Spread of $\alpha$-synuclein pathology through the brain connectome is modulated by selective vulnerability and predicted by network analysis

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Studies of patients afflicted by neurodegenerative diseases suggest that misfolded proteins spread through the brain along anatomically connected networks, prompting progressive decline. Recently, mouse models have recapitulated the cell-to-cell transmission of pathogenic proteins and neuron death observed in patients. However, the factors regulating the spread of pathogenic proteins remain a matter of debate due to an incomplete understanding of how vulnerability functions in the context of spread. Here we use quantitative pathology mapping in the mouse brain, combined with network modeling to understand the spatiotemporal pattern of spread. Patterns of $\alpha$-synuclein pathology are well described by a network model that is based on two factors: anatomical connectivity and endogenous $\alpha$-synuclein expression. The map and model allow the assessment of selective vulnerability to $\alpha$-synuclein pathology development and neuron death. Finally, we use quantitative pathology to understand how the G2019S LRRK2 genetic risk factor affects the spread and toxicity of $\alpha$-synuclein pathology.

The synaptic protein $\alpha$-synuclein misfolds in Parkinson’s disease (PD)1–3 and related diseases and aggregates into large intraneuronal inclusions known as Lewy bodies (LBs)4. Recent evidence suggests that $\alpha$-synuclein not only accumulates in LBs but can itself act as an agent of disease, templating the aggregation of $\alpha$-synuclein in anatomically connected neurons throughout the brain5. While the cell-to-cell transmission of $\alpha$-synuclein explains much of the pathology and symptom progression seen in PD and related synucleinopathies, certain neuron populations are clearly more vulnerable than others6, which results in the specific degeneration of certain neurons while sparing others nearby7. Therefore, the extent to which intrinsic cell vulnerability or cell-to-cell spread of $\alpha$-synuclein pathology drives PD pathogenesis is still a matter of debate.

The injection of exogenous misfolded $\alpha$-synuclein into wild-type mice induces misfolding of endogenous $\alpha$-synuclein into phosphorylated, aggregated inclusions that resemble human LBs8,9. These inclusions are found in many brain regions directly or indirectly connected to the injection site, and the inclusions induce a time-dependent degeneration of the inclusion-bearing neurons10,11. Previous work has demonstrated that pathogenic $\alpha$-synuclein is transmitted extracellularly, since spread can be blocked by an anti-$\alpha$-synuclein antibody12 or by blocking a receptor for misfolded $\alpha$-synuclein13. Thus, injection of misfolded $\alpha$-synuclein into mice is an ideal model to understand the spatiotemporal pattern of pathogenic protein spread and how network connectivity and neuronal vulnerability affect spreading dynamics.

In this study, we performed a quantitative assessment of $\alpha$-synuclein pathology in non-transgenic (NTG) mice injected with $\alpha$-synuclein pre-formed fibrils (PFFs), and the data were used to generate a spatiotemporal map of pathology spread and neuron death. A network diffusion model based on anatomical connectivity explained much of the variation in pathological burden. Furthermore, $\alpha$-synuclein gene (Snca) expression correlated well with the differential vulnerability observed between regions, which suggests that anatomical connectivity and $\alpha$-synuclein protein expression are major contributors to pathogenic protein spread. Finally, we sought to determine how genetic risk factors affect the network dynamics of pathologial $\alpha$-synuclein spread. We performed quantitative pathology mapping in transgenic mice carrying the Gly2019Ser (G2019S) leucine-rich repeat kinase (LRRK2) risk factor for PD14 and assessed how transgene expression affects network properties of $\alpha$-synuclein spread, neuron vulnerability and neuron toxicity. The quantitative pathology maps and network model presented here represent an important framework for understanding and treating progressive neurodegenerative diseases.

Results

Brain-wide quantification of $\alpha$-synuclein pathology. A model for PD pathogenesis has recently been described in which $\alpha$-synuclein pathology can be induced in NTG mice following a single injection of $\alpha$-synuclein PFFs5, but pathology is not induced by injection of either monomeric $\alpha$-synuclein or PBS5. While the $\alpha$-synuclein pathology in these mice has been described qualitatively, we...
sought to understand the spatiotemporal spread of α-synuclein pathology quantitatively and to use these data to develop a network model of spread. To accomplish these aims, we injected 5 μg of α-synuclein PFFs into 3-month-old NTG (C57BL/6) mice in the dorsal striatum and then allowed these mice to age to 1, 3 or 6 months post-injection (MPI) (Fig. 1a). Brains embedded in paraffin were sectioned and stained using traditional immunohistochemical techniques for pathological misfolded (Syn506) and phosphorylated (pS129) α-synuclein. These sections were then scanned into a digitized format, allowing for automated quantitation of α-synuclein pathology burden. An expert in mouse neuroanatomy, but blinded to treatment, manually annotated 172 regions displayed. The ipsilateral SN of this brain is shown below with and without an analysis mask overlaid. Scale bars, 1 mm (upper), 100 μm (lower).

Fig. 1 | Quantitation of α-synuclein pathology allows for brain-wide analysis of pathology spread. a, Experiment schematic: mice were injected in the dorsal striatum with α-synuclein at 3 months of age. The mice were then aged 1, 3 or 6 MPI and assessed for motor behaviors during that time period. The brains of mice were used for quantitative pathology analyses. b, Representative images of brain sections (similar for 16 wild-type mice), with manual annotation of 172 regions displayed. The ipsilateral SN of this brain is shown below with and without an analysis mask overlaid. Scale bars, 1 mm (upper), 100 μm (lower).

