice showed small punctates in some neurons of the myenteric plexus (Fig. 2B enlarged; arrows), however, these structures were not observed in the saline- or normal extract-injected mice. At 90 days, α-synuclein punctates grew larger in DLB extract-injected mice (Fig. 2C enlarged; arrows). Finally, after 4 months of injection, the number of the myenteric plexus neurons with α-synuclein punctates were increased, and the size of individual punctates became larger (Fig. 2D). Some of the saline- and the normal extract-injected mice started to show small punctates after 4 months. The size and prevalence of these structures were similar to those observed in the DLB-injected mice at 30 days. In contrast to the results in α-synuclein tg mice, wild type C57BL6 mice injected with the DLB extract did not show the punctate α-synuclein patterns even after 4 months (Fig. 5 upper panel). Therefore, the α-synuclein aggregates found in the A53T tg mice injected with the DLB extract are the result of the induced aggregation of neuronal α-synuclein and not the result of simple deposition of the exogenously injected proteins. The graph in Fig. 3 summarizes the changes in the patterns of α-synuclein staining

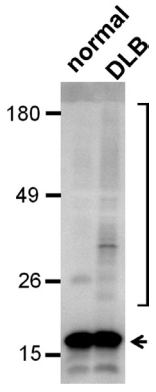

Fig. 1. α-Synuclein proteins in the brain extracts of a DLB patient and a control subject. Total brain extracts (10 μg) from a normal control and a DLB patient were immunoblotted for α-synuclein. The arrow indicates α-synuclein monomers and a bracket shows high MW α-synuclein aggregates.

