By Dag Forssell, Jan 23, 2021
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What Causes Parkinson's Disease?

As discussed in

THE CARNIVORE CODE

by Paul Saladino, M.D.

CHAPTER 7: OF KIDNEY BEANS AND PARKINSON'S DISEASE

Text excerpted from this chapter, followed by a current paper and the four references listed in these four pages, downloaded using scholar.google.com.

The Jan 1, 2021 email compelled me to insert next page.

This pdf page 7

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This pdf page 15

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This pdf page 39

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Recent, related information on gut health

Stanford Medicine - Department of Neurology & Neurological Sciences

http://med.stanford.edu/neurology.html

features extensive Parkinson's support information

http://med.stanford.edu/parkinsons.html

with pointers to support groups, exercise resources, mailing lists, and more.

A group of staffers review webinars offered on the web.

Most deal with treatment, but on November 17 2020, the subject was

Webinar Notes: *How the Brain Progresses with Parkinson's Disease*

Neurologist Yasar Torres-Yaghi shared how the pathology of Parkinson's disease begins in the gut, then progresses to the olfactory bulb before invading the entire brain.

The webinal is available here: www.youtube.com/watch?v=wA2uxnT2cfk

On Jan 1, 2021, the subject was

Webinar Notes: *Inflammation, Immunity, and the Gut: Microbiome in Parkinson's Risk and Progression*

This webinar explored the relationship between inflammation, gut health, Parkinson's disease (PD) risk, and PD progression. The guest speaker was Malú Gámez Tansey, Ph.D. Dr. Tansey gave a high-level overview of the immune system, inflammation, and its effects on PD. The webinar be viewed here:

https://youtu.be/ODrVG1K6aw4https://youtu.be/ODrVG1K6aw4

This second webinar was the more impressive to me, with clear illustrations, but as with the first, there was no suggestion of how to achieve gut health.

Could Lectins Play a Role in Parkinson's Disease?

So far, we've seen the havoc that lectins from plants like legumes, wheat, nightshades, and peanuts can wreak on our gut and our immune system, but lectins also appear to be able to negatively affect our brains and have been hypothesized to play a role in Parkinson's disease. This illness is a neurodegenerative condition in which dopamine-producing neurons from a region known as the basal ganglia are damaged, leading to problems with movement, speech, and cognitive processing. Many Parkinson's patients progress to dementia and experience depression as their illness worsens. The impact of this disease on quality of life is substantial. The cause of neuronal cell death and depletion of dopamine signaling in the midbrain is not fully understood, but characteristic aggregates known as Lewy bodies occur and are composed of the protein alpha-synuclein.

In 2015, an intriguing study was done in Denmark showing that people who had undergone surgical severing of the vagus nerve during the previous forty years developed Parkinson's disease at a much lower rate than the general population.[23] In order to understand what this may have to do with lectins, let's dig a bit deeper into what could be going on here.

The vagus nerve originates in the brain stem and sends signals to many portions of the body and much of the digestive tract, including the stomach, liver, pancreas, and intestines. It is a large nerve and serves as a bidirectional information superhighway between our gastrointestinal organs and our brain, with signals from the brain traveling through the vagus nerve to the digestive organs, and back again. You may have heard the term "gut-brain axis" in reference to the notion that our gut and brain communicate in many ways. In addition to the signals transmitted to the brain through cytokines in the bloodstream, the vagus nerve is also a big part of this cross talk.

Within surgical medicine, this nerve is sometimes cut to treat patients with severe peptic ulcer disease because signals from the vagus nerve to the stomach cause the release of acid during the digestive process. In the aforementioned study from Denmark, investigators compared people who had truncal vagotomy, a complete severing of the vagus nerve, with those who had superselective vagotomy in which only the vagus nerve's connection to the stomach was cut. Reduction in the incidence of Parkinson's disease was only observed with truncal rather than superselective vagotomy, suggesting that it was the connection between *both* the intestines and the stomach that accounted for this finding. Why would severing the neural connection between the gut and the brain affect a neurodegenerative condition like Parkinson's disease? Is it possible that a substance from the gastrointestinal tract could move to the brain through the vagus nerve?

This is truly a fascinating question, and I believe that a couple of studies in animal models of Parkinson's disease will help shed light on this mystery.

