Preclinical Detection of Alpha-Synuclein Seeding Activity in the Colon of a Transgenic Mouse Model of Synucleinopathy by RT-QuIC

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Abstract: In synucleinopathies such as Parkinson’s disease (PD) and dementia with Lewy body (DLB), pathological α-synuclein (α-syn) aggregates are found in the gastrointestinal (GI) tract as well as in the brain. In this study, using real-time quaking-induced conversion (RT-QuIC), we investigated the presence of α-syn seeding activity in the brain and colon tissue of G2-3 transgenic mice expressing human A53T α-syn. Here we show that pathological α-syn aggregates with seeding activity were present in the colon of G2-3 mice as early as 3 months old, which is in the presymptomatic stage prior to the observation of any neurological abnormalities. In contrast, α-syn seeding activity was not detectable in 3 month-old mouse brains and only identified at 6 months of age in one of three mice. In the symptomatic stage of 12 months of age, RT-QuIC seeding activity was consistently detectable in both the brain and colon of G2-3 mice. Our results indicate that the RT-QuIC assay can presymptomatically detect pathological α-syn aggregates in the colon of G2-3 mice several months prior to their detection in brain tissue.

Keywords: RT-QuIC; alpha-synuclein; synucleinopathy; Parkinson’s disease

1. Introduction

Alpha-synuclein (α-syn) is a neuronal protein of 140 amino acids predominantly localized to presynaptic nerve terminals [1,2]. α-synucleinopathies (or synucleinopathies) are a group of neurodegenerative diseases characterized by the accumulation of pathological α-syn aggregates in the brain [3,4]. The three major phenotypes include Parkinson’s disease (PD), dementia with Lewy bodies (DLB) and multiple system atrophy (MSA), in addition to several rarer diseases such as pure autonomic failure (PAF) [5,6]. While α-syn deposits appear in neuronal soma and processes (known as Lewy bodies and Lewy neurites, respectively) in PD and DLB, α-syn-positive glial cytoplasmic inclusions in oligodendrocytes are the neuropathological hallmark of MSA [4,7–9]. In patients affected by the various synucleinopathies, pathological α-syn deposits develop not only in the central nervous system (CNS) but also in the peripheral nervous system (PNS) of multiple organs, including stellate and sympathetic ganglia, the gastrointestinal (GI) tract, the heart and the adrenal gland [10–13]. Moreover, α-syn deposits in the enteric nervous system (ENS) of the GI tract from PD patients were reported to develop up to 20 years prior to the onset of motor symptoms [14]. Nonetheless, a number of immunohistochemistry (IHC)-based studies aiming to detect pathological α-syn inclusions in GI biopsies of PD patients have yielded conflicting results regarding the diagnostic accuracy of this approach [15–20]. The reported discrepancies between studies that examined GI biopsies of PD patients might be associated with variable amounts of pathological α-syn aggregates in the examined samples, methodological variation in IHC and the potentially variable expertise in the interpretation of results [21–24].
Real-time quaking-induced conversion (RT-QuIC) is a seeded cyclic amplification technique developed to detect very small amounts of disease-specific prion protein aggregates (PrP\textsubscript{Sc}) with high sensitivity and specificity [25,26]. Due to its ability to detect femtogram (10\textsuperscript{-15}) levels of PrP\textsubscript{Sc} [27], RT-QuIC has been extensively investigated during the last decade for diagnostic applications using cerebrospinal fluid (CSF) or other tissue outside the brain [28–36]. Recently, based on the prion-like properties of misfolded α-syn aggregates [37–40], RT-QuIC was successfully adapted to synucleinopathies [41,42]. Subsequently, a number of studies employing this assay successfully detected α-syn seeding activity with high sensitivity and specificity in CSF or peripheral tissues from patients affected by a variety of synucleinopathies [43–49].

In this study, using the α-syn RT-QuIC assay that we reported recently [50], we comparatively investigated the presence of α-syn seeding activity in the brain and colon of transgenic mice expressing an A53T mutant form of human α-syn. Our results show that α-syn seeding activity occurs in the colon of presymptomatic mice, and precedes the appearance of seeding activity in the brain.

