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SARS-CoV-2-mimicking pseudoviral particles accelerate alpha-synuclein aggregation *in vitro*

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Abstract (150 words)

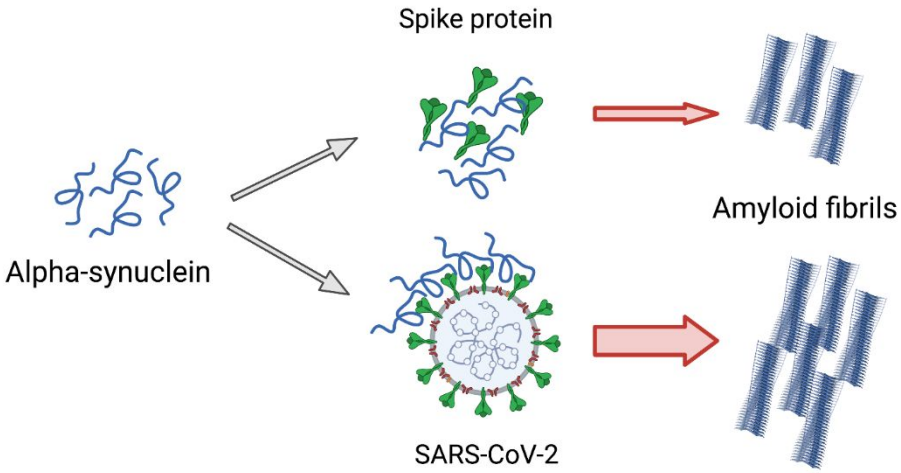
Since SARS-CoV-2 virus started spreading worldwide, evidence pointed towards an impact of the infection on the nervous system. COVID-19 patients present neurological manifestations and have an increased risk of developing brain-related symptoms in the long term. In fact, evidence in support of the neuroinvasive potential of SARS-CoV-2 has emerged.

Considering that viral parkisonism was observed as a consequence of encephalopathies caused by viral infections, it has been already suggested that COVID-19 could affect the dopaminergic neurons and contribute to neurodegeneration in Parkinson's disease (PD), by promoting the formation of amyloid fibrils constituted by the PD-related protein alpha-synuclein. Here, we not only observe that SARS-CoV-2 viral Spike protein and Nucleocapsid protein can alone promote alpha-synuclein aggregation, but also that the Spike protein organization in a corona shape on the viral envelope may be crucial in triggering a fast amyloid fibrils formation, thus possibly contributing to PD pathogenesis.

Keywords (6 words)

COVID-19, Parkinson's disease, alpha-synuclein, coronavirus, Spike, aggregation

Graphical abstract



Introduction

In the last four years, since the first case was officially reported in Wuhan, China [1], COVID-19 has been a dramatic global health emergency, with more than half a billion cases reported worldwide and patients still reporting a broad variety of symptoms for up to more than a year after onset [2].

Other than its most common respiratory symptoms, ranging from mild to severe and fatal[3], patients can also present a series of neurological signs, including encephalopathies and inflammatory central nervous system (CNS) syndromes, but most commonly the insurgence and persistence of anosmia and dysgeusia in COVID-19 patients [4], common non-motor features of Parkinson's disease (PD) [5].This spiked the interest of the scientific community, raising the question of whether this infection could represent a risk factor for the insurgence of neurodegenerative diseases, as shown for example in the past, following Influenza infections [6]. Clinically this concern deserves credit, a very large cohort of patients were analysed six months after the infection showing an overall increased risk of developing brain-related symptoms, which includes both mental health issues and neurological defects [7].

Recovered patients have also been affected by small fiber peripheral neuropathy, similarly to other neuronal degenerative diseases, even months after recovery [8].

This concern was also substantiated by an emerging body of evidence in support of the neuroinvasive potential of SARS-CoV-2 with multiple studies reporting the presence of viral RNA in different brain sections in post-mortem patients, and immunostaining analysis confirming the presence of SARS-CoV-2 proteins in cranial nerves originating from the lower brainstem, Human Brainstem Nuclei and cortical neurons [9,10]. Interestingly, the angiotensin-converting enzyme 2 (ACE2) receptor, which mediates SARS-CoV-2 entry into target cells [11], plays a role in brain function and development and it has been detected at the level of different neuronal and glial cell types, including dopaminergic neurons [12,13]. Moreover, the Neuropilin 1 receptor (NRP1), which facilitates SARS-CoV-2 cell entry [14], is also expressed in neurons [15]. In this frame, using human brain organoids and mice overexpressing human ACE2, the virus was also confirmed to be able to directly target neurons for invasion [11,16] and some studies also report traces of the virus S-protein (Spike protein) within neurons of the olfactory mucosa and endothelial cells of small CNS vessels [14,17], indicating two possible entryways that the virus could exploit to reach the brain.

Our avenue of investigation in the realm of neurodegeneration is Parkinson's disease (PD), which is characterized by the progressive loss of dopaminergic neurons of the *substantia nigra pars compacta* [18]. Indeed, viral parkinsonism is a well-known secondary consequence of encephalopathies caused by viral infections, like those caused by influenza, Coxsackie, Japanese encephalitis B, St. Louis, West Nile and HIV viruses [19]. Possible scenarios of how COVID-19 would affect the dopaminergic system have already emerged in the literature [20]. Among these, of great interest is the study of alpha-synuclein (asyn) aggregates formation, whose presence inside proteinaceous deposits called Lewy bodies is a well-known hallmark of PD [18]. The fact that asyn aggregation can be promoted by other proteins has been already established [21,22]. In more recent studies the same was demonstrated for SARS-CoV-2 proteins, which

were shown to interact with asyn *in vitro* and when overexpressed in cell models, suggesting that they can contribute to the formation of amyloid aggregates [23,24]. Moreover, intracellular asyn aggregates were also identified in the *substantia nigra* of macaques infected with SARS-CoV-2 virus [25].