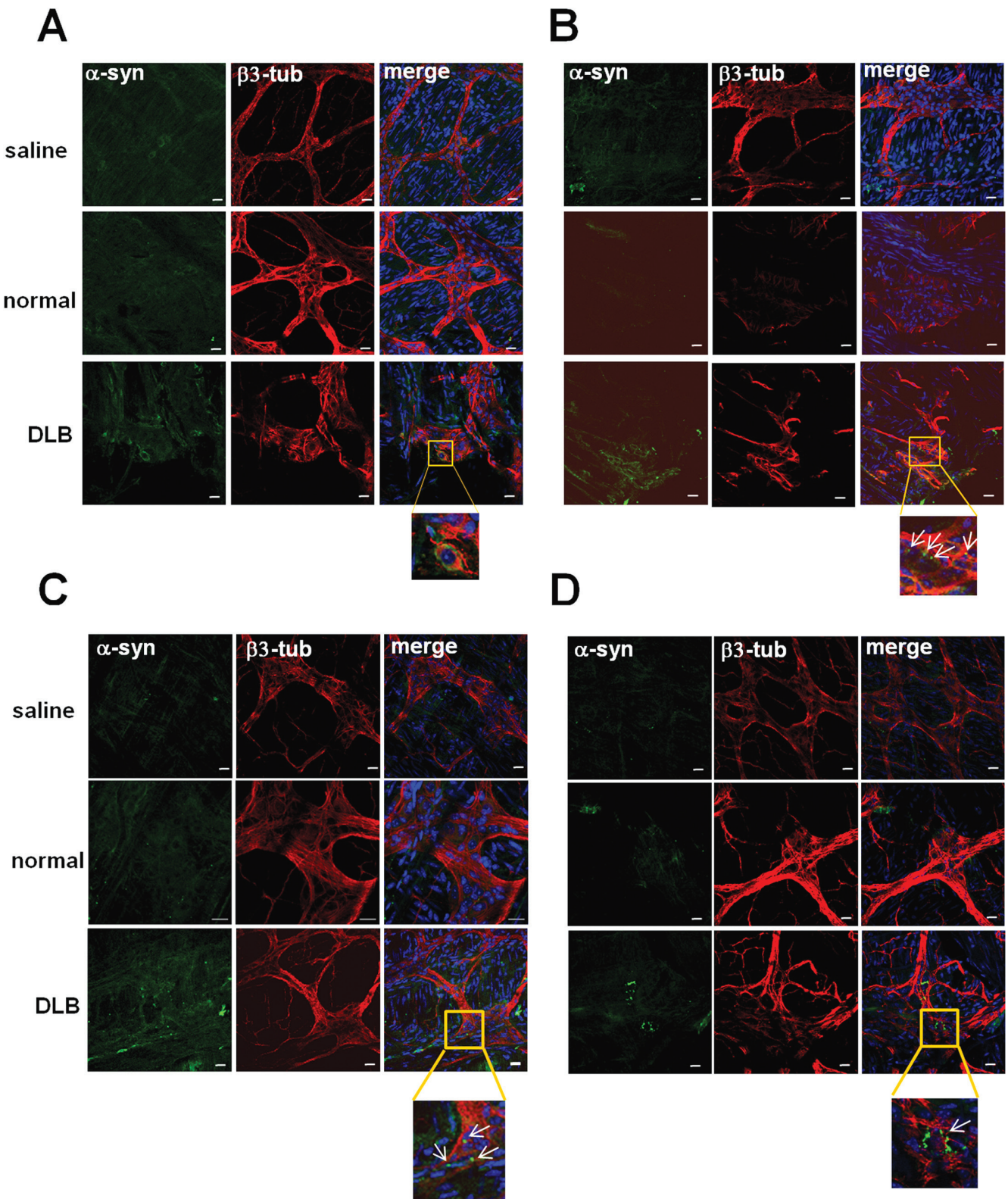

Fig. 2. Accumulation of α-synuclein aggregates in the myenteric plexus of injected A53T mice. Normal and DLB brain extracts or saline were injected to the stomach walls of the A53T tg mice. The whole-mount staining of stomach tissue was performed at (A) 1 month, (B) 2 months, (C) 3 months, and (D) 4 months. Antibodies for β3-tubulin as a neuronal marker and human α-synuclein (274) were used. Arrows in the enlarged images indicate α-synuclein aggregates (Scale bar: 20 μm).

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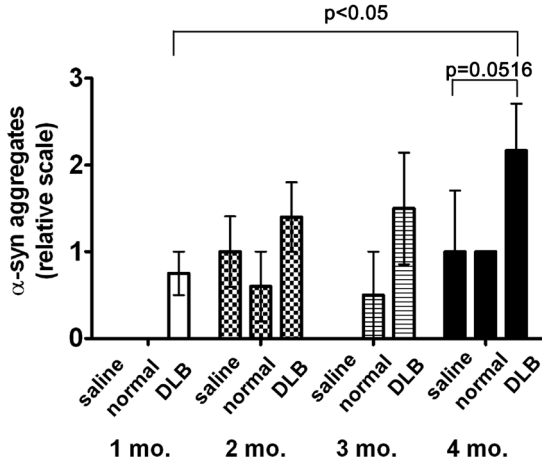

Fig. 3. Quantification of α-synuclein deposition in the myenteric neurons. The graph represents the degree of α-synuclein aggregation shown in Fig. 2. The abundance and size of α-synuclein aggregates are represented in a scale of 0 to 3, with 0 being no aggregates.

from slight, diffused staining (scale 0) to larger aggregates (scale 3) in the DLB extract-injected mice. Only the mice injected with the DLB extract showed an increase in α-synuclein aggregates over time. These results suggest that exogenous α-synuclein aggregates could induce the aggregation of the endogenous protein in the myenteric neurons, thereby propagating synucleinopathy lesions within the enteric nervous system.

Changes in the inflammatory responses in the DLB extract-injected A53T mice

To determine if inflammatory responses are related to the changes in the α-synuclein aggregation, activation of macrophage was evaluated by the immunostaining for major histocompatibility complex class 2 (MHCII) (Fig. 4). There was slight increase in the MHCII staining in the DLB extract-injected mice at 30 days (Fig. 4A). MHCII expression increased further at 60 days in the DLB extract-injected mice (Fig. 4B). However, 90 and 120 days after injection, the expression levels for MHCII dropped to the levels of saline and normal extract controls (Fig. 4C, D). There were little changes in the MHCII immunoreactivity in the saline and normal extract-injected mice (Fig. 4) and in wild-type mice injected with the DLB extract (Fig. 5 lower panel) during the entire period of the experiment. There were no significant changes in glial fibrillar acidic protein (GFAP) immunoreactivity, suggesting little effect on enteric astrocytes (Fig. 4). This result shows that the inflammatory responses precede the changes in the α-synuclein aggregation and may have an important role in the transmission of synucleinopathies.