2. Materials and Methods

Transgenic mice overexpressing human α-syn with an A53T mutation under the control of mouse prion protein promoter (line G2-3) was described previously [51]. The G2-3 mice develop neurological disease at ~12 months of age, which is characterized by rapidly progressive motor dysfunction leading to death within a few weeks after the clinical onset [51,52]. The mice display a neuropathological phenotype including loss of motor neurons and neuronal accumulation of pathological α-syn aggregates [51,53]. Ethical approval for the use of mice was obtained from the Institutional Animal Care and Use Committee of Korea Brain Research Institute (KBRI). Mice transgenic for human A53T α-syn and non-carrier controls were euthanized at 1, 3, 6 and 12 months of age. At each timepoint, brain and colon tissue samples were recovered. Subsequently, a caudal part of the brain (brainstem and midbrain) and a distal portion of the colon were separated and then homogenized in phosphate-buffered saline (PBS, pH 7.4) containing complete EDTA-free protease inhibitors and phosphatase inhibitors (Roche Applied Science, Upper Bavaria, Germany), by using the Precellys 24 tissue homogenizer (Bertin Instrument, Frankfurt, Germany). The 5% (w/v; colon) or 10% (w/v; brain) homogenates were clarified by centrifugation at 2000 \times g for 2 min and the supernatants were kept in aliquots at −80 °C until needed.

RT-QuIC analysis for brain or colon homogenates was performed as described previously [41] with some modifications [42]. Recombinant full-length human α-syn protein (140 amino acids) carrying an A53T mutation (rPeptide) was used as substrate. Regarding seeds, tissue homogenates kept at −80 °C were thawed on the day of analysis and sonicated as described [50]. Sonicated tissue homogenates were serially diluted and then used as seeds at indicated dilutions. Dilutions were expressed in relation to brain or colon tissue; for example, 10\textsuperscript{-2} dilution is equivalent to 1% tissue homogenate. A 96-well black plate with clear bottom (Nalgene Nunc.) was used for this assay. Each well of the plate was preloaded with 4 glass beads (1.0–1.25 mm in diameter) before the addition of 100 µL reaction mixture containing 10 µM recombinant α-syn, 10 µM thioflavin T (ThT) and 2 µL seeds in 100 mM phosphate buffer (pH 8.2). Individual samples were prepared in quadruplicate. The plate was sealed and then incubated at 37 °C with alternating 1 min shake and 1 min rest cycles (400 rpm, double orbital) in a FLUOstar Omega plate reader (BMG Labtech, Ortenberg, Germany). The plate reader measures ThT fluorescence in relative fluorescence units (RFU) and is saturated at 260,000 RFU. ThT fluorescence was measured at the starting point of the assay and then at one-hour intervals from the bottom of the wells (440 nm excitation and 480 nm emission, gain of 1750, 20 flashes per well). Fluorescence values at each measurement were shown as average for quadruplicate wells on the graph. A ThT fluorescence threshold in each plate was determined by averaging the fluorescence of the first five measurements for all samples plus 10 standard deviations
Samples were considered positive when at least two of four replicates crossed this threshold. \( RFU_{max}/RFU_{initial} \) ratios were calculated by dividing maximal ThT fluorescence over the 40-h reaction \( (RFU_{max}) \) by ThT fluorescence at the starting point \( (RFU_{initial}). \)

3. Results

Given that G2-3 mice are known to develop rapidly progressive neurological symptoms at \( \sim \)12 months, we first investigated \( \alpha \)-syn seeding activity in brain and colon tissues of 12-month-old mice using RT-QuIC. Positive RT-QuIC responses were seen in all samples recovered from 12-month-old mice. For brains, \( \alpha \)-syn seeding activity was identified up to \( 10^{-5} \) dilution in two of three mice (Figure 1A,E) or up to \( 10^{-8} \) dilution in the remaining mouse (Figure 1C). In colon tissue, RT-QuIC seeding activity was detectable up to \( 10^{-3} \) dilution in all three mice (Figure 1B,D,F). Further dilutions of colon tissue samples did not show any positive response in all three mice and were not investigated further (data not shown). While the two tissue types in 12M-#1 and #3 mice showed similar levels of \( \alpha \)-syn seeding activity, its level in the brain of 12M-#2 mouse was \( 10^{3} \) times higher than in the colon (Table 1). Responses rising above the threshold were usually observed after 14–20 h in reactions seeded with \( 10^{-3} \) to \( 10^{-5} \) dilutions of both tissue homogenates, except for the brain of 12M-#1 mouse that required 22–27 h of lag phase to cross the threshold at indicated dilutions (Figure 1A). There were longer lag phases up to 26 h in reactions seeded with \( 10^{-6} \) to \( 10^{-8} \) dilutions of brain tissue from 12M-#2 mouse (Figure 1C). We observed that reactions seeded with the \( 10^{-3} \) dilution of tissue homogenates often gave weaker responses with longer lag phases and lower \( RFU_{max}/RFU_{initial} \) ratios than those seeded with \( 10^{-4} \) or even higher dilutions, which were more prominent in colon samples (Figure 1 and data not shown). This observation is thought to be associated with the presence of inhibitory components in tested samples that interfere with the RT-QuIC reaction [26,54]. It appears that \( 10^{-5} \) dilution was not enough to fully reduce the reaction inhibitors to subinhibitory concentrations in some samples, particularly colon samples, and further dilution to \( 10^{-4} \) is required for full removal of inhibitors as described previously [55].