Finally, viral particles themselves have been shown to facilitate the formation of amyloid aggregates, as in the case of the Alzheimer's disease related protein amyloid-beta [26], leading to the question of whether a similar outcome could be expected following the interaction of the SARS-CoV-2 virus with αsyn.

In this study, we compared, both *in vitro* and in a cell model, the different contributions that soluble SARS-CoV-2 viral S-protein, soluble SARS-CoV-2 viral N-protein (Nucleocapsid protein) and SARS-CoV-2 pseudo-viral particles have on asyn aggregation kinetics and products, to understand the importance of the organized distribution of the S-protein on the viral particle surface for the aggregation of αsyn.

Results and Discussion

We first set out to investigate how the interactions between recombinant S-protein and asyn may affect the amyloid fibrils formation kinetics in *in-vitro* experiments. Since a recent report suggested that SARS-CoV-2 N-protein can accelerate asyn fibrils formation [23], we also studied the N-protein impact on this process. For this purpose, we exploited an asyn seed amplification assays (SAAs), called RT-QuIC, typically used in diagnostic to detect abnormally folded prion proteins [27] (**Fig. 1a**).

Aggregation lag time normalized to the control is significantly reduced in the presence of either viral protein (**Fig. 1b**). Specifically, in both tested conditions, the presence of the viral proteins has a shortening effect on the lag time, resulting in intermediate values as compared to the positive controls for asyn aggregation (1 ng and 1 pg of PFFs). The process was further studied by characterizing the slopes of the exponential growth phase of the aggregation curves (**Fig. 1c**). Strikingly, only the presence of the viral N-protein has a significant effect on the derivative of the curve, which is steeper, suggesting a faster elongation of the fibrils. A decrease in the aggregation lag time was previously reported to occur only because of the interaction between N-protein and asyn [23] and not with S-protein, suggesting that the different methods used to assess their effects (ThT kinetics vs RT-QuIC) may allow to identify different behaviours.

To further characterize the possible role of the S-protein assembled on the SARS-CoV-2 virus in the promotion of asyn amyloid fibrils formation, we verified whether SARS-CoV-2-mimicking VSVΔG pseudoviral particles would have a similar effect on asyn aggregation compared to the viral proteins. Indeed, recent reports indicate that some viruses interact with amyloidogenic peptides though their protein corona, functioning as nucleation points for amyloid aggregation [26]. With this aim, we used the RT-QuIC setup to test three serial dilutions of both S-protein exposing VSVΔG pseudoviral particles (VSVΔG-S-protein) and control VSVΔG pseudoviral particles presenting the stomatitis virus proper surface glycoprotein (VSVΔG-G). Once again, we measured both the aggregation lag time and slope of the exponential growth phase of the aggregation curves. (**Fig. 1d-f**).

The two highest concentrations used of VSVΔG-S-protein significantly reduce the aggregation lag time compared to the control, while VSVΔG-G show no significant effect on asyn aggregation lag time (**Fig. 1e**).

Similarly, under the same two VSVΔG-S-protein aggregation conditions, the exponential growth phase of the aggregation curves presents a significantly higher derivative compared to the control. The same occurs for the higher concentration of VSVΔG-G, which also shows a significant increase of the slope of the curve (**Fig. 1f**). When comparing VSVΔG-S-protein result to the one obtained in the presence of the S-protein alone, we found that the relative reduction in the lag time was similar: 0.68 ± 0.14 and 0.79 ± 0.17 in the case of the two highest concentrations of VSVΔG-S-protein and 0.77±0.12 in the presence of only viral S-protein. To further compare these two conditions, we quantified by western blot the level of the S-protein present in the VSVΔG-S-protein samples used for the aggregation assays and compared it to the amount used in the assays where the S-protein alone was present (**Fig. 1g**). While we were able to detect a signal from the serial dilutions of recombinant S-protein, the same was not possible in the pseudoviral particle samples. These data suggest that the amount of S-protein in the VSVΔG-S-protein samples is lower than in the aggregation assay where only the recombinant S-protein was used, supporting the notion that when ordered in a "corona" shape, S-protein is much more efficient in speeding up the aggregation process.

To understand whether the observed variations on the kinetics of fibril formation due to the presence of VSVΔG-S-protein could also affect fibril elongation, amount and morphology, we performed steady-state Thioflavin T (ThT) fluorescence measurement and atomic force microscopy (AFM) imaging. ThT fluorescence emission allowed to evaluate the presence of beta-sheet positive amyloid fibrils, which were particularly significant in the seed-induced fibrils, as expected, but were also present in the VSVΔG-S-protein and VSVΔG-G fibrils (**Fig. Suppl. 1a**). Accordingly, AFM imaging (**Fig. 2**) performed on aliquots taken at the same time points of aggregation runs, performed either in the presence of asyn pre-formed seeds, of VSVΔG-S-protein or of VSVΔG-G, evidenced substantially higher amounts of fibrillar material with respect to the aggregation of asyn alone. In particular, aggregations only containing asyn were observed to be almost devoid of fibrils after 168h of incubation, whereas abundant fibrils with the diameter typically associated with mature asyn amyloids observed in similar conditions (~7.5 nm) [28] were present in the remaining three samples.

Overall, these data support the concept that unbound S-protein can impact on the aggregation of αsyn, but its effect is dramatically increased upon inclusion on the surface of VSVΔG-S-protein pseudoviral particles. This suggests that S-protein can act as a nucleating factor for the aggregation of αsyn, but more efficiently when ordered or simply locally more concentrated on the particle surface.