DISCUSSION

The discovery of abnormal deposition of α-synuclein in the ENS of PD patients early in the disease [15-17] has raised the speculation that the ENS might be an initiation site for the α-synuclein pathology, which then propagates to the CNS possibly via the vagus nerve. Supporting this, in a transgenic mouse model, accumulation of α-synuclein aggregates in the ENS precedes the changes in the CNS [19]. In the present study, we have shown that injection of the total brain extract of a DLB patient into the gastric walls of α-synuclein A53T transgenic mice resulted in an accumulation of α-synuclein aggregates in the enteric neurons. In contrast, injection of control brain extract failed to produce the aggregates. The spreading of aggregates was much more robust in the transgenic mice than in non-transgenic control mice, suggesting the interaction between the exogenous and neuronal endogenous α-synuclein proteins. Our results suggest that once α-synuclein aggregates are formed in some neurons in the GI system, they can spread to a larger neuronal population in the ENS, thereby increasing the chance to propagate the aggregates to the CNS.

The GI system is one of the most susceptible systems to environmental stresses in the body as it is in direct contact with environmental agents. Recently, intragastric administration of rotenone in mice resulted in a progressive deposition of α-synuclein in both the ENS and the CNS neurons that are affected by PD, such as the neurons in the myenteric plexus, the dorsal motor nucleus of vagus (DMV), the spinal cord, and the SN [22]. Injection of proteasome inhibitors to the ventral wall of the stomach also led to the formation of α-synuclein-immunopositive aggregates in the DMV in rats [23]. These studies suggested that environmental stresses to the GI system could lead to the α-synuclein pathology in the CNS. The mechanism by which environmental agents induce α-synuclein aggregation is unknown. However, a recent study showed that α-synuclein expression in the ENS could be upregulated by agents that cause depolarization and increase the levels of cyclic AMP [24].

We and several other groups have previously shown that a small amount of α-synuclein could be secreted from neuronal cells via exocytosis [13]. A significant portion of the released α-synuclein is in oligomeric states [25, 26]. After the release, the extracellular α-synuclein can be internalized into neighboring neurons, astrocytes, and microglia via endocytosis [27, 28]. These findings inspired the studies, in which direct cell-to-cell transfer of α-synuclein has been demonstrated in both cell culture and animal models [29-32]. Transfer of α-synuclein resulted in the formation of LB-like inclusion bodies and neurodegeneration [29]. The direct cell-to-cell transfer of α-synuclein might be the underlying