One of the coolest innovations in molecular biology over the last twenty years is the use of a bioluminescent protein from jellyfish, called green fluorescent protein (GFP). This molecule can be used to visualize the movement of proteins within living organisms. When a savvy group of investigators fed the invertebrate worm *C. elegans* green-fluorescent-protein labeled PHA and other lectins, they were able to visualize exactly where these lectins went in that organism after they were ingested. Their findings were striking. Not only did a number of lectins appear to be transported from the *guts to the brains of these worms through the vagus nerve*, the lectins then *clustered in the dopamine secreting neurons*. In the case of PHA, this lectin appeared to be toxic to neurons, reducing their number and function.[24] Referencing the aforementioned Danish study, the authors concluded:

> *"These observations suggest that* ***dietary plant lectins are transported to and affect dopaminergic neurons in C. elegans****, which support Braak and Hawkes' hypothesis, suggesting* ***one alternate potential dietary etiology of Parkinson's disease*** *(PD). A recent Danish study showed that vagotomy resulted in 40% lower incidence of PD over 20 years. Differences in inherited sugar structures of gut and neuronal cell surfaces may make some individuals more susceptible in this conceptual disease etiology model."*

As mentioned in the previous excerpt, Braak and Hawkes, had previously hypothesized that an ingested "unknown pathogen" enters the gastrointestinal tract and transports via the vagus nerve to the brainstem, inducing a spreading neural dysfunction.[25] The authors of this study suggest, based on the Danish vagotomy cohort and their own findings, that ingested lectins may be damaging the gut and traveling through the vagus nerve to the brain, where they appear to be toxic to dopaminergic neurons.

These researchers make another very interesting observation:

> *"Lectins from dietary plants have been shown to enhance drug absorption in the gastroin-testinal tract of rats,* ***be transported trans-synaptically as shown by tracing of axonal and dendritic paths****...and other carbohydrate-binding protein toxins are known to* ***traverse the gut intact*** *in dogs."*

Enhanced drug delivery sounds like a good thing, until we realize that the way lectins do this is by increasing the permeability of the gut lining! As noted by these investigators, lectins have been found to damage the gut and traverse the small intestinal epithelium in animals like dogs, and the appearance of peanut, kidney bean, and tomato lectins in samples of human blood indicates that lectins are doing the same things in us.

C. elegans is not the only organism in which ingestion of lectins has been linked to damage in dopaminergic neurons in the brain. In a study with rats, researchers administered a lectin from peas and looked for Parkinsonian behavior or changes in gastric motility.[26] Their findings were striking and were published in the prestigious journal *Nature* in 2018:

> *"**These data demonstrate that co-administration of subthreshold doses of paraquat and lectin induces progressive, L-dopa-responsive parkinsonism that is preceded by gastric dysmotility.** This novel preclinical model of environmentally triggered Parkinson's disease provides functional support for Braak's staging hypothesis of idiopathic Parkinon's disease."*

Paralleling the study from Denmark discussed earlier, these authors also included a group of rats that underwent vagotomy prior to exposure to pea lectins, and these rats did not demonstrate any of the neuronal damage or gastric dysmotility issues observed in the other experimental groups.

The possibility of plant lectins contributing to the pathogenesis of this disease is striking and is a potential paradigm shift in the world of neurodegenerative illness. It is important to highlight, however, that certain individuals are more likely to be susceptible to this type of neuronal injury. Not everyone who eats beans, tomatoes, or peanuts develops Parkinson's disease, but in those with genetics susceptible to this type of lectin-induced damage, consumption of plant foods could be a contributing factor to the development and progression of neurodegenerative illness.

More studies are needed here, but based on the these findings, it seems reasonable to hypothesize the following: lectins in the gut could cause damage there, travel to the brain through the vagus nerve in a retrograde fashion, and trigger injury of dopamine-secreting neurons in the regions of the basal ganglia that control movement and other complex tasks. Scary! Maybe this will make us think twice about adding beans to that chili.

The Plinko Model of Autoimmune Disease

You may be familiar with Plinko from the game show, *The Price Is Right*. I used to love this show when I was a kid and always hoped someone would win the car. When playing Plinko, participants stand over a slanted board full of pins and release a disk, which slides down through the pins in a random fashion before ending up in one of the bottom wells that are each marked with different dollar amounts. I think of the combination of our genetic susceptibility to disease and our environment like the game of Plinko.