We finally wanted to verify whether the VSVΔG-S-protein pseudoviral particles were able to induce the accumulation of asyn aggregates in a cellular model. To this aim, we exploited an inducible HEK293T cell line, which allows to induce the over-expression of asyn by the addition of doxycycline (dox) in cell culture medium in a concentration-dependent manner (**Fig. Suppl. 2a**). In this model, we developed two different paradigms to investigate the role of SARS-CoV-2 proteins or infection in cells: (i) we over-expressed the VSV Glycoprotein or the S-protein, which we showed are localized not only at the plasma membrane, but also in the cytoplasm, in immortalized cell lines (**Fig. Suppl. 2b**), thus being possibly able to interact with the over-expressed asyn; (ii) we induced the assembly of pseudo-viral particles within the HEK293T cells by

over-expressing either the VSV Glycoprotein or the S-protein, and then by infecting the cells with the VSVΔG-G pseudoviral particles. The latter allowed to produce VSVΔG-G and VSVΔG-S-protein pseudoviral particles within the cells, that were also released in the medium and could be taken up by HEK293T cells (**Fig. Suppl. 2c**) possibly via the classical interaction with the ACE2 and NRP1 receptors, which are expressed by this cell type (**Fig. Suppl. 2d**). Despite being artificial, we predicted that this way it would be more likely to have good levels of VSV Glycoprotein or S-protein, or of the respective pseudoviral particles, able to promote their interaction with the overexpressed αsyn, within the cells, at the plasma membrane or within the endo-lysosomal compartment.

Thus, the proposed experimental approach allowed to compare the amount of asyn aggregates in αsyn-dox cells, either when overexpressing the S-protein or in the presence of VSVΔG-S-protein pseudoviral particles, with different control conditions (VSV Glycoprotein overexpression or VSVΔG-G pseudoviral particles presence, αsyn-overexpressing or naïve cells) (**Fig. 3a**). The expression of the different proteins (αsyn, VSV Glycoprotein or S-protein) was evaluated by western blot in the different conditions (**Fig. 3b**), while to quantify the amount of asyn aggregates we exploited a custom-made ELISA assay [29], using specific antibodies against asyn aggregates, which provided a larger sensitivity compared to other techniques. The outcome of the measurements, which were performed on cellular lysates at the end of the experimental procedure shown in **Fig. 3a**, suggested that the impact of the S-protein overexpression or the presence of VSVΔG-S-protein pseudoviral particles on the amount of asyn aggregates in HEK293T cells is negligible compared to the asyn overexpressing cells, at least under our experimental conditions (**Fig. 3c**). Seemingly in contrast with the *in vitro* results, this could be explained by the fact that the measurement was performed at a fixed point in time, dictated by the use of a cell model. Thus, we could neither monitor the development of aggregates in real time nor observe changes that may have occurred at later stages, or even at earlier stages, but undetected due to cell death and dispersion of possible asyn aggregates in the medium. The possible compartmentalization of the viral proteins, the pseudoviral particles or of asyn (at the membranes or in different intracellular compartments) could also interfere with the interaction and the possible impact on the aggregation. Moreover, in the intracellular environment other processes, such as autophagy, are likely to interfere with the asyn aggregation process, but also with the behaviour of the pseudoviral particles and of the S-protein itself. Lastly, to confirm our observations regarding the Spike protein corona effect on the aggregation of asyn, further study using a non-functionalized particle as a negative control would be ideal. The replication-restricted rVSV-ΔG pseudotypes used as our model for the virus are versatile, but their production process does not allow to easily obtain particles with no surface protein. Introducing a surface protein presenting a cleavage site for the excision of any binding domain from the particle surface after assembly and collection is for sure a possibility but requires further study and fine tuning. An alternative option would be to adopt a different virus mimicking model, for example maleimide labelled lipid nanoparticles to be later functionalized with the Spike protein [30], adapting our experimental design to accommodate for the lack of replication ability of the nanoparticles and introducing the particles inside the cells by other means, like microinjection or electroporation [31].

These results and considerations suggest that further studies would be needed to understand whether the SARS-CoV-2 proteins or the virus itself can work as nucleation centres and accelerate asyn aggregation in cell models. Overall, our work showed that both N-protein and S-protein can accelerate the *in vitro* formation of asyn amyloid fibrils when in their soluble unbound forms. Interestingly, in recently published papers S-protein fragments are shown to be able to stimulate the aggregation of asyn, both in molecular dynamics simulations studies [32] and in *in vitro* experiments [33], and some of these fragments also possess amyloidogenic properties themselves in *in vitro* aggregation experiments [34]. One of these fragments in particular is generated when the S-protein is processed by neutrophil elastase, suggesting that neuroinflammation could contribute to the aggregation process during the viral infection [35].

In light of these findings we have to take into account that part of the promoting effect on aggregation that we observe in our experiments could be due to S-protein fragmentation during the aggregation process. This is certainly an avenue worth exploring in future projects.

Strikingly, less S-protein is sufficient to promote similar kinetic effects when it is assembled on pseudoviral particles, thus suggesting that its ordered or locally confined distribution is a contributing factor that has a similar or bigger relevance to the aggregation than the S-protein structural properties alone. This is in good agreement with the fact that intracellular aggregates were found in the midbrain of macaques after SARS-CoV-2 infection [25]. A clinical trial is ongoing to measure the presence of pathological asyn aggregates in the olfactory mucosa of patients with COVID-19, using an RT-QuIC method similar to the one we applied (https://ichgcp.net/clinical-trials-registry/NCT05401773), confirming that selecting the approach presented here was ideal. It should also be mentioned that RT-QuIC relies on the use of very low concentrations of asyn (7uM) compared to the concentration chosen when applying other biophysical or biochemical methodologies to study asyn aggregation in-vitro [36]. This means that also the seeds, being proteins, fragments of asyn fibrils or viral particles, present very low concentrations in our assay. These experimental conditions allow us to avoid molecular crowding effect that normally occurs because of the high concentration of macromolecules present in solution, which causes the solvent to be less available to the molecules thus increasing their local concentrations. Since we have low concentrations of the molecules under investigation in the RT-QuIC samples, we are aware that we are distant from the conditions of the intracellular environment [37]. On the other hand, this approach allowed us to single out the mechanisms unrelated to molecular crowding that could possibly determine the increase in the asyn aggregation rates in the different conditions.