Dynamic spread patterns in NTG mice. Regional pathological burden values differed by greater than 1,000-fold and revealed dynamic patterns of regional α-synuclein pathology spread over time. Several distinct patterns of spread were observed that are consistent with pathological progression and neuron death (Fig. 2a). (1) Slow initiation of pathology but constant progression after initiation, as seen in the ipsilateral caudoputamen (CPu) and ipsilateral hippocampus. (2) Delayed initiation but constant progression, as seen in the contralateral CPu, which is likely due to the trans-synaptic nature of pathology spread. (3) Rapid initiation and slow progression, as seen in the directly connected ipsilateral secondary motor cortex (M2) cortex. (4) Rapid initiation and rapid decrease, as seen in the vulnerable ipsilateral substantia nigra (SN)4–6, which has robust cell death by 6 MPI and a commensurate reduction in pathology. (5) Constant initiation and slow progression, as seen in the ipsilateral primary somatosensory cortex. The pathological burden of each region at all three time points is displayed as heat maps overlaid onto the brain anatomy (Fig. 2b) and by region over time (Supplementary Fig. 2). Whole-brain quantitation of α-synuclein pathology facilitates the macroscopic observation of network patterns, including the overall increase in pathology over time and the most substantial spread to highly connected ipsilateral regions. While pathological α-synuclein load is informative, we also sought to develop a brain-wide measure of neuron death.

Estimating neuron death. Pathology can take multiple morphological forms. Soon after injection, the majority of the α-synuclein pathology is neuritic (Fig. 3a). By 3 MPI, much α-synuclein pathology has consolidated into large LB-like cell body inclusions. By 6 MPI, some regions have diminished pathology, which is possibly due to the consolidation of neuritic pathology into cell body inclusions and/or neuron death. Previous work has demonstrated that in the SN4, the cortex4 and the accessory olfactory nucleus9, loss of cell body pathology is indicative of the death of the inclusion-bearing neurons. Therefore, we proposed to obtain estimates of neuron loss in each region by measuring cell body inclusion levels throughout the brain and using the difference between time points as an estimate of neuron loss (Fig. 3a).

To validate the neuron loss measure, sections from 3 MPI mice between the two sections used for pathology quantitation were stained for tyrosine hydroxylase (TH), and the number of neurons in the SN were counted (Fig. 3d). The mean estimated neuron loss for this region from 3 to 6 MPI was then subtracted from the TH cell counts to give an estimated 35% neuron loss on the side ipsilateral to the injection side compared with the contralateral side (Fig. 3c). To test the accuracy of this estimate, every tenth section through the midbrain of 6 MPI mice was stained for TH, and the number of TH cells was counted (Fig. 3f,g). The actual TH cell loss in the ipsilateral SN of 6 MPI mice was 34% compared with the contralateral side. The concordance of the estimated and actual neuron loss suggests that loss of cell body inclusions is a valid proxy for neuron loss.

Network model of pathological α-synuclein spread. Observationally, many of the regions to which α-synuclein spreads have high direct anatomical connectivity to the injected CPu6 (Supplementary Fig. 3). However, this connectivity hypothesis...
has not been tested using quantitative pathology data. Here, we experimentally validated a network diffusion model that is based on anatomical connectivity (Supplementary Fig. 4; see Methods for details). Using this model, we estimated pathology as a function of time given the introduction of misfolded protein into the ipsilateral CPu. The model specified that pathological α-synuclein would propagate retrogradely along synapses at a rate that is proportional to the density of axonal projections, and it explained much of the overall variability in the mean regional pathology across all mice at 1, 3 and 6 MPI (Fig. 4a). These results suggest that linear dynamics imposed on a network of synaptic connections can explain pathological α-synuclein spread over time.

To further test the ability of anatomical connectivity to predict the spread of pathology, each of the other brain regions was used as the seed region, and the model was propagated forward to generate predictions of pathological spread at 1, 3 and 6 MPI. The fit of the predicted pathology to the actual pathology displayed a range of accuracies, but an ipsilateral CPu seed produced either the best or second-best fit at all time points (Fig. 4b). The other seed regions that showed a high fit to the empirical data had high in-projection similarity to the CPu (Supplementary Fig. 5), which suggests that seed regions exhibiting high degrees of topological similarity yield similar predictions.

To explore the directionality of spread, retrograde and anterograde connectivity values were separately inserted into the model. While retrograde connectivity had r correlation values of 0.56–0.69 (Fig. 4a), anterograde alone gave r values of 0.32–0.43 (Supplementary Fig. 6), which suggests that α-synuclein spreads primarily in a retrograde manner or that retrograde spread to cell bodies is more easily detected than anterograde spread to nerve terminals.

We further tested the specificity of the network diffusion model by evaluating the ability of proximity to the injection site and higher-order topological features to explain the observed α-synuclein pathology. A network defined by the Euclidean distance between regions poorly explained the observed pathology (Pearson’s r = −0.066–0.1, Supplementary Fig. 7a), and disruption of higher-order topological features also abrogated the predictive capabilities of the model (Pearson’s r = 0.14–0.18, Supplementary Fig. 7b).

Notably, the model requires only a single parameter to be fit, which is a time constant that simply scales the location of the observed time points along the trajectory of the model. We selected the time constant that produced the best mean fit across all time points with the empirical data. To ensure that the time constant we selected was externally valid, we performed split reliability tests. Specifically, we fit the time constant on a random sample composed of half of the mice, and tested its ability to explain the mean regional α-synuclein pathology values in the remaining half of the mice. This procedure was repeated 100 times, and the fits observed when using every mouse (Fig. 4a) did not lie outside the distribution of out-of-sample fits (Supplementary Fig. 8). These results suggest that there is a high reliability of the estimated time constants and low variability between mice in the time course of the spread of pathological α-synuclein.

Differential vulnerability of regions is correlated with α-synuclein expression. Intrinsic vulnerability is postulated to be a critical factor that affects the development of pathology at any given time, and the network model provides a framework to understand which regions are more or less vulnerable than expected if anatomical connectivity was the sole driver of pathological spread (Supplementary Fig. 9). We therefore defined the difference, or residual, between the connectivity-based prediction and observed pathology to be a measure of relative vulnerability. Regions where α-synuclein pathology is exactly predicted by the model have a vulnerability value of 0. Regions that have more pathology than predicted have vulnerability values greater than 0, and regions that have less pathology than predicted have vulnerability values less than 0 (Supplementary Fig. 10a).