In this model, our genetics create a unique pattern of pins on the board through which the disk (inflammation) travels in a unique pattern leading to one of a variety of chronic diseases. As I mentioned previously, not everyone will be susceptible to Parkinson's disease when they eat lectins, but some might be. We all possess different genetics that make us uniquely susceptible to different diseases. We've all got holes in our armor—they're just in different places. I strongly believe that inflammation is at the root of most chronic disease but is manifested differently in each person based on their own unique genetic susceptibilities. Thus, eating lectins and other plant toxins might contribute to Parkinson's disease in some people, heart disease in others, and skin issues or joint pain for others still. All of these diseases have a common root trigger, which is inflammation, but it shows up differently in all of us based on our individual genetic Plinko board of susceptibilities.

Because we've been eating tons of plants and living in toxic environments for most of our lives, we've all been exposed to inflammatory stimuli, but the results show up in different ways. When I am exposed to such stimuli, I get asthma and eczema and become a bit irritable. These are my genetic weaknesses, not yours. When you are exposed to inflammatory insults, you might develop autoimmune thyroid disease, like Hasimoto's thyroiditis, or you might develop lupus, rheumatoid arthritis, or diabetes. Western medicine becomes flummoxed when it tries to think of these diseases as thousands of different entities, often leaving it powerless to bring real change in the lives of those who suffer with such maladies. **The critical error of judgment that Western medicine is making is in imagining that there are thousands of different chronic illnesses, when in fact there's really only one big one—inflammation.**

So how do we correct this one major cause of chronic illness? We search for its roots and remove them! One of the radical notions this book advances is that, because of all of their intrinsic toxins, plants might be triggering such inflammation in ways never before considered. Wild, right? Let's continue our journey through the lectin jungle; there's more to see!

Could Lectins Be Causing Us to Gain Weight?

One of the more positive effects experienced by those on a carnivore diet is weight loss. There are now thousands of stories of people easily losing weight when plants are eliminated from their diet and high-quality animal foods become the focus. Many of these people began with ketogenic diets, which still include some plants, but they found weight loss even easier and

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https://journals.lww.com/neurotodayonline/Fulltext/2020/11190/Two_Types_of_Parkinson_s_Disease_Pathologies_.12.aspx