The outcome of the clinical study mentioned above will hopefully allow us to better understand the contribution of SARS-CoV-2 infection in PD and other synucleinopathies, in order to evaluate the possibility that the pandemic may have (possibly in a large timeframe) an impact on PD predisposition in the former COVID-19 patients similarly to other epidemiological and environmental risk factors (**Fig. 4**).

In conclusion, viral parkinsonism was already observed as a secondary consequence of encephalopathies caused by viral infections [19] and asyn aggregation was shown to be promoted by other proteins [21,22,23],

but no previous work that we could find focused was on viral particle themselves as a seeding agent for asyn aggregation, as was done instead in the case of Alzheimer's amyloid-beta protein [26].

With all this considered we believe that our work builds on previous publications on the topic, confirming the role of SARS-CoV-2 proteins in asyn aggregation and suggesting an interesting avenue of research on viral particle themselves as seeding agents, not only in the case of SARS-CoV-2, but also all those viral infections linked to PD in which direct seeding by viral particles protein coronas themselves has not yet been investigated.

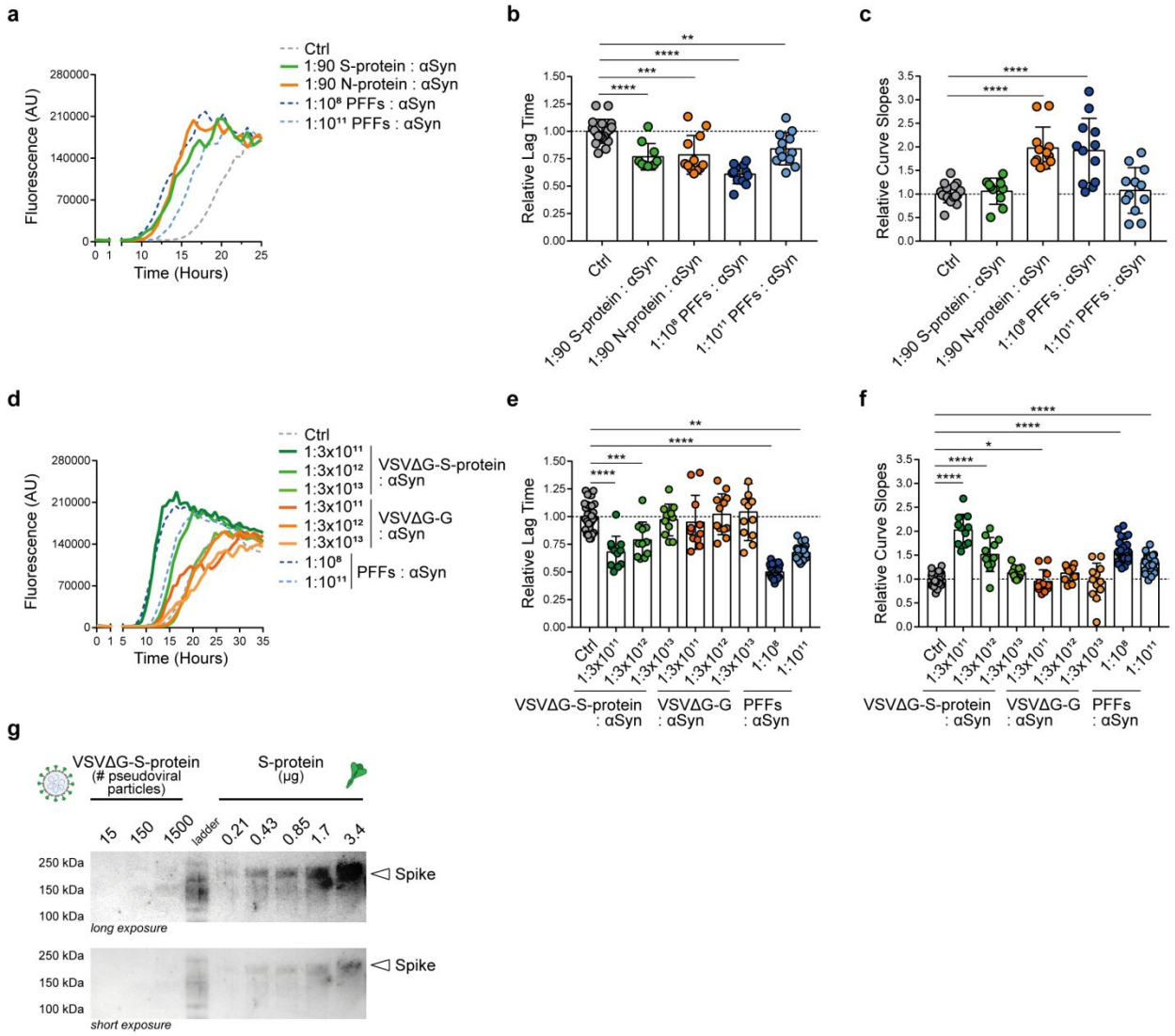


Figure 1. aSyn in-vitro aggregation is enhanced by SARS-CoV-2 proteins and SARS-CoV-2-mimicking VSVΔG pseudoviral particles.