We then investigated brains and colons of 6-month-old mice using RT-QuIC, given previous studies in which pathological \( \alpha \)-syn was not yet detectable in the brain of G2-3 mouse at this age but appeared at 7–8 months of age [56]. As shown in Figure 2A,E, \( \alpha \)-syn seeding activity was not detectable in the brains of two 6-month-old mice. Positive responses were seen only in reactions seeded with \( 10^{-3} \) dilution of the brain tissue from the 6M-#2 mouse (Figure 2C). In contrast to the brain, colon samples from all three 6-month-old mice had detectable seeding activity at \( 10^{-3} \) dilution (Figure 2F), and even up to \( 10^{-5} \) dilution (Figure 2B,D). Therefore, \( \alpha \)-syn seeding activity occurred first in the colon at least in two of the three 6-month-old mice, and its colon levels were higher than in the brains of all three mice (Table 1). The lag times varied between samples and degrees of dilution, ranging from 15 to 30 h.

Next, considering that GI deficits similar to those occurring in the prodromal phase of PD were reported to develop at 3 months of age [57], brains and colons recovered from G2-3 mice of this age were examined for \( \alpha \)-syn seeding activity. As shown in Figure 3 (left column), all reactions seeded with \( 10^{-3} \) to \( 10^{-5} \) dilutions of brain tissue from 3-month-old mice gave flat responses in the assay. In contrast, colon tissue from all three 3-month-old mice contained \( \alpha \)-syn seeding activity detectable only at \( 10^{-3} \) dilution (3M-#2 and -#3) or up to \( 10^{-4} \) dilution (3M-#1) (Figure 3, right column). Further dilutions of colon tissue of 3M-#1 mouse did not show any positive response (data not shown). The lag phases varied from 18 to 34 h between colon samples. Positive responses at \( 10^{-3} \) dilution of colon tissue of 3M-#2 or 3M-#3 mouse were observed only in partial wells (two or three of four), indicating low seeding doses in those samples that were barely detectable in this assay (Figure 3D,F). Additionally, reactions seeded with \( 10^{-4} \) dilution of colon tissue of these two mice were determined to be negative, since only one of four wells gave responses rising above the threshold (Figure 3D,F; Table 1). We occasionally observed such a spontaneous positive response, as described previously [50]. Lastly, colon tissue recovered from 1-month-old
G2-3 mice were examined for α-syn seeding activity. All reactions seeded with $10^{-3}$ or $10^{-4}$ dilution of colon tissue from 1-month-old mice gave flat responses in the assay (Figure 4), indicating the absence of α-syn seeding activity in the colon of G2-3 mice at this age.

Figure 1. RT-QuIC detection of α-syn seeding activity in both the brain and colon of 12-month-old G2-3 mice. Serial dilutions of brain homogenates ($10^{-3}$ to $10^{-8}$) or colon homogenates ($10^{-3}$ to $10^{-5}$) from 12-month-old G2-3 mice were used to seed RT-QuIC reactions with A53T recombinant α-syn as substrate. RT-QuIC responses to brain samples were shown in the left column (A, C, E) and those to colon samples were shown in the right column (B, D, F). As controls, reactions were also seeded with $10^{-3}$ dilution of brain tissue or colon tissue from a non-carrier mouse of the same age. ThT fluorescence was measured at one-hour intervals and average values from quadruplicate wells were plotted as a function of time.
Figure 2. RT-QuIC analysis of brain and colon samples from 6-month-old G2-3 mice. Serial dilutions of brain or colon homogenates ($10^{-3}$ to $10^{-5}$) from 6-month-old G2-3 mice were used to seed RT-QuIC reactions with A53T recombinant α-syn as substrate. RT-QuIC responses to brain samples were shown in the left column (A,C,E) and those to colon samples were shown in the right column (B,D,F). As controls, reactions were also seeded with $10^{-3}$ dilution of brain tissue or colon tissue from a non-carrier mouse of the same age. ThT fluorescence was measured at one-hour intervals and average values from quadruplicate wells were plotted as a function of time.
Figure 3. Alpha-synuclein seeding activity is present in the colon but absent in the brain of 3-month-old G2-3 mice. Serial dilutions of brain homogenates ($10^{-3}$ to $10^{-5}$) or colon homogenates ($10^{-3}$ to $10^{-4}$) from 3-month-old G2-3 mice were used to seed RT-QuIC reactions with A53T recombinant α-syn as substrate. RT-QuIC responses to brain samples were shown in the left column (A,C,E) and those to colon samples were shown in the right column (B,D,F). As controls, reactions were also seeded with $10^{-3}$ dilution of brain tissue or colon tissue from a non-carrier mouse of the same age. ThT fluorescence was measured at one-hour intervals and average values from quadruplicate wells were plotted as a function of time.
Figure 4. Alpha-synuclein seeding activity is absent in the colon of 1-month-old G2-3 mice. Colon homogenates were prepared from three G2-3 mice of 1 month of age and one non-carrier mouse of the same age. Colon homogenates at $10^{-3}$ dilution (A) or $10^{-4}$ dilution (B) were used to seed RT-QuIC reactions with A53T recombinant $\alpha$-syn as substrate. ThT fluorescence was measured at one-hour intervals and average values from quadruplicate wells were plotted as a function of time.