To account for noise in the estimation of intrinsic neuron vulnerability, we computed a summary measure of vulnerability based on two principles that can be enforced on the residuals between the model and the empirical data. (1) Intrinsic vulnerability should

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**Fig. 2** | The spread of α-synuclein occurs in a dynamic spatiotemporal pattern throughout the mouse brain. a, Representative quantitative pS129 α-synuclein pathology plots and images are shown for 1-, 3- and 6-month time points. Scale bar, 50 μm. Plots display the mean ± s.e.m. b, Heat map of regions affected by α-synuclein pathology (asterisks indicate the injection site). The names of the associated areas are plotted onto identical maps in Supplementary Fig. 2. n = 4 (1 MPI), 6 (3 MPI) and 6 (6 MPI) mice.
Fig. 3 | Select quantification of cell body pathology allows for assessment of neuron loss. a. Theoretical framework for assessing neuron loss. Pathology appears at 1 month as primarily neuritic pathology, which consolidates over time into large LB-like inclusions. If these large inclusions could be measured as sequential time points, it would allow an approximation of the number of neurons that are lost during that time period. b. An example of a region that has both high neuritic and cell body pathology burden. The total pathology mask identifies both forms of pathology, while the cell body pathology mask excludes neuritic pathology and identifies only cell body pathology. Scale bar, 100 µm. c. Heat map of the estimated number of neurons lost in each anatomical region between 1 and 3 (1–3 MPI) and between 3 and 6 (3–6 MPI) months post-injection (asterisks indicate the injection site). d. One section in between the two sections used for pathology quantitation was stained with an anti-TH antibody and used for quantification of SN neurons in each 3 MPI mouse (two-tailed paired t-test, P = 0.0026). Con, contralateral; Ips, ipsilateral. e. The mean estimated neuron loss between 3 and 6 months from the SN was subtracted from the TH+ cell counts in 3 MPI mice (two-tailed paired t-test, P = 0.0075). f. Every tenth section through the SN was stained with an anti-TH antibody, and SN neurons were counted to estimate the total number of neurons present in NTG mice 6 MPI (two-tailed paired t-test, P = 0.0003). g. Representative images of the contralateral and ipsilateral SN from NTG mice 6 MPI. Scale bar, 500 µm. Plots display the mean ± s.e.m. with individual values also plotted; n = 4 (1 MPI), 6 (3 MPI) and 6 (6 MPI) mice.
through the CPus to cortical regions, which is analogous to what has been observed in the staging of human cases. Seeding other regions, including the hippocampus and the M2 (Supplementary Fig. 13), showed very distinct patterns of spread. Intriguingly, injections into the M2 resulted in rapid decreases in pathology in frontal cortical structures in conjunction with progressive increases in pathology in subcortical structures (Supplementary Fig. 13b). In silico injection into the CPus demonstrated the ability of the network model to recapitulate the empirical spread pattern, but also highlighted regions in which spread was not fully recapitulated by the model (Supplementary Fig. 13c). Future in vivo injections at other sites will be important to quantitatively validate this model and to determine other factors influencing pathological protein spread.

**Understanding changes in network parameters due to G2019S LRRK2 expression.** While theoretical manipulations of the network model can provide a powerful means of hypothesis testing, we sought to directly assay how a genetic risk factor for PD alters the spread of pathogenic α-synuclein and to use network modeling to understand the properties underlying these alterations. While most cases of PD are idiopathic, autosomal-dominant mutations in LRRK2 are responsible for 1–2% of all PD cases. Patients with PD who also harbor LRRK2 mutations have a similar onset and slower progression of disease than patients with idiopathic PD and a similar incidence of pathological α-synuclein inclusions. The most common mutation G2019S LRRK2 leads to elevated kinase activity, and a recent report has suggested that LRRK2 kinase activity is elevated even in patients with idiopathic PD. Despite the strong evidence implicating LRRK2 in PD pathogenesis, only 25–42.5% of G2019S carriers will develop disease, which suggests that mutations in LRRK2 may modulate the pathogenesis of PD. We therefore hypothesized that while G2019S LRRK2 expression may not initiate α-synuclein pathogenesis in mice, it may alter the vulnerability of neurons, alter the toxicity of α-synuclein pathology and/or change the rate of pathology progression. Using the approach described thus far for NTG mice, we quantitatively measured and modeled α-synuclein pathology spread in the brains of G2019S LRRK2 mice to understand the contribution of this genetic risk factor to disease.

The BAC transgenic G2019S LRRK2 mice used for this study exhibited similar expression levels of α-synuclein as wild-type (NTG) mice (Supplementary Fig. 14a,b) and showed no accumulation of pathogenic α-synuclein without α-synuclein PFF
injection up to at least 12 months of age (Supplementary Fig. 14c,d). Interestingly, G2019S mice weighed less than NTG mice (Supplementary Fig. 14e), but showed similar grip strength (Supplementary Fig. 14f) and enhanced motor performance on the rotarod test at early ages (Supplementary Fig. 14g), which is consistent with previous reports.

G2019S LRRK2 mice were injected with the same α-synuclein PFFs in the dorsal striatum at the same time as C57BL/6J mice, so the two groups of mice could be directly compared. Injection of α-synuclein PFFs caused reduced grip strength in both NTG and G2019S mice compared with non-injected controls, but no difference between the genotypes (Supplementary Fig. 14f). Injection of α-synuclein PFFs did not cause a change in the latency to fall in the rotarod assay for NTG mice, but abrogated the enhanced performance seen in G2019S mice (Supplementary Fig. 14g).

The overall pattern of pathological spread in NTG and G2019S mice was similar (Fig. 6; \( n = 4 \) (1 MPI-NTG), 6 (1 MPI-G2019S), 6 (3 MPI-NTG), 6 (3 MPI-G2019S), 6 (6 MPI-NTG) and 7 (6 MPI-G2019S) mice). In fact, the spread pattern in many regions was nearly identical (ipsilateral and contralateral CPu, and ipsilateral M2; Fig. 6a), thus validating the replicability of this quantitative pathology method. However, there were large regional differences in the degree and rate of α-synuclein pathology accumulation (ipsilateral hippocampus, SN and primary somatosensory cortex). These regional differences led to an overall alteration in the dynamics of pathological α-synuclein spread (Fig. 6b), which is best understood at the network level.

