LETTER TO THE EDITOR

Progression of phosphorylated α-synuclein in *Macaca fuscata*

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Abstract
Prion-like spreading of abnormal proteins is proposed to occur in neurodegenerative diseases, and the progression of α-synuclein (α-syn) deposits has been reported in the brains of animal models injected with synthetic α-syn fibrils or pathological α-syn prepared from patients with Parkinson’s disease (PD) and dementia with Lewy bodies (DLB). However, α-syn transmission in nonhuman primates, which are more similar to humans, has not been fully clarified. Here, we injected synthetic human α-syn fibrils into the left striatum of a macaque monkey (*Macaca fuscata*). At 3 months after the injection, we examined neurodegeneration and α-syn pathology in the brain using α-syn epitope-specific antibodies, antiphosphorylated α-syn antibodies (pSyn#64 and pSer129), anti-ubiquitin antibodies, and anti-p62 antibodies. Immunohistochemical examination with pSyn#64, pSer129, and α-syn epitope-specific antibodies revealed Lewy bodies, massive α-syn-positive neuronal intracytoplasmic inclusions (NCIs), and neurites in the left putamen. These inclusions were also positive for ubiquitin and p62. LB509, a human-specific α-syn antibody targeting amino acid residues 115–122, showed limited immunoreactivity around the injection site. The left substantia nigra (SN) and the bilateral frontal cortex also contained some NCIs and neurites. The left hemisphere, including parietal/temporal cortex presented sparse α-syn pathology, and no immunoreactivity was seen in olfactory nerves, amygdala, hippocampus, or right parietal/temporal cortex. Neuronal loss and gliosis in regions with α-syn pathology were mild, except for the left striatum and SN. Our results indicate that abnormal α-syn fibrils propagate throughout the brain of *M. fuscata* via projection, association, and commissural fibers, though the progression of α-syn pathology is limited.

KEYWORDS
animal model, *Macaca fuscata*, pathology, progression, propagation, α-synuclein
INTRODUCTION

Misfolded α-synuclein (α-syn) accumulates in neurons and neurites of patients with Parkinson's disease (PD) (1) and dementia with Lewy bodies (DLB) (2), and the resulting pathological α-syn inclusions appear to progress through the brain, following neuronal connections via axonal projections (3). In support of this notion, the spread of α-syn pathology has been observed in many animal models after injection of synthetic α-syn fibrils and pathological α-syn derived from the brains of patients with PD/DLB, suggesting that α-syn pathology is transmitted in the brain in a prion-like manner (4,5). However, it is unclear whether the observed pathogenic effects in rodents are directly relevant to the α-syn accumulation associated with PD/DLB in humans. In this regard, studies in macaque monkeys, which are considered an excellent nonhuman primate model in brain aging research caused by their anatomical and physiological similarities to humans (6), might be informative. In the present study, we investigated the pathological consequences of intracerebral injection of synthetic human α-syn fibrils in a macaque, Macaca fuscata, by examining the immunoreactivity of whole brain sections to various α-syn antibodies, including anti-phosphorylated α-syn antibody and 10 epitope-specific antibodies covering the whole α-syn molecule (7).

METHODS

2.1 Animals and preparation

Recombinant human wild-type α-syn and fibrils were prepared as described previously (5,8). We designed the experiment in line with current animal protection considerations based on the 3R (reduce, reuse, and recycle) principle. One male 15-year-old M. fuscata was used for this experiment. This monkey had previously been the subject of several experiments on brain electrophysiology. The monkey was anesthetized, then human α-syn fibrils (200 μl/site, total 400 μg/brain) were injected into the left putamen (interaural +29 mm, lateral +17 mm, and depth −10 mm) and nucleus accumbens (interaural +33 mm, lateral +11 mm, and depth −9 mm) in the brain in a biological safety level 2 (BSL-2) environment. Immunohistological studies were carried out 3 months later. All experiments were approved by the Animal Research Ethics Committee of Tamagawa University (experiment protocol; H29-53/R2-53) and were carried out in accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiments in Neuroscience (Japan Neuroscience Society).