(a) Representative RT-QuIC assay aggregation curves in the presence of viral proteins as seeding agents. Samples consist in asyn-only control, asyn in the presence of two serial dilutions of asyn preformed fibrils (PFFs) to act as positive controls [asyn : PFFs molar ratio of $1:10^8$ and $1:10^{11}$], asyn in the presence of viral S-protein [asyn : S-protein molar ratio of 1:90] and asyn in the presence of viral N-protein [asyn : N-protein molar ratio of 1:90]. **(b)** Aggregation curves lag times normalized to the control (multiple comparison ordinary one-way ANOVA; S-protein, **** $p < 0.0001$; N-protein, *** $p = 0.0001$; PFFs 1ng, **** $p < 0.0001$; PFFs 1pg, ** $p = 0.0049$; n = 10-20). **(c)** Slopes of the exponential growth phases of the aggregation curves normalized to the control (multiple comparison ordinary one-way ANOVA; N-protein, **** $p < 0.0001$; PFFs 1ng, **** $p < 0.0001$; n = 10-20). **(d)** Representative RT-QuIC assay aggregation curves in the presence of VSVΔG pseudoviral particles as seeding agents. Samples consist in αsyn-only control, asyn in the presence of two serial dilutions of asyn preformed fibrils (PFFs) to act as positive controls [asyn : PFFs molar ratio of $1:10^8$ and $1:10^{11}$], asyn in the presence of three serial dilutions of S-protein exposing VSVΔG pseudoviral particles (VSVΔG-S-protein) [asyn molecules : VSVΔG-S-protein particles ratio of $1:3x10^{11}$, $1:3x10^{12}$ and $1:3x10^{13}$] and asyn in the presence of three serial dilutions of VSVΔG pseudoviral particles presenting the stomatitis virus proper surface glycoprotein (VSVΔG-G) [asyn molecules : VSVΔG-G particles ratio of $1:3x10^{11}$, $1:3x10^{12}$ and $1:3x10^{13}$]. **(e)** Aggregation curves lag times normalized to the control (multiple comparison ordinary one-way ANOVA; VSVΔG-S-protein 1:3x10^14, **** $p < 0.0001$; VSVΔG-S-protein 1:3x10^15, *** $p = 0.0006$; PFFs 1ng, **** $p < 0.0001$; PFFs 1pg, **** $p < 0.0001$; n = 10-20). **(f)** Slopes of the aggregation curves exponential growth phases normalized to the control (multiple comparison ordinary one-way ANOVA; VSVΔG-S-protein 1:3x10^14, **** $p < 0.0001$; VSVΔG-S-protein 1:3x10^15, **** $p < 0.0001$; VSVΔG-G 1:3x10^14, * $p = 0.0167$; PFFs 1ng, **** $p < 0.0001$; PFFs 1pg, **** $p < 0.0001$; n = 10-20). **(g)** Detection by western blot of the level of S-protein present in the SARS-CoV-2-mimicking VSVΔG pseudoviral particles samples used for the aggregation assays (three serial dilutions containing respectively 1500, 150 and 15 particles) compared to the S-protein amount used in the protein-only assays.

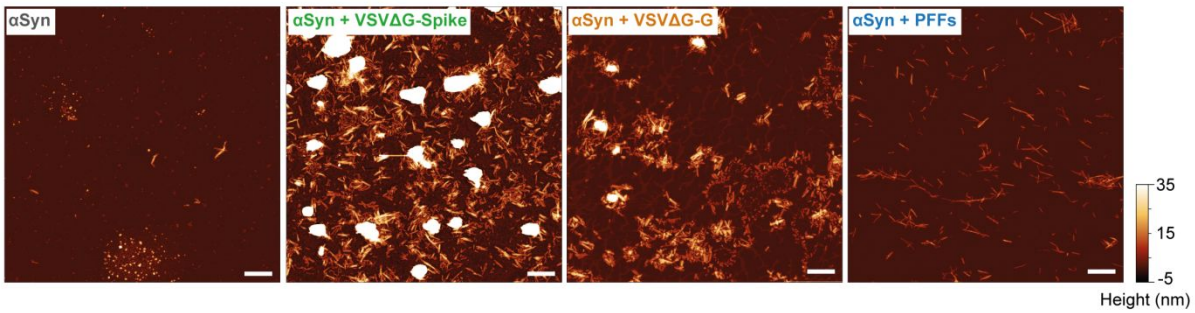


Figure 2. SARS-CoV-2 pseudoviral particles accelerate the formation of mature asyn fibrils *in vitro*. Representative AFM micrographs of aliquots taken after 168h of incubation without additives (αsyn), 1ng asyn preformed fibrils (PFFs), S-protein-exposing VSVΔG pseudoviral particles (VSVΔG-S) and pseudoviral particles presenting the stomatitis virus proper surface glycoprotein (VSVΔG-G). Scale bar: 1 µm.