We first fit the G2019S pathology data with the same network model as we had previously done for NTG mice. Specifically, we used a network that is based on anatomical connectivity weighted by Snca expression and fit a time constant to the G2019S pathology data. The model showed a similar predictive power for the G2019S mice as we had already established for the NTG mice (Fig. 7a), which suggests that anatomical connectivity and Snca expression are powerful predictors of pathological spread. The model that included Snca expression also had a similarly elevated fit over a model that only incorporated anatomical connectivity (Supplementary Fig. 15). However, the mean of the bootstrapped time constants was lower for G2019S than for NTG mice. This finding suggests that the rate of connectivity-based spread of α-synuclein pathology is globally reduced in G2019S mice.

To test the hypothesis that the G2019S mutation would systematically affect vulnerability to connectivity-based spread, we visually compared the difference in α-synuclein pathology between the NTG and G2019S plotted out on anatomical coordinates (Fig. 7b) to vulnerability values in NTG mice (Fig. 4c). It appeared that many of the regions with elevated pathology in G2019S mice were those that were resilient in NTG mice. Furthermore, many of these regions also underwent enhanced neuron loss in the G2019S mice (Fig. 7c), including the ipsilateral SN, a phenomenon that was confirmed by counts of TH-stained cells (Supplementary Fig. 16). To further explore the relationship between the vulnerability of regions to α-synuclein pathology as established in NTG mice and the pathology observed in G2019S mice, we plotted vulnerability values against the difference in log(pathology), that is, log(G2019S/NTG pathology) (Fig. 7d), for each time point. There was a negative correlation at all time points, which suggests that G2019S LRRK2 expression selectively enhances α-synuclein pathology in otherwise resilient populations of neurons.

Discussion

With limited neuropathology data, there appear to be islands of vulnerable neurons that are susceptible to developing pathogenic protein inclusions. However, meticulous neuropathological examination throughout the brain at different stages of disease has revealed patterns that suggest pathology spreads between anatomically connected regions over time. Mouse models have further indicated that exogenous misfolded proteins can be taken up by neurons and induce the misfolding of endogenous protein, eventually leading to neuron death. Here, we employed absolute quantification paired with network diffusion modeling to explore the factors that dictate the rate and severity of pathogenic α-synuclein spread.

The current study takes advantage of several features of pathological protein spread in mice. First, the disease factor (pathological α-synuclein) can be directly imaged in tissue. Second, the origin and timing of the disease factor is known, since α-synuclein PFFs were injected in the dorsal striatum at 3 months of age and allowed to age a further 1, 3 or 6 months. Third, anatomical connectivity

**Fig. 5 | In silico seeding of alternative regions in the mouse brain. a, b.** Heat maps of regions affected by α-synuclein pathology with in silico propagation of α-synuclein pathology after seeding in either the piriform cortex (a) or the SN (b). Asterisks indicate the in silico injection sites.
of the mouse brain has been extensively characterized at a similar scale, providing accurate anterograde and retrograde connectivity measures for the model. Comparison of the network diffusion model developed in this study to the empirical data set found that anatomical connectivity is a major pathway of pathogenic protein spread. Studies using human imaging data sets have demonstrated the utility of similar network diffusion models to explain the degree of atrophy seen in human patients and even to extrapolate an initiation site for the observed atrophy. Future development of ligands that bind misfolded proteins in the human brain will allow...
longitudinal in vivo imaging of pathogenic protein and direct comparison between mouse and human neuropathology.

We were particularly interested in regions that showed higher or lower pathology than would be expected given the anatomical-connectivity-based prediction, which may represent vulnerable and resilient populations of neurons, respectively. We found that Snca gene expression showed a remarkably similar pattern to inferred vulnerability, thus suggesting that resilient regions are those with very low α-synuclein expression and that vulnerable regions have relatively higher α-synuclein expression. This finding is consistent with...
work from human brains showing that regions with low α-synuclein expression are resilient to LB pathology41. While other factors such as neurotransmitter type, spike rate, reactive oxygen species production and hyperbranching axons may shape the susceptibility of neurons to cell death1, much of the variance in α-synuclein spread was explained purely by anatomical connectivity and α-synuclein expression. It should be noted that Snca mRNA expression was utilized due to the public availability of these data and the correspondence of in situ hybridization data to neuronal cell bodies. Future studies should confirm whether other pathogenic proteins, such as tau, may spread based on anatomical connectivity and endogenous protein expression and whether incorporating other factors such as neuron type into the network model can explain even more of the variance in pathological protein spread.

The current study also found a high correspondence between clearing of LB-like inclusions and neuron death in the SN, thus confirming previous studies that found that neurons bearing LB-like inclusions eventually die10,26. This observation allowed us to use LB-like inclusion loss as a proxy for neuron loss. Measurement of LB-like inclusion number throughout the brain of mice at different time points indicated that neuron loss mostly occurs after long time periods and is related to the pathological burden in that region. Although the data presented here are based on a single injection site, the factors dictating the network diffusion model are independent of the pathology data set. As intrinsic components of the network, these factors can be similarly applied to any injection site. Future empirical data are necessary to fully assess the generalizability of the model. In the interim, we believe that modulation of the factors controlling pathogenic protein spread will allow the assessment of the likely efficacy of therapeutic treatments and will aid in the understanding of the impact of genetic risk factors. This method can be merged with tools from network control theory34,35 to analytically identify the optimal approach to effect a targeted change in the progression of pathology, either by modifying network nodes12 (therapies targeted to regions) or edges11 (therapies targeted to connections).