2.2 Neuropathological examination

The monkey was deeply anesthetized by injection of pentobarbital and euthanized by perfusion fixation with a mixture of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. After fixation, the left striatum and midbrain were sectioned coronally at 50 μm using a vibratome (Leica, Wetzlar, Germany). Other regions were fixed in 10% formalin, embedded in paraffin, and sectioned at 8 μm. Hematoxylin–eosin (HE) and Klüver-Barrera (K-B) stains were applied. We investigated the bilateral cerebrum, cerebellum, midbrain, pons, and medulla. For high-sensitivity detection, brain sections were treated with formic acid for 20 min, washed, and boiled at 100°C for 20 min. Sections were then incubated with 0.5% H₂O₂ in methanol for 30 min to inactivate the endogenous peroxidases, blocked with 10% calf serum in PBS for 20 min, and incubated overnight with appropriate antibodies. After incubation with biotinylated secondary antibody for 2 h, labeling was detected with DAB, using an ABC staining kit (Vector). Sections were counterstained with hematoxylin. Immunohistochemistry was performed with antibodies directed against phosphorylated α-syn (pSyn#64, mouse monoclonal, 1:1,000 Wako), phosphorylated α-syn (abS1253, pS129, rabbit monoclonal, 1:1,000 Abcam), human α-syn (LB509, mouse monoclonal, 1:1,000; gift from T. Iwatsubo), α-syn (42 α-syn, mouse monoclonal, 1:1,000 BD, Transduction Laboratories) tyrosine hydroxylase (TH, MAB318, mouse monoclonal, 1:1,000, Millipore), p62 (GP62-C, guinea pig polyclonal, 1:1,000 Progen), and Ubiquitin (Z0458, rabbit poly 1:1,000, Dako). We also used polyclonal antibodies directed against synthetic peptides corresponding to residues 1–10, 11–20, 21–30, 31–40, 41–50, 51–60, 61–70, 75–81, and 131–140 of human α-syn (Cosmo Bio) (7). Some α-syn epitope-specific antibodies are known to detect the conformational changes that distinguished monomers from fibrils (7). For double-label immunofluorescence detection, Alexa Fluor 488-conjugated goat anti-mouse or anti-rabbit IgG and Alexa Fluor 568-conjugated goat anti-rabbit IgG (Molecular Probes) were used. Sections were coverslipped with nonfluorescent mounting media with DAPI (VECTASHIELD; Vector Laboratories) and observed with a BZ-X710 fluorescence microscope (Keyence).

We also counted TH-immunoreactive (IR) cells in the bilateral SN of the macaque to evaluate the influence of the injection. Ten sections were randomly selected in each region and quantified by BZ-H3C Hybrid Cell Count Software (Keyence).

GROSS AND MICROSCOPIC PATHOLOGY

Physically, the monkey presented no apparent parkinsonism or behavioral abnormalities after injection. Neuropathologically, the brain weighed 980 g after fixation and showed slight enlargement of the left lateral ventricle. No atrophy was apparent in other regions, including the cerebrum/cerebellum, hippocampus,
amygdala, brainstem, and spinal cord. Discoloration was seen at the bilateral nucleus accumbens (NAc), globus pallidus (GP), putamen (Pu), and substantia nigra (SN). The left SN showed decreased pigmentation compared with the right one (Figure 1Aa). The right occipital cortex presented slight damage caused by previous surgery.

Microscopically, the left striatum including the two injection sites was damaged diffusely and filled with massive macrophages containing hemosiderin. The regions presented neuronal loss and gliosis. Some ballooned neurons were seen in HE stains (Figure 1Ab) and a few neurons with Lewy bodies (LB) were present (Figure 1Ac). Staining of α-syn with antibodies such as ab51253 and pSyn#64 revealed massive neuronal intracytoplasmic inclusions (NCIs) and neurites in the left Pu (Figure 1Ad,e). NCIs and neurites in the affected region were also stained by all of the α-syn epitope-specific antibodies, though there were subtle differences of intensity (Figure 1Af,g, Syn 1–10 h, Syn 41–50 i, and Syn 131–140). Results for α-syn antibodies directed against the other peptides are not shown. The immunopositive components were also stained with p62 and ubiquitin antibodies (Figure 1Aj,k). LB509 showed mild immunoreactivity around the injection site (Figure 1Al). Mild α-syn pathologies are seen in the left parietal/temporal cortex and bilateral frontal cortex, (Figure 1Am). In the left SN, several NCIs/neurites were detected with

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**FIGURE 1**  
(A) Microscopic findings of *Macaca fuscata*. (a) Macroscopic photographs of the monkey, *Macaca fuscata*. The two injection sites are indicated by white arrows. The inserted right photo shows the bilateral mid brain. The left substantia nigra (indicated by an asterisk) exhibits decreased pigmentation compared with the right side. (b) The injection site in the left putamen. The upper left photo shows macrophages containing hemosiderin in the injection scar. Some ballooned neurons are seen in the area (black arrow). HE stain. (c), Neuron with Lewy body-like inclusion in the left putamen near the injection site. HE stain. (d)–(i), Numerous α-syn-positive NCIs and neurites in the left putamen near the injection site. (d) ab51253 (e) pSyn#64 (f) 42α-syn (g) Syn 1-10 (h) Syn 41-50 (i), and Syn131-140. (j) p62-positive neurons in the left putamen. (k) Ubiquitin-positive neurons in the left putamen. (l) LB509 staining around the injection site of the left putamen. (m) NCI and neurites in the left medial frontal cortex. (n) Mild neuronal loss in neuromelanin-containing neurons of the substantia nigra. K-B stain. (o) Neurites (black arrow) are seen in the substantia nigra. pSer129. (p and q) Immunofluorescence staining in the left putamen (p) and the left substantia nigra (q). sSer 129 positive NCIs are seen in regions with many TH-positive neurons in the left putamen. In the left substantia nigra, some of pS129-positive neurites are colocalized with TH staining. (Photos from left to right: DAPI, anti-α-syn antibody, anti-TH antibody, merge). Scale bar, a: 1 cm, b, d–q: 50 μm, c: 25 μm. (B) Distributions of α-syn pathology in the brain of *Macaca fuscata*. The two injection sites are indicated by gray arrows. CN, caudate nucleus; GP, globus pallidus; MD, mediodorsal nucleus of the thalamus; NAc, nucleus accumbens; Pu, putamen; SN, substantia nigra
mild neuronal loss (Figure 1An,o). Quantification of TH-IR neurons gave a median value for the left SN of 35.23 (/mm²), which is less than in the right SN, 48.24 (/mm²), though the difference is not significant (Mann–Whitney U test, p = 0.0657) (see also Figure S1 in the data supplement).