Two Types of Parkinson's Disease Pathologies: Body-First Versus Brain-First

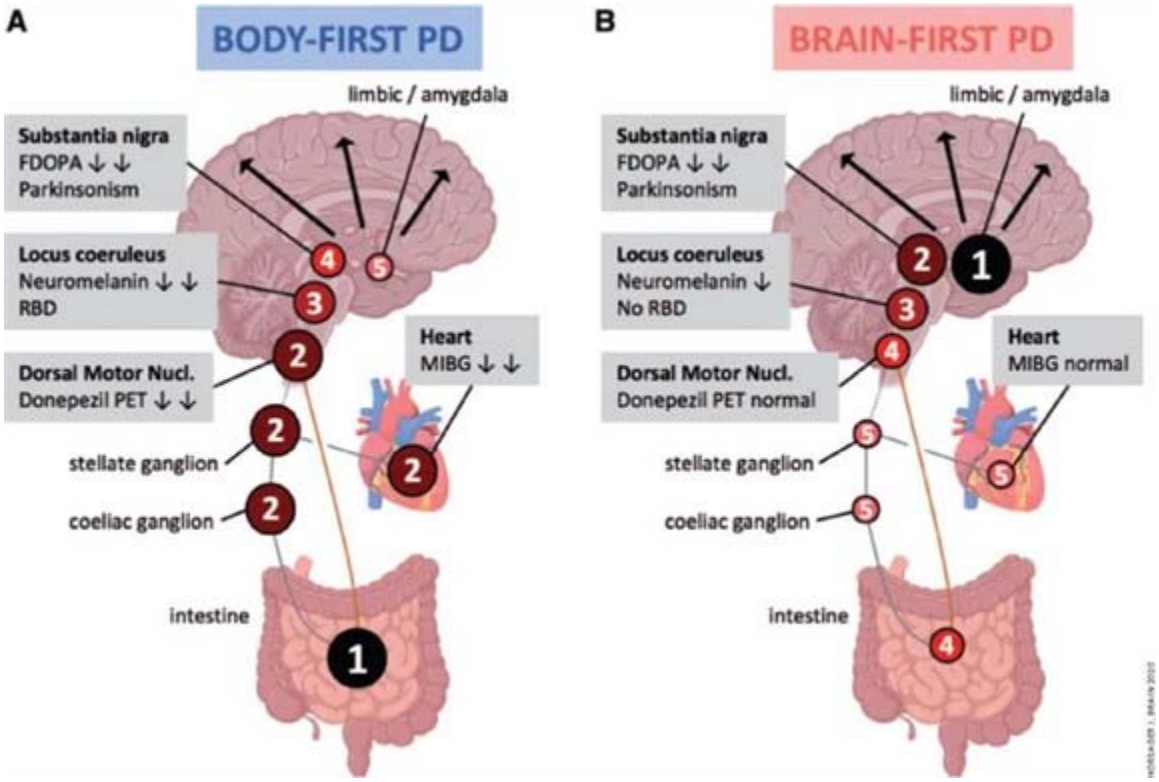
- DISEASE MECHANISMS

By Dan Hurley

November 19, 2020

Article In Brief

New research findings suggest that two different underlying mechanisms for Parkinson's disease—a "brain-first" neuropathology that begins in the brain and then shifts downward and a "body-first" version that follows the standard trajectory of Braak staging.



The figure depicts two contrasting spreading routes for Parkinson disease: the body-first type (A) and the brain-first type (B). Numbered circles depict the starting point and subsequent propagation of pathology in the two subtypes.

Rather than always beginning in the enteric or peripheral autonomic nervous system and moving upward toward the central nervous system, Parkinson's disease (PD) neuropathology begins in the brain itself about one-third of the time and then heads downward, according to a study published online August 24 in the journal *Brain* by a team of Danish researchers.

They call this new subtype a “brain-first” version of the disease, in contrast to what they call a “body-first” version that follows the standard trajectory of Braak staging.

The new schema is based on multimodal imaging of three groups: patients with newly diagnosed PD who did not also have REM sleep behavior disorder (RBD); those with RBD but not PD; and those with both. Functional imaging studies focused on the colon, the gut, the heart, striatal dopaminergic targets, and the locus coeruleus.

“What we have shown is that there are quite clearly two types of Parkinson's disease,” said the senior author of the paper, Per Borghammer, MD, PhD, MSc, clinical professor in the department of nuclear medicine and PET at Aarhus University in Denmark.

“In the first type, you have quite dramatic damage to the peripheral nervous system, the sympathetic and the cholinergic system, but a normal dopamine system. In the second type, you see a normal peripheral autonomic system, but quite severe damage to the dopamine system in the brain.”

The theory, Dr. Borghammer said, should be understood as postulating two major subtypes of Parkinson's PD based on the initial starting point and subsequent spreading of Lewy pathology, but that other factors such as selective neuronal vulnerability are also important.

Reaction to the paper by movement-disorder specialists familiar with Parkinson's subtyping efforts was mixed. Most described the paper as elegant and important and said it could have implications for ongoing efforts to study the role of the microbiome in the early stages of the disease. Cases that begin in the brain, they said, would likely not benefit from any intervention that targets the gut.

Others, however, said there are likely to be still other subtypes and questioned whether any treatment implications could be drawn.

“This is solid work, this is great work,” said Ronald B. Postuma, MD, MSc, professor of neurology and neurosurgery at McGill University School of Medicine. “They use REM sleep behavior disorder as their main wedge for distinguishing the two subtypes. It's a very powerful wedge. But it's not the only model that can explain the patterns we see.”

So many models seeking to classify the ways that PD can present and progress have been developed that the International Parkinson and Movement Disorder Society (MDS) has established a task force on PD subtypes. Dr. Postuma is a member of the task force and co-authored a commentary on the new study.

While acknowledging the limitations of the paper, in particular its cross-sectional design, the commentary applauded its focus on biological correlates rather than on symptoms alone. The findings "shed important new light on the concept of top-down versus bottom-up RBD that will augur new studies in this area," the commentary stated.

"This is solid work, this is great work. They use REM sleep behavior disorder as their main wedge for distinguishing the two subtypes. Its a very powerful wedge. But its not the only model that can explain the patterns we see."—DR. RONALD B. POSTUMA

Study Details

Dr. Borghammer and colleagues first proposed the brain-first vs. body-first hypothesis last October in the *Journal of Parkinson's Disease*. In that paper, they also hypothesized that isolated REM sleep behavior disorder is a prodromal phenotype for the body-first type.

They sought to test the hypothesis by quantifying neuronal dysfunction in structures corresponding to Braak stages I, II, and III involving three patient groups: 24 newly diagnosed PD patients who did not have the REM sleep disorder (PDRBD-), 13 new PD patients who also had the sleep disorder (PDRBD+), and a comparator group of 22 patients who had isolated RBD (iRBD).