The current study found that G2019S mice exhibit higher pathology in some regions, yet lower pathology at other regions, relative to NTG mice, which perhaps explains some of the conflicting data suggesting that either increases12,36–38 or decreases39,40 in LRRK2 activity can lead to α-synuclein aggregation and dopaminergic neuron death. The increase in α-synuclein pathology in the SN at 3 MPI and increased neuron death at 6 MPI in G2019S mice are consistent with what has been observed in G2019S BAC mice injected with PFFs42,43 or G2019S knock-in mice overexpressing A53T α-synuclein via adeno-associated virus44. However, our work reveals that broader network dynamics of pathogenic protein spread are changed. The global rate of pathogenic α-synuclein spread is reduced in G2019S mice, and resilient populations of neurons show enhanced α-synuclein pathology and toxicity. It will be interesting to integrate these novel findings relating G2019S LRRK2 to pathological α-synuclein network spread with the known involvement of LRRK2 in vesicle trafficking and network physiology45.

One limitation of the data and model presented here is the mesoscale of analysis, which was chosen to allow comparison to anatomical connectivity data. Pathology in cortical regions shows a clear laminar distribution, and future detailed investigations of the layer and neuron types affected will provide additional information about pathology spread. Another limitation is the sparseness of the regions sampled. Given current technological limitations, it was not possible to map quantitative pathology in the whole brain. However, the sparse data provide sufficient constraints of the network model to allow remarkably accurate reconstruction of pathological α-synuclein spread. The further ability of in silico injection in an independent brain region to capture the pattern of spread suggests that the data captured are sufficient to develop highly informative predictions. Possible differences in anatomical connectivity between strains of mice is also an important caveat to keep in mind in future modeling studies. Future work will also focus on improving the performance of the model by incorporating factors such as regional protein turnover rate, neuron type and functional connectivity.

The network model presented here is informed by quantitative empirical data, but it is simple and uses only intrinsic network properties, including anatomical connectivity and gene expression, for its predictions. Therefore, the model is also easily manipulated by seeding in different regions, predicting the effects of known genetic risk factors and modeling therapeutic intervention by changing parameters of the model (for example, reducing diffusivity to mimic α-synuclein immunotherapy).

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at https://doi.org/10.1038/s41593-019-0457-5.

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Author contributions

M.X.H conceived and designed the experiments, performed experiments, analyzed results and wrote the manuscript. E.J.C. conceived and designed the experiments, analyzed results and wrote the manuscript. All authors reviewed and approved the manuscript.

Competing interests

The authors declare no competing interests.

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Methods

Animals. All housing, breeding, and procedures were performed according to the NIH Guide for the Care and Use of Experimental Animals and approved by the University of Pennsylvania Institutional Animal Care and Use Committee. C57BL/6J (NTG, JAX 000666, RRID: IMSR_JAX:000666) and B6.Cg-Cre-+/+ (Tg(Lrkr2*G2019S)2Yve1/J (G2019S, JAX 012467, RRID: IMSR_JAX:012467) mice have been previously described\(^\text{17}\). The current G2019S BAC line was backcrossed to C57BL/6J mice for more than ten generations and breed to homozygosity at loci as determined by quantitative PCR and outbreeding. The expression level of G2019S LRRK2 was thereby stabilized in this line of mice. All experiments shown used homozygous G2019S mice. Both male and female mice were used for this study.

Behavior. Mouse all-limb grip strength was measured using the animal grip strength test (ITTC 2200). For this test, a grid mesh is attached to a digital force transducer. Mice were moved to a quiet behavioral testing suite and allowed to acclimate for 1 h. A grid mesh was held by one of the tail and allowed to rasp the grid mesh. Once the mouse clasped the grid, the mouse was slowly moved backwards, in line with the force transducer, until the mouse released the grid. The maximum grip force was recorded. The mouse was allowed to rest for several seconds and then was placed on the grid again. The maximum grip strength of five tests was recorded. No fatigue was observed during the test period, so the average of all five measures is reported.

An accelerating rotarod (MED-Associates) was used to assess motor coordination. Mice received two training sessions and two tests sessions. During the training sessions, mice were placed on a still rod. The rod then began to accelerate from 4 to 40 rotations per minute over 5 min. Mice were allowed to rest at least 1 h between training and testing sessions. During the testing sessions, mice were treated as before, and the latency to fall was recorded. Time was also stopped if a mouse gripped the rod and rotated with it instead of walking. Mice were allowed a maximum of 10 min on the rod.

Quantitative pathology. All section selection, annotation and quantification were done blinded to genotype. For quantification of α-synuclein pathology, computer algorithms automatically matched the right and left hemispheres. Measurement of the area occupied by α-synuclein pathology and the number of cell body inclusions. Standardized annotations were drawn to allow independent quantification of 172 regions throughout the brain. Each set of annotations was imported onto the desired section and modified by hand to match the designated brain regions. After annotation, the analysis scripts were applied to the brain to make sure that no non-pathology signal was detected. After annotation of all brains, analysis algorithms were applied to all stained sections, and data analysis measures for each region were recorded.

Two analysis algorithms were applied to the tissue. The first detects the total signal above a minimum threshold. Specifically, the analysis included all diaminobenzidine signals that were above a 0.157 optical density threshold, which was empirically determined to not include any background signal. This signal was then normalized to the total tissue area. A minimal tissue optical density of 0.62 was used to exclude any areas of tissue that was split, and a tissue area thickness of 25.2 µm was applied to exclude any edge effect staining. The second analysis was designed to detect only cell body inclusions. This analysis first classified staining into two broad classes: thin versus thick pathology based on size and texture inputs. The analysis used only the class of thick pathology and excluded objects smaller than 40 µm and greater than 400 µm² to remove small inclusions and to separate multiple inclusions from each other, respectively.

Every tenth slide through the midbrain was stained with TH. TH-stained sections were used to annotate the SN, and cell counting was performed manually in a blinded manner for all sections. The sum of all sections was multiplied by ten to estimate the total count that would be obtained by counting every section.