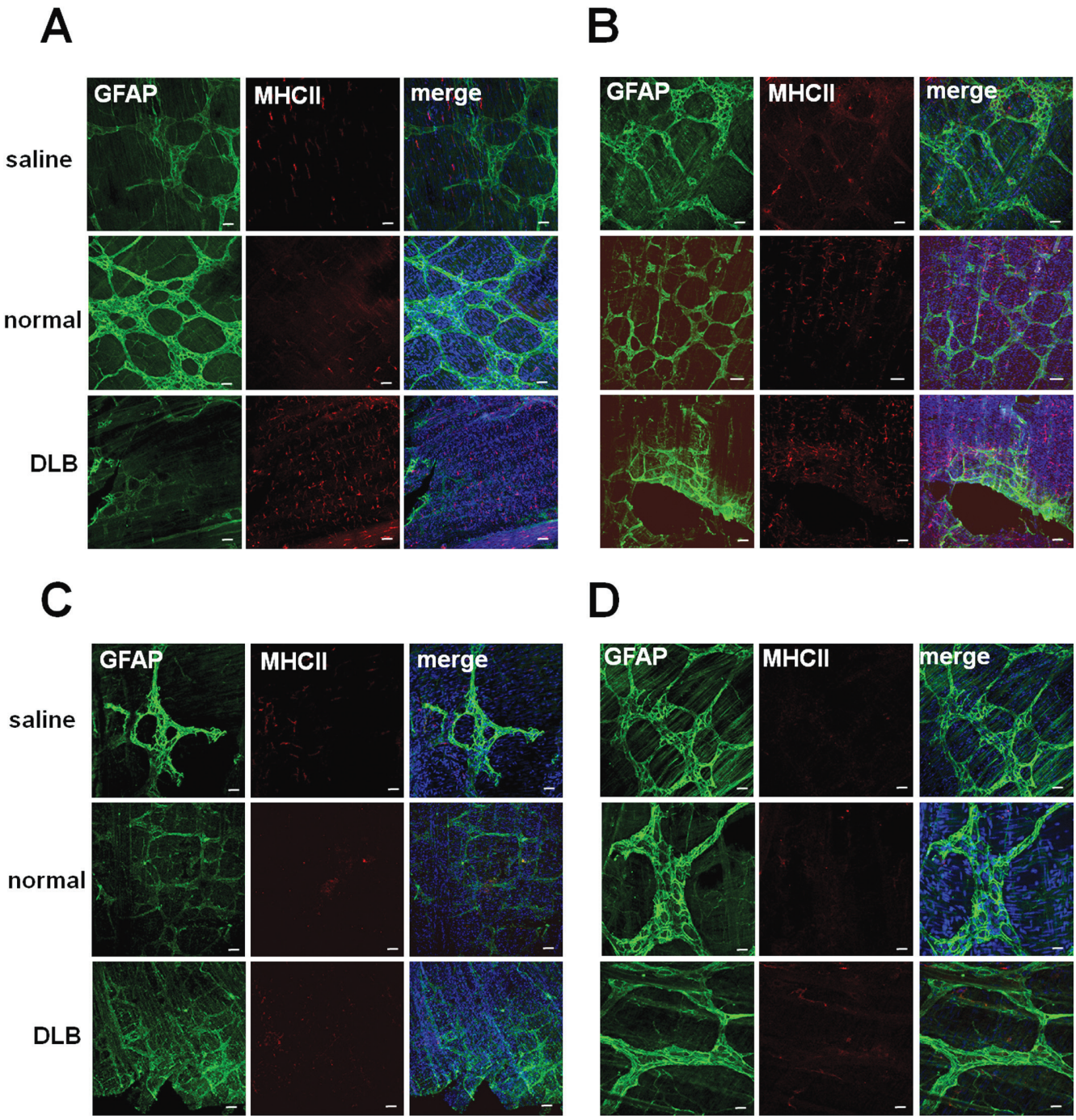

Fig. 4. Inflammatory responses precedes the accumulation of α-synuclein in the DLB extract-injected A53T mice. Normal and DLB Brain extracts or saline were injected to the stomach walls of the A53T tg mice. The whole-mount staining of stomach tissue was performed at (A) 1 month, (B) 2 months, (C) 3 months, and (D) 4 months with antibodies for astrocyte marker GFAP and a marker for activated macrophage MHCII. Note that the MHCII staining was increased 1 month after injection, and more so at 2 months in the DLB extract-injected mice, but decreased afterwards (Scale bar: 20 μm).

mechanism for the aggregate spreading in the ENS observed in the current study.

Another interesting finding from our experiments was the transient activation of macrophages at earlier time points (1~2 months). Chronic inflammation is a critical component in the progression of PD and might be an inducer of protein aggregation [33]. We have previously shown that α-synuclein can be directly transferred from neurons to astroglia, and as a result, pro-inflammatory responses were induced [34]. Microglia and macrophages were also activated by ectopically administered

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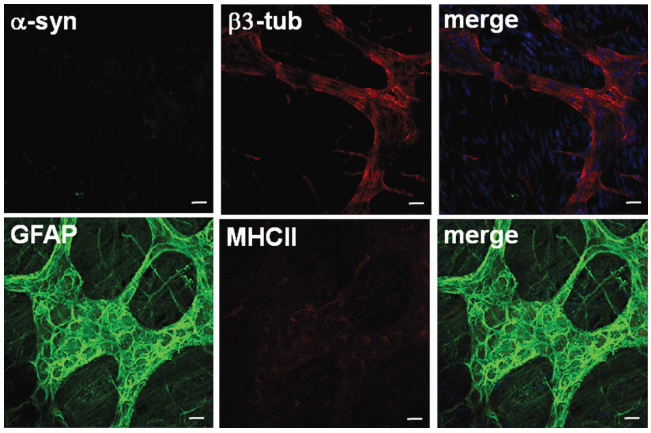

Fig. 5. Lack of α-synuclein accumulation and inflammation in the myenteric plexus of the DLB extract-injected wild type mice. Wild type C57BL6 mice injected with the DLB extract, and 4 months after injection, the whole-mount staining of stomach tissue was performed as in Figs. 2 and 4 (Scale bar: 20 μm).

α-synuclein proteins [35, 36]. Microglial activation was detected in early stages of PD, suggesting that the neuroinflammatory responses are significant factors in the disease progression [37]. In the current study, we did not detect any changes in astroglia in the ENS after injection of the DLB extract. However, macrophage activation was observed transiently prior to accumulation of α-synuclein aggregates. These results suggest that inflammatory responses in the GI tissues may contribute to the initiation of α-synuclein aggregation in the ENS and perhaps to the propagation of aggregates within the ENS.

In conclusion, we have demonstrated that when exogenously introduced, pathological α-synuclein aggregates present in the brain of DLB patient can induce the aggregation of endogenous α-synuclein in the ENS neurons. These results would be an important foundation for future research to verify the progressive transmission of α-synuclein pathology from the ENS to the CNS in PD and related disorders.

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