Tests included 11C-donepezil PET/CT to assess cholinergic (parasympathetic) innervation; 123I-metaiodobenzylguanidine (MIBG) scintigraphy to measure cardiac sympathetic innervation; neuromelanin-sensitive MRI to measure the integrity of locus coeruleus pigmented neurons; and 18F-dihydroxyphenylalanine (FDOPA) PET to assess putaminal dopamine storage capacity. In addition, colon volume and transit times were assessed with CT scans and radiopaque markers.

As hypothesized, both PD groups were found to have similarly sharp reductions in putaminal FDOPA-specific uptake, whereas two-thirds of the non-PD patients with iRBD had normal putaminal scans.

On the other hand, both the PD patients who had RBD and the non-PD patients with the sleep disorder had reduced heart and colon functions compared with the PDRBD- group. At the same time, “in comparison to the other groups, the PDRBD + group also had enlarged colon volumes and delayed colonic transit times,” the paper stated.

In addition, the group with PD plus the sleep disorder also trended toward a reduced mean MRI locus coeruleus: pons ratio compared to the group that had PD without RBD.

Dr. Borghammer and colleagues concluded that both groups with RBD had the body-first trajectory, “characterized by initial loss of cardiac MIBG signal and 11C-colonic donepezil signal followed by loss of putaminal FDOPA uptake.” However, the newly diagnosed Parkinson's patients who did not have RBD were considered to have the brain-first trajectory.

“In Braak's staging scheme from 2003, he postulated that the pathology starts in the peripheral nervous system, especially the gut, then ascends via the vagus and then reaches the bottom of the brainstem, where it causes RBD,” Dr. Borghammer told *Neurology Today*. “I think Braak was correct, but that his hypothesis only applies to some of the patients, and those are the body-first patients. But we have known for more than ten years that this is an incomplete hypothesis. In his 2003 paper, Braak only studied patients who had pathology at the bottom of the brainstem. That was his inclusion criteria. For that reason, it's no surprise that all of his patients were of the same type.”

He cited a 2008 paper in *Acta Neuropathologica*, which found many PD patients who, on autopsy, had no evidence of pathology at the bottom of the brainstem. He also cited another autopsy paper published last year in the same journal finding that that two-thirds of Lewy-body positive patients aged 85 or older had a brainstem-predominant pattern, while the remaining one-third had an amygdala-predominant pattern with much less pathology in the lower brainstem.

If it's true that a significant portion of PD cases begin in the brain rather than in the gut, Dr. Borghammer said, then studies presuming that all cases begin in the gut will produce murky results.

“If the microbiome is the culprit, it would probably only be so in the body-first patients,” he said. “Your brain-first patients most likely have a normal microbiome.”

Expert Commentary

The co-chair of the MDS task force on PD subtypes said he welcomed the study's focus on physiology rather than on clinical symptoms.

“Most of the PD subtypes are rooted in clinical features, which might not address the questions we need to address, regarding the pathophysiology of the disease, the prognosis, and ultimately the possibility of disease-modifying therapies.”—DR. TIAGO A. MESTRE

“Most of the PD subtypes are rooted in clinical features, which might not address the questions we need to address, regarding the pathophysiology of the disease, the prognosis, and ultimately the possibility of disease-modifying therapies,” said Tiago A. Mestre, MD, MSc, associate professor in the division of neurology at the University of Ottawa and an associate scientist at the Ottawa Brain and Mind Research Institute.

Although the study was relatively small, Dr. Mestre added, “it definitely warrants further investigation.”

Eduardo De Pablo Fernandez, PhD, MD, a neurologist and clinical research associate at University College London's Queens Square Brain Bank, said he thought the case for two subtypes, while “elegant and with sound imaging methods,” was not entirely convincing.

“Two subtypes are probably simplistic,” he said. “There are probably more than two subtypes of Parkinson's based on ways of pathology progression.”

Alberto J. Espay, MD, MSc, FAAN, professor of neurology, director and endowed chair of the James J and Joan A Gardner Center for Parkinson's Disease and Movement Disorders at the University of Cincinnati Academic Health Center, also expressed skepticism about the two disease subtypes.

“We all know that Parkinson's is not one disease,” said Dr. Espay, who is also a member of the MDS task force on subtypes. “