Computational model. To generate a computational model of pathological spread, we required anatomical connectivity, gene expression and α-synuclein pathology measures that were in the same anatomical space. We used the previously generated data set for synaptic connectivity and Snca gene expression\(^\text{18}\). The mean value for all pixels in a given region was taken as the expression energy for that region. The α-synuclein pathology measures generated for this study were calculated as described above and are reported in Supplementary Table 1. The anatomical structure was correspondingly corresponded by the three data sets so that they did not, the average for the regions in the corresponding atlas was taken to create an accumulated score for that region. For example, the thalamus was broken into several large regions for α-synuclein pathology analysis due to the fact that there is little pathology there and the several nuclei that encompass it are difficult to manually demarcate. Therefore, the ventromedial thalamus in the pathology data actually encompasses the following nuclei from the connectivity data: ventral anterior-lateral, ventral posterior medial, ventral posterolateral and reticular nuclei of the thalamus. In the other direction, the piriform area, while only having one connectivity value, actually spans several of the sections in which pathology was quantified. Therefore, the mean of all pixels of α-synuclein pathology values was used to compare to the connectivity of the piriform area.

Linear diffusion model of pathological spread. Previous studies have used linear diffusion models to predict patterns of atrophy observed in various neurodegenerative diseases\(^\text{19}\), thus supporting the use of these three data sets as a cross-referential means to understand the connectivity of the α-synuclein pathology proteins along large white matter fibers. In the present work, we aimed to validate the ability of these linear diffusion models to predict the spread of misfolded α-synuclein throughout the mouse brain from an injection site in the right ipsilateral CPu. We modeled the pathological spread of α-synuclein as a diffusion process on a 3D grid, where each grid point represents pixels of high expression density and subcortical, respectively, grey matter regions and whose edges \(E\) represent an axonal projection initiating in \(V_I\) and terminating in \(V_E\), where \(I \neq E\) for all \(E\). Edge strength was quantified by the Allen Brain Institute using measures of fluorescence intensity from retrograde viral tract tracing\(^\text{20}\). We defined the weighted adjacency matrix of the network as \(A = [A_{ij}]\). Rows and columns of \(A\) were averaged to generate a final percentile of 116 regions, in accordance with our quantitative measurements of regional synuclein pathology at time \(t\), which were obtained at \(t = [136]\) months.

Stereotactic injections of α-synuclein PFFs. Purification of recombinant α-synuclein and generation of α-synuclein PFFs were conducted as described elsewhere\(^\text{21}\). All of the surgery and experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. Mouse α-synuclein PFFs, which were generated at a concentration of 5 mg ml\(^{-1}\), were vortexed and diluted with Dulbecco’s PBS to 2 mg ml\(^{-1}\). They were sonicated on ice for 10 cycles of 30 on, 30 off (Diagenode Rioruptor UC30-300 bath sonicator).

Mice were injected when 3–4 months old. Mice were evenly assigned to one of two surgeries at random. Even groups of NTG and G2019S mice were injected concurrently to minimize any differences due to surgery date or α-synuclein PFF preparation. Mice were injected unilaterally by insertion of a single needle into the right forebrain (coordinates: +0.2 mm relative to bregma, +2.0 mm from midline) targeting the dorsal striatum (2.6 mm beneath the dura) with 5 µg of α-synuclein PFFs (2.5µl). Injections were performed using a 10-µl syringe (Hamilton) at a rate of 0.4 µl min\(^{-1}\). After 1, 3 or 6 months, mice were perfused transcardially with PBS, brains were removed and underwent overnight fixation in 70% ethanol in 150 mM NaCl, pH 7.4.

Immunohistochemistry. After perfusion and fixation, brains were embedded in paraffin blocks, cut into 6-µm sections and mounted on glass slides. Slides were then stained using standard immunohistochemistry as described below. Slides were de-paraffinized with two sequential 5-min washes in xylene, followed by 1-min washes in a descending series of ethanol as follows: 100%, 95%, 80% and 70%. Sections were then incubated in deionized water for 1 min before antigen retrieval as noted. After antigen retrieval, slides were incubated in 5% hydrogen peroxide in methanol to quench endogenous peroxidase activity. Slides were washed for 10 min in running tap water, 5 min in 0.1 M Tris buffer, then blocked in 0.1 M peroxide in methanol to quench endogenous peroxidase activity. Slides were washed for 5 min in 0.1 M Tris, then incubated with an α-synuclein antibody (Syn506, ref. \(\text{22}\)) was used at 0.4 µg ml\(^{-1}\) final concentration with microwave antigen retrieval (95°C for 15 min with citric-acid-based antigen unmasking solution (Vector H-3000)). For pathologically phosphorylated α-synuclein, pS129 α-synuclein (EP1536Y; Abcam, ab51253) was used at 1:2000 with microwave antigen retrieval. To stain midbrain dopaminergic neurons, TH (TH-16; Sigma-Aldrich, T2928) was used at 1:5000 with formic acid antigen retrieval.

Primary antibody was rinsed off with 0.1 M Tris for 5 min, then incubated with goat anti-rabbit (Vector BA1000) or horse anti-mouse (Vector BA2000) biotinylated IgG in 0.1 M Tris/2% FBS/1:1000 for 1 h. Biotinylated antibody was rinsed off with 0.1 M Tris for 5 min, then incubated with avidin-biotin solution (Vector PK-6100) for 1 h. Slides were then rinsed for 5 min with 0.1 M Tris, then developed with ImmunPACT diaminobenzidine peroxide substrate (Vector SK-4105) and briefly counterstained with hematoxylin. Slides were washed in running tap water for 5 min, dehydrated in ascending ethanol (for 1 min each) as follows: 70%, 80%, 95%, 100%, 100%. Slides were then washed twice in xylene for 5 min and cover-slipped in Cytoseal Mounting Media (Fisher, 23-24-256).

All mice were initially stained with Syn506 for quantification of pathology. It was noted that several mice had perfusion artifacts, which were recognized by secondary antibody staining with Syn506 as Syn506 is a mouse antibody. Since absolute quantification relies on no background staining, these mice were excluded from analysis. The recently developed pS129 α-synuclein antibody (EP1536Y) is a high sensitivity antibody that allows confident detection of subcortical pathology in areas where other antibodies prevent the recognition of endogenous mouse antibodies present in perfusion artifacts. Direct comparison of EP1536Y staining to Syn506 staining revealed a high correspondence of the two stains; therefore, the remaining mice were stained with EP1536Y. The fact that analyzing mice in iterative groups found good correspondence of time constant fitting (Supplementary Fig. 8) also supported the high concurrence of the two stains.