Double immunostaining with TH and α-syn in the left Pu/SN revealed α-syn-positive inclusions and neurites in the areas with TH-positive cells, and some neurons/neurites showed colocalization (Figure 1Ap,q). The distribution of α-syn pathology in the brain is shown in Figure 1B, based on the monkey brain atlas (9).

As regards with neurodegenerative changes, the hippocampus, amygdala, cerebellum, and brainstem showed no remarkable histopathological changes and immunopositivity. Neurofibrillary tangles/Aβ stages around the hippocampal region and other regions were extremely mild, Braak & Braak NFT stage 0 and Aβ stage 0. TDP-43, one of the aging-related proteins, was not detected.

4 | DISCUSSION

We found that α-syn pathology in the monkey brain spread from the striatum to multiple brain regions known to be affected in PD/DLB patients’ brains. Even though the interval after injection was short, α-syn neuronal inclusions and neurites were detected mainly in SN and related neocortical regions, away from the injection site. SN receives a strong axonal projection (striatoniigral fibers) from the collateral striatum and the frontal/parietal cortex is connected via corticostrial projection fibers. The contralateral neocortices are connected by commissural fibers. Thus, the distribution of α-syn pathology corresponds to regions closely linked to the putamen. We also found neurons bearing Lewy bodies, one of the pathological hallmarks of human PD/DLB, in severely affected regions in HE-stained sections. Some neurons resemble ballooned cells, indicating axonal damage over a wide range of connected regions. Overall, the results strongly indicate the progression of α-syn pathology via axonal connections through synaptically coupled networks, thereby supporting the idea that this intracellular amyloid-like protein has prion-like properties.

We clarified in detail the α-syn-positive components in the brain of the injected monkey. All the α-syn epitope-specific antibodies detected abnormal α-syn fibrils in neurons and dendrites/axons at the injection site. Previous work has demonstrated that antibodies to the C-terminal region of α-syn recognize monomers and fibrils almost equally, in contrast to antibodies to the N-terminal regions, which label fibrils more strongly than monomers (7). We also confirmed that the intracellular amyloid-like proteins are stained for ubiquitin and p62, which are common pathological features in human patients’ brains.

Macaca fuscata and human α-syn share 99% amino acid sequence homology, with only two amino acid differences, at positions 95 and 114. Interestingly, position 114 is close to the epitope of LB509 (amino acid residues 115–122), which is a human-specific α-syn antibody. As we observed immunoreactivity in a limited region around the injection site, this result might suggest that exogenous α-syn can be converted into endogenous α-syn via prion-like propagation.

As a limitation, we have to consider the influence of the previous surgeries in the brain. For this reason, we could not evaluate definitively whether the pathology was associated with behavioral changes.

In conclusion, we observed a limited distribution of α-syn pathology in this monkey in comparison with other animal models. This might imply that the progression of α-syn within the brains of individual PD/DLB patients is not a simple process, bearing in mind that pathology in the human brain is governed by regional, cell-autonomous factors (10) and is heterogeneous. Studies of the progression of abnormal protein accumulations in primate models with higher brain functions indicate the existence of considerable extranigral pathology, which may have implications for future therapy.

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AUTHOR CONTRIBUTIONS

IK performed microscopy, data analysis, and wrote the manuscript. AM helped with the microscopy analysis and participated in study design. AS, RO, and MT helped with the microscopy analysis. MHashimoto, MK, and KS conducted animal surgery. MM-S provided key information about the fibrils used. MHashimoto, MM-S, and MHashigawa provided helpful advice for the interpretation of data. MHashigawa supervised the study design and its coordination. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the Supporting Information of this article. Further data are available from the corresponding author upon reasonable request.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.

**Fig S1** Quantification of TH-IR neurons in the bilateral SN. The median for the left side was 35.23 (/mm²), which is less than on the right side, 48.24 (/mm²), but the difference is not significant according to the Mann–Whitney U test ($p = 0.0657$).

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