Table 1. Summary of the $\alpha$-syn RT-QuIC assay for brains and colons of G2-3 mice.

| Age at Death | ID      | Detectable Dilution $^{(1),(2)}$ | Comments |
|--------------|---------|---------------------------------|----------|
|              |         | Brain $^{(3)}$ | Colon $^{(4)}$ |
| 3 months     | 3M-#1   | ND $^{(5)}$     | $10^{-4}$ |          |
|              | 3M-#2   | ND                | $10^{-3}$ |          |
|              | 3M-#3   | ND                | $10^{-3}$ |          |
|              | 3M-Con  | ND                | ND       | non-carrier |
| 6 months     | 6M-#1   | ND                | $10^{-5}$ |          |
|              | 6M-#2   | $10^{-3}$         | $10^{-5}$ |          |
|              | 6M-#3   | ND                | $10^{-3}$ |          |
|              | 6M-Con  | ND                | ND       | non-carrier |
| 12 months    | 12M-#1  | $10^{-5}$         | $10^{-5}$ |          |
|              | 12M-#2  | $10^{-8}$         | $10^{-5}$ |          |
|              | 12M-#3  | $10^{-5}$         | $10^{-5}$ |          |
|              | 12M-Con | ND                | ND       | non-carrier |

$^{(1)}$ The fluorescence threshold in each plate was in the range of 15,000–25,000 RFU, which was the basis of determining positive RT-QuIC response. $^{(2)}$ RT-QuIC seeding activity was detected at least twice from $10^{-3}$ to indicated dilution(s) in each sample. $^{(3)}$ Brainstem and midbrain areas were used for RT-QuIC analysis. $^{(4)}$ Distal part of the colon was used for RT-QuIC analysis. $^{(5)}$ ND = not detectable at $10^{-3}$ dilution.

4. Discussion

In this study, we show that our RT-QuIC assay was able to detect pathological $\alpha$-syn aggregates with seeding activity in the colon of 3-month-old G2-3 mice, which is in the preclinical stage prior to any motor impairments or CNS pathology [51]. In contrast, $\alpha$-syn seeding activity was not detectable in the brain at 3 months of age and appeared at 6 months of age only in one of three mice. At 12 months, which is in the clinical stage presenting with neurological abnormalities [51,53,56], RT-QuIC seeding activity was found in both types of tissue in all three mice. Collectively, our results clearly demonstrate that $\alpha$-syn seeding activity in the colon precedes its occurrence in the brain of this transgenic mouse model of synucleinopathy. In agreement with our results, GI dysfunction including constipation and $\alpha$-syn deposits in enteric neurons was described in the G2-3 mice from 3 months of age [57]. Therefore, given the presence of GI abnormalities for several months prior to the emergence of pathological changes in the brain, this G2-3 line can be a useful...
model in developing treatments to stop or delay disease progression during the prodromal phase of PD or other synucleinopathies.

Pathological α-syn aggregates are also known to appear in the GI tract in PD patients even decades prior to the onset of motor impairments, but multiple IHC-based studies using GI biopsies have produced conflicting results regarding diagnostic accuracy [15–17,19,20]. Therefore, it is of interest whether the α-syn RT-QuIC assay that has demonstrated its diagnostic potential in multiple studies [43–46,49,58] could detect pathological α-syn aggregates in GI biopsies of PD patients, particularly at the prodromal phase. While the application of α-syn RT-QuIC into GI biopsies has not yet, to the best of our knowledge, been reported, our successful results in detecting α-syn seeding activity in the colon of presymptomatic G2-3 mice strongly support the diagnostic potential of this approach. Additionally, biopsy sites within the GI tract of PD patients could be important for successful detection of α-syn aggregates with seeding activity [20,24]. RT-QuIC-based detection and quantitation of α-syn seeding activity in GI biopsies would be useful not only in the early diagnosis of PD and other synucleinopathies, but also in monitoring disease progression and assessing treatment efficacy [48].

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**Institutional Review Board Statement:** In association with the use of mice, the approval of KBRI IACUC was described in M & M section. Additionally, we use the term of IRB for human study and use the term of IACUC for animal study.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

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