Slides were scanned into digital format on a Luminar scanner (Perkin Elmer) at ×20 magnification. Digitized slides were then used for quantitative pathology.

ARTICLES
We represented the magnitude of observed α-synuclein pathology of all N nodes at time t as the vector x(t). We computed the predicted regional α-synuclein pathology x(0) as a function of A and seed region s ∈ V:

\[
\hat{x}(t) = e^{-\lambda t} x_0
\]

where \( \lambda \) is:

\[
\lambda_{ij} = \begin{cases} -\lambda_i & \text{if } s = ij \\ \nu_i \lambda_{ij} & \text{if } s = ij^{-1} \\ 0 & \text{otherwise} \end{cases}
\]

and \( \sigma \) is a constant to tune the time scale of the system. We selected the time constant \( \sigma \), which maximized the model fit, defined as Pearson correlation between log(x(t)) and log(\hat{x}(t)) for all non-zero values of x. However, we considered the resulting time course (Fig. 1D) and in-sample performance. Note that in the out-degree Laplacian, we used a version of the well-characterized graph Laplacian designed for directed graphs39. This model posits that the rate of change of in pathology at region i is proportional to a weighted sum of the pathology at all other regions and the strength of outgoing projections arriving at those regions from region i, minus the sum of incoming connections from region i times the amount of pathology at region i. Notably, as a property of all Laplacian matrices, \( \hat{x}(t) \) has a single eigenmode with an eigenvalue equal to 0, which describes the steady-state behavior of the system.

To accomplish this, we modified A (ref. 50. Asymmetric graphs also have eigenmodes with complex eigenvalues, such that \( \hat{x}(t) \) oscillates, or in the case of our particular matrix A, exhibits a single, damped oscillation over time. Thus, the time constant \( \sigma \) identifies where along the damped oscillation curve of the system \( x(t) \) provides the best fit to the observed data, with large values of \( \sigma \) indicating the best fit lies closer to the static end behavior. All computations were performed in R and Matlab.

Model incorporating α-synuclein expression. In addition to the spread of the misfolded protein along anatomical connections, we also hypothesized that the pathogenicity of misfolded synuclein would be dependent on the presence of endogenous α-synuclein at each brain region. We observed that the residuals of \( x(t) \) as a linear predictor of \( x(t) \) were correlated with A, a vector of length N containing the estimated levels of α-synuclein mRNA at each brain region (https://www.brain-map.org). Thus, we sought a way to explicitly incorporate \( \hat{x}(t) \) to our linear diffusion model as a measure of the intrinsic vulnerability of each region. To accomplish this, we modified A such that \( A = S A \), where S is the diagonal matrix of R such that \( S = \begin{cases} R_i & \text{for } i = j \\ 0 & \text{otherwise} \end{cases} \). This step weights the outgoing connections of each region in A by its level of synuclein expression, and can be viewed as an “update rate” in our context-dependent systems theory40. All subsequent steps, including generation of the Laplacian, fitting of the time constant \( \sigma \) and propagation of the model as a continuous linear system, were performed according to the procedures described above.

Statistical analysis. Quantification of model specificity. After identifying that our connectome-based linear diffusion model could explain substantial variance in the spread of misfolded α-synuclein from the ipsilateral CPu over time, we next sought to test the specificity of this model to the use of the ipsilateral CPu as the seed site s, which defines the vector x(t). To test the model specificity, we fit the time constant \( \sigma \) to the observed data as described above using every region as s except for the ipsilateral CPu, generating a distribution of nonspecific fits for each time point. We assessed the specificity of the ipsilateral CPu as the seed region by computing the number of alternative seed regions that yield a better fit than the fit obtained using the ipsilateral CPu. While the ipsilateral CPu seed produced nearly the best possible fit at all time points, we observed that several models utilizing alternative seed regions still fit the observed data relatively well. We hypothesized that seed regions with similar connectivity profiles to that of the ipsilateral CPu would perform similarly in explaining the observed data. We computed the similarity of incoming connectivity to the ipsilateral CPu as the Pearson’s correlations between the column of \( A \), where j is the ipsilateral CPu, and the columns of \( A \) where j is all other regions except the ipsilateral CPu. Conversely, we computed the similarity of outgoing connectivity to ipsilateral CPu as the Pearson’s correlations between the row of \( A \), where i is the ipsilateral CPu, and the rows of \( A \) where i is all other regions except the ipsilateral CPu.

Because the relationship between alternative seed model fit and similarity of incoming connectivity to the ipsilateral CPu appeared to have a non-linear relationship, we used a general additive model to fit the relationship using the gam function from the mgcv package in R41. A general additive model is a generalized linear model in which the linear predictor is defined by unknown smooth functions of predictor variables42. We fit the following nonlinear model to predict the fit of the alternative seed model from a smooth function of the connection similarity as follows:

\[
y = \hat{y}(C) + \epsilon,
\]

where y is the fit of the alternate seed model, \( \hat{y} \) is the connection similarity of the respective seed region to the ipsilateral CPu (described above) and \( \epsilon \) is an error term.

Evaluating split-reliability and generalizability of model fit. The data presented in the body of the paper utilize values for the time constant \( \sigma \) derived from data from all mice at every time point (\( x_{null}(t) \)). To ensure that this approach did not result in overfitting, we randomly sampled without replacement the available mice at each time point to generate \( x_{null}(t) \) and \( x_{null}(t) \) for each time point. The time constant \( \sigma \) was determined on \( x_{null}(t) \), and the model was evaluated based on its fit with \( x_{null}(t) \). This process was repeated 100 times, thus generating a distribution of out-of-sample fits for each time point. We computed a nonparametric P value for the generalizability of the model as the number of times the fit generated using \( x_{null}(t) \) exceeded the fit generated by \( x_{null}(t) \), thus quantifying the difference between in-sample and out-of-sample performance.