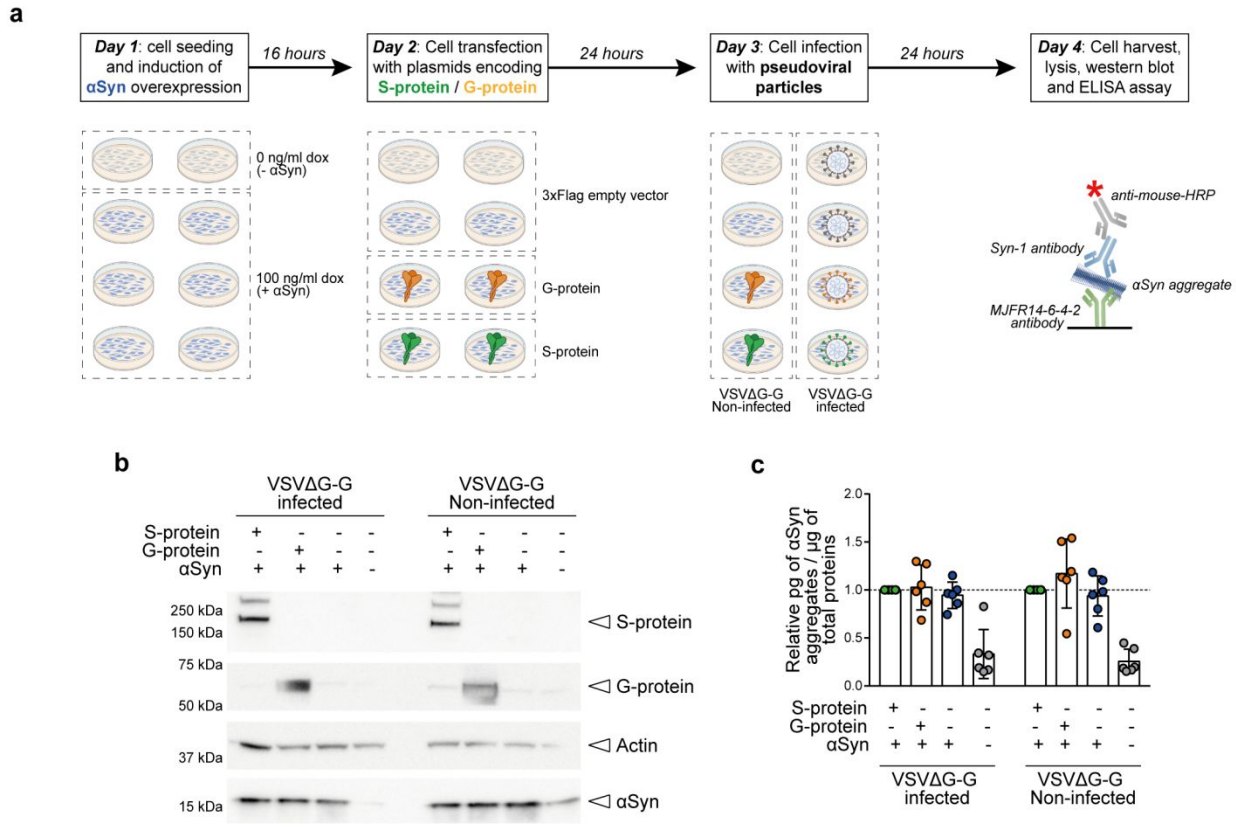


Figure 3. S-protein or S-protein pseudoviral particles do not impact on asyn aggregation in cell models.
(a) Schematic representation of the experimental setup used to test the ability of S/G-proteins alone or assembled on pseudoviral particles to induce asyn aggregation in a cellular environment. Briefly, asyn overexpression was induced by the addition of 100 ng/ml dox in the cell medium of stable and inducible HEK293T cells, while non-induced cells were used as negative control. Cells were then transfected with either S-protein or G-protein encoding plasmids, or 3xFlag empty vector used as control. On the third day, half of the cells were infected with VSVΔG-G pseudoviral particles to promote S/G-protein assembly on their surface. Non-infected cells were used to compare the effect of S/G-proteins alone on asyn aggregation. **(b)** 24-hour post-infection, asyn protein levels were analyzed by western blot in all conditions, S-protein and G-protein expression was tested by specific antibodies and Actin was used as loading control. **(c)** The presence of asyn aggregates in the cell lysate was detected by a sandwich ELISA using the anti-aggregated asyn MJFR14-6-4-2 antibody and the Syn-1 antibody (total αsyn) as detection and capturing antibody, respectively. Data are pooled together from three independent experiments with two technical replicates each. Data are presented as Mean +/- SD and normalized for the S-protein-transfected condition within VSVΔG-G infected and non-infected groups.

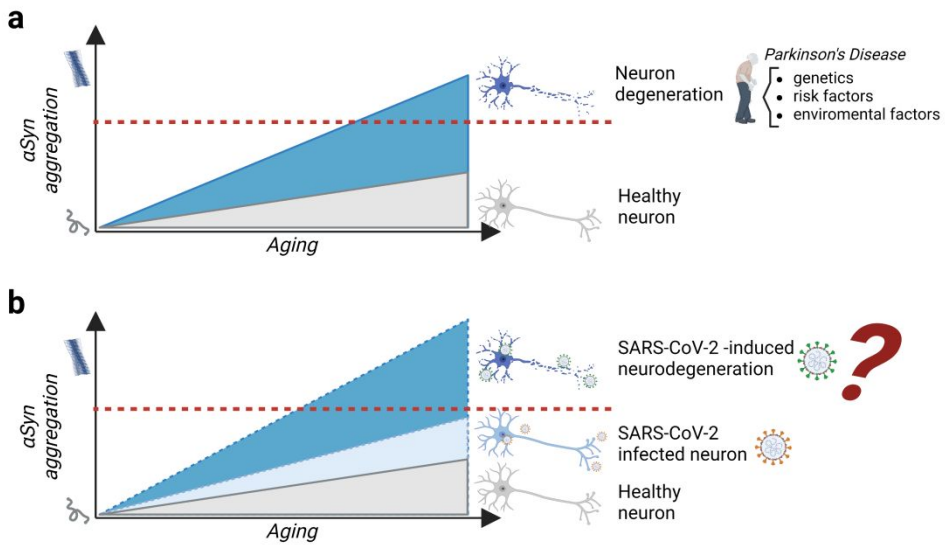


Figure 4. Schematic representation of the possible impact of SARS-CoV-2 infection on the predisposition to develop PD or other synucleinopathies.

a) A large variety of causative genes were associated to the etiology of PD, as well exposure to toxins, by promoting asyn oligomerization and aggregation process. Moreover, genetic or environmental risk factors, including aging, were suggested to increase the predisposition to PD or may be involved in the observed differences in PD onset, progression or symptoms.

b) SARS-CoV-2 infection, which can be associated to the presence of the virus in the infected neurons due to its neurotrophic factors, may be one of the possible risk factors that in combination with the aging and other risk factors, can predispose to PD by affecting the aggregation of αsyn.

Supporting Information

Supplementary file contains Supplementary Table S1, which contains aSyn and seeds final mix concentrations and relative ratios used in RT-QuIC assay. Supplementary figure S1, representing the study of asyn aggregation using ThT and the details of the purification of the spike protein. It also contains Supplementary figure S2, which shows the characterization of the used cells at the level of protein expression (asyn, spike and glycoprotein and spike receptors).

Finally, the supplementary file also contains the materials and methods section, detailing: the production of recombinant His-asyn, recombinant S-protein, and pseudoviral particles. The Real-time quaking-induced conversion (RT-QuIC) Aggregation Assay, asyn aggregation for AFM imaging and ThT assay. Generation of inducible stable cell line and in-cell pseudoviral particle induced asyn aggregation. Western blot, aggregated asyn ELISA assay and immunocytochemistry

Author Information

Authors contribution:

G. Z. prepared asyn stocks, performed the aggregation experiments, prepared the cells, performed the infections and the imaging experiments, and wrote the manuscript; A. M. performed the Elisa assay; M. S. and A. A. helped with the RTquiC experiment; A. C. and L. C. optimized and performed the S-protein purification; F. M. provided insights into the pseudo-viral particles use; A. K. M. provided the pseudo-viral particles and helped preparing the manuscript; M. B. performed the AFM imaging; N. P. conceived and supervised the work and wrote the manuscript; L. B. conceived the work and revise the manuscript.

Notes

The authors declare no competing financial interest.

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References

1) Zhu, N., Zhang, D., et al. A Novel Coronavirus from Patients with Pneumonia in China, 2019, N Engl J Med 2020; 382:**727**-733 (2020). https://doi.org/10.1056/NEJMoa2001017

2) Davis, H. E., McCorkell, L., *et al.* Long COVID: major findings, mechanisms and recommendations. *Nat Rev Microbiol* **21**, 133–146 (2023). https://doi.org/10.1038/s41579-022-00846-2

3) Docherty, A. B., Harrison, E. M., et al. Features of 20 133 UK patients in hospital with covid-19 using the ISARIC WHO Clinical Characterisation Protocol: prospective observational cohort study *BMJ*, 369 :m1985 (2020). https://doi.org/10.1136/bmj.m1985

4) Paterson, R. W., Brown, R. L., et al. The emerging spectrum of COVID-19 neurology: clinical, radiological and laboratory findings, *Brain*, Volume 143, Issue 10, October 2020, Pages 3104–3120. https://doi.org/10.1093/brain/awaa240

5) Tarakad, A., & Jankovic, J., Chapter Seventeen - Anosmia and Ageusia in Parkinson's Disease. *International Review of Neurobiology. Volume 133 Pages 541-556 (2017).* https://doi.org/10.1016/bs.irn.2017.05.028

6) Cocoros, N. M., Svensson, E., et al. Long-term Risk of Parkinson Disease Following Influenza and Other Infections. *JAMA Neurol.*; 78(12):1461–1470 (2021). doi:10.1001/jamaneurol.2021.3895

7) Al-Aly, Z., Bowe, B., et al. Long COVID after breakthrough SARS-CoV-2 infection. *Nat Med* **28**, 1461–1467 (2022). https://doi.org/10.1038/s41591-022-01840-0

8) Midena, E., Cosmo, E., et al. Small Fibre Peripheral Alterations Following COVID-19 Detected by Corneal Confocal Microscopy. *J. Pers. Med.*, *12*, 563 (2022). https://doi.org/10.3390/jpm12040563

9) Matschke, J., Lütgehetmann, M., et al. Neuropathology of patients with COVID-19 in Germany: a post-mortem case series. Lancet Neurology, The, 2020-11-01, Volume 19, Issue 11, Pages 919-929. https://doi.org/10.1016/S1474-4422(20)30308-2

10) Emmi, A., Rizzo, S., *et al.* Detection of SARS-CoV-2 viral proteins and genomic sequences in human brainstem nuclei. *npj Parkinsons Dis.* **9**, 25 (2023). https://doi.org/10.1038/s41531-023-00467-3

11) Song, E., Zhang, C., et al. Neuroinvasion of SARS-CoV-2 in human and mouse brain. *J Exp Med* 1 March 2021; 218 (3): e20202135. https://doi.org/10.1084/jem.20202135

12) Rodriguez-Pallares, J., Quiroz, C. R., et al. Angiotensin II increases differentiation of dopaminergic neurons from mesencephalic precursors via angiotensin type 2 receptors. European Journal of Neuroscience, Volume 20, Issue 6, 1489-1498 (2004). https://doi.org/10.1111/j.1460-9568.2004.03621.x

13) Emmi, A., Tushevski, A., et al. ACE2 Receptor and TMPRSS2 Protein Expression Patterns in the Human Brainstem Reveal Anatomical Regions Potentially Vulnerable to SARS-CoV-2 Infection. ACS Chem Neurosci. 2023 Jun 7;14(11):2089-2097. https://doi.org/10.1021/acschemneuro.3c00101

14) Cantuti-Castelvetri, L., Ojha, R., et al., Neuropilin-1 facilitates SARS-CoV-2 cell entry and infectivity.*Science***370**, 856-860 (2020). https://doi.org/10.1126/science.abd2985

15) Reza, J. N., Gavazzi, I., et al. Neuropilin-1 Is Expressed on Adult Mammalian Dorsal Root Ganglion Neurons and Mediates Semaphorin3a/Collapsin-1-Induced Growth Cone Collapse by Small Diameter Sensory Afferents, Molecular and Cellular Neuroscience, Volume 14, Issues 4–5, Pages

317-326, ISSN 1044-7431, (1999). https://doi.org/10.1006/mcne.1999.0786

16) Ramani, A., Müller, L., et al. SARS-CoV-2 targets neurons of 3D human brain organoids. The EMBO Journal, 39:e106230 (2020). https://doi.org/10.15252/embj.2020106230