Network null models. To ensure that our results were specific to the retrograde spread of misfolded synuclein along neuronal processes, we repeated our analyses using several network null models. To demonstrate a general specificity of the model for the topology of the synaptic connectome represented by A, we carried out a procedure that rewires the edges of G while exactly preserving the out-degree and in-degree sequence. That is, \( \sum_{i=1}^N x_i \) and \( \sum_{i=1}^N x_i 

Empirical data. For the α-synuclein pathology data, a two-way analysis of variance (ANOVA) was run on each region, and the months were compared with each other using Tukey’s multiple comparison test, while the genotypes were compared with each other using Sidak’s multiple comparison test. For counts of TH-stained cells in the midbrain, the side ipsilateral to the injection site was compared with the side contralateral to the injection site, and the genotypes were compared with each other using a two-way ANOVA with Sidak’s multiple comparison test. All behavioral data compared both non-injected mice to α-synuclein PFF injection and NTG to G20195 cohorts using two-way ANOVA with Sidak’s multiple comparison test. Data distribution was assumed to be normal, but this was not formally tested. No statistical methods were used to predetermine sample sizes, but our sample sizes were similar to those reported in previous publications43-45.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
All primary pathology data are available in Supplementary Table 1 and on GitHub (https://github.com/ajcorn/connectome_diffusion). Any other data used to generate the figures in this study are available from the corresponding author upon reasonable request.

Code availability
All code is available at https://github.com/ajcorn/connectome_diffusion. See also the Supplementary Software.

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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection
Slides were scanned into digital format on a Lamina scanner (Perkin Elmer) at 20x magnification. Digitized slides were then used for quantitative pathology. The digitized images were imported into HALO software to allow annotation and quantification of the percentage area occupied by α-synuclein pathology and the number of cell body inclusions.

Data analysis
Prism 7.0 (GraphPad) was used for statistical analyses of primary pathological data. All computational modeling and analysis was performed in R, using custom code relying on open source packages for statistics, matrix algebra, and data visualization. All code necessary to generate modeling-based figures will be made available at upon publication at https://github.com/ejcorn.

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**Sample size**
No statistical methods were used to pre-determine sample sizes but our sample sizes are similar to those reported in previous publications cited in the main text (Luk et al., 2012; Henderson et al., 2019). For main network analysis, modeling was validated at the level of individual mice.

**Data exclusions**
In analyzing the fit of our model, we computed the correlations between log(observed) and log(predicted) pathology. We excluded a small number of regions (see figure legends) with detected pathology equal to 0, because the log of 0 is -infinity and thus cannot be included in a correlation. These criteria were pre-established by the methodology.

**Replication**
To assess the reproducibility of our pathological spread measures, we performed split reliability tests. Specifically, we fit the time constant on a random sample composed of half of the mice, and tested its ability to explain the mean regional α-synuclein pathology values in the remaining half of the mice. This procedure was repeated 100 times, and the fits observed when using every mouse did not lie outside of the distribution of out-of-sample fits. This analysis demonstrates that our findings are robust in multiple held-out subsamples of our data. This analysis confirms the reproducibility of experiments and is further described and demonstrated in the Supplementary material.

**Randomization**
Stereotaxic surgeries are routinely carried out by two surgeons in parallel. The two genotypes of mice being injected were assigned equally to each of the two surgeons to reduce variability by surgeon.

**Blinding**
All manual annotation of brain regions and counting of tyrosine hydroxylase-positive neurons was done by an experimenter blinded to the genotype of animals.

Reporting for specific materials, systems and methods

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### Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ☑ | Antibodies |
| ☑ | Eukaryotic cell lines |
| ☑ | Palaeontology |
| ☑ | Animals and other organisms |
| ☑ | Human research participants |
| ☑ | Clinical data |

### Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☑ | ChIP-seq |
| ☑ | Flow cytometry |
| ☑ | MRI-based neuroimaging |

### Antibodies

#### Antibodies used

Primary antibody name (clone; company name, catalog number) dilution for IHC (lot numbers not available):
- pS129 α-synuclein (EP1536Y; Abcam, ab51253) 1:20,000
- Misfolded α-synuclein (Syn506; Center for Neurodegenerative Disease Research) 1:5,000
- Tyrosine hydroxylase (TH-16; Sigma-Aldrich, T2928) 1:5,000

Secondary antibody name (company name, catalog number) dilution for IHC
- Goat anti-rabbit (Vector, BA1000) 1:1,000
- Horse anti-mouse (Vector BA2000) 1:1,000

#### Validation

Each antibody was validated for the respective species and application by the correspondent manufacturer, and is publicly available on its website with indicated catalogue numbers. Syn506 was generated and validated initially in 2002 (Duda et al., 2002) and has been extensively validated in previous publications. EP1536Y is a newer antibody, but has been highly validated as one of the most selective pS129 α-synuclein antibodies available (Delic et al., 2018) and we validate it in our own manuscript by showing it doesn’t bind non-injected mouse brains (Supplementary Fig. 14c, d). TH-16 is a highly reliable antibody which has been used extensively for staining of tyrosine hydroxylase (Luk et al., 2012, Henderson et al., 2017, Henderson et al., 2018, Henderson et al., 2019).
### Laboratory animals
The species, strain, sex and age of laboratory animals used in this study have been described in the main text. Briefly, male and female C57BL/6J (NTG, JAX 000664, RRID: IMSR_JAX:000664) and B6.Cg-Tg(Lrrk2*G2019S)2Yue/J (G2019S, JAX 012467, RRID: IMSR_JAX:012467) mice were used in this study. The age of mice is noted in all figures and ranged from 3 months (the age of injected mice) to 12 months of age.

### Wild animals
No wild animals were used.

### Field-collected samples
This study did not use samples collected from the field.

### Ethics oversight
All housing, breeding, and procedures were performed according to the NIH Guide for the Care and Use of Experimental Animals and approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Note that full information on the approval of the study protocol must also be provided in the manuscript.