17) Meinhardt, J., Radke, J., et al. Olfactory transmucosal SARS-CoV-2 invasion as a port of central nervous system entry in individuals with COVID-19. *Nat Neurosci* **24**, 168–175 (2021). https://doi.org/10.1038/s41593-020-00758-5

18) Kalia, L. V, & Lang, A. E.; Parkinson's disease, Lancet, The, 2015-08-29, Volume 386, Issue 9996, Pages 896-912, (2015). https://doi.org/10.1016/S0140-6736(14)61393-3

19) Jang, H., Boltz, D. A., et al. Viral parkinsonism, Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease, Volume 1792, Issue 7, Pages 714-721 (2009). https://doi.org/10.1016/j.bbadis.2008.08.001

20) Verkhratsky, A., Li, Q., et al. Can COVID-19 pandemic boost the epidemic of neurodegenerative diseases?. *Biol Direct* **15**, 28 (2020). https://doi.org/10.1186/s13062-020-00282-3

21) Antony, T., Hoyer, W., et al., Cellular Polyamines Promote the Aggregation of α-Synuclein. Journal of biological chemistry. Volume 278, Issue 5, p3235-3240, (2003). https://doi.org/10.1074/jbc.M208249200

22) Gerard, M., Debyser, Z., et al. The aggregation of alpha-synuclein is stimulated by FK506 binding proteins as shown by fluorescence correlation spectroscopy. The FASEB Journal, 20: 524-526, (2006). https://doi.org/10.1096/fj.05-5126fje

23) Semerdzhiev, S. A., Fakhree, M. A. A., et al. Interactions between SARS-CoV-2 N-Protein and α-Synuclein Accelerate Amyloid Formation, *ACS Chemical Neuroscience*, *13* (1), 143-150, (2022). https://doi.org/10.1021/acschemneuro.1c00666

24) Wu, Z., Zhang, X., et al. SARS-CoV-2 Proteins Interact with Alpha Synuclein and Induce Lewy Body-like Pathology In Vitro. *Int. J. Mol. Sci.*, *23*, 3394 , (2022). https://doi.org/10.3390/ijms23063394

25) Philippens, I. H. C. H. M., Böszörményi, K. P., et al. Brain Inflammation and Intracellular α-Synuclein Aggregates in Macaques after SARS-CoV-2 Infection. *Viruses*, *14*, 776, (2022). https://doi.org/10.3390/v14040776

26) Ezzat, K., Pernemalm, M., et al. The viral protein corona directs viral pathogenesis and amyloid aggregation. *Nat Commun* **10**, 2331 (2019). https://doi.org/10.1038/s41467-019-10192-2

27) Rossi, M., Candelise, N., et al. Ultrasensitive RT-QuIC assay with high sensitivity and specificity for Lewy body-associated synucleinopathies. *Acta Neuropathol* **140**, 49–62 (2020). https://doi.org/10.1007/s00401-020-02160-8

28) Sweers, K. K. M., Segers-Nolten, I. M. J., et al. Structural model for α-synuclein fibrils derived from high resolution imaging and nanomechanical studies using atomic force microscopy, Soft Matter, 8, 7215-7222, (2012). https://doi.org/10.1039/C2SM25426C

29) Masato, A., Plotegher, N., et al. DOPAL initiates αSynuclein-dependent impaired proteostasis and degeneration of neuronal projections in Parkinson's disease, NPJ Parkinsons Dis. 2023; 9: 42.

https://doi.org/10.1038/s41531-023-00485-1

30) Menon, I., Zaroudi, M., Zhang, Y., et al. Fabrication of active targeting lipid nanoparticles: Challenges and perspectives. *Materials Today Advances, 16*, 100299, (2022). https://doi.org/10.1016/j.mtadv.2022.100299

31) Alkilany, A. M., Farokhzad, O. C., et al. Cellular uptake of nanoparticles: journey inside the cell. *Chemical Society Reviews, 46*(14), 4218–4244, (2017). https://doi.org/10.1039/C6CS00636A

32) Jana, A. K., Lander, C. W., et al. Effect of an Amyloidogenic SARS-COV-2 Protein Fragment on α-Synuclein Monomers and Fibrils. *Journal of Physical Chemistry B*, *126*(20), 3648–3658, (2022). https://doi.org/10.1021/ACS.JPCB.2C01254

33) Mesias, V. S. D., Zhu, H., Tange t al. Moderate Binding between Two SARS-CoV-2 Protein Segments and α-Synuclein Alters Its Toxic Oligomerization Propensity Differently. *Journal of Physical Chemistry Letters*, *13*(45), 10642–10648, (2022). https://doi.org/10.1021/ACS.JPCLETT.2C02278

34) Nyström, S., & Hammarström, P, Amyloidogenesis of SARS-CoV-2 Spike Protein. *Journal of the American Chemical Society*, *144*(20), 8945–8950, (2022). https://doi.org/10.1021/JACS.2C03925

35) Chesney, A. D., Maiti, B., et al. SARS-COV-2 spike protein fragment eases amyloidogenesis of α-synuclein,*J. Chem. Phys*, *159*, 15103, (2023). https://doi.org/10.1063/5.0157331

36) Munishkina, L. A., Cooper, E. M., et al. The effect of macromolecular crowding on protein aggregation and amyloid fibril formation. *Journal of Molecular Recognition*, *17*(5), 456–464, (2004). https://doi.org/10.1002/JMR.699

37) Rivas, G., & Minton, A. P., Macromolecular Crowding In Vitro , In Vivo , and In Between. *Trends in Biochemical Sciences*, *41*(11), 970–981, (2016).. https://doi.org/10.1016/j.tibs.2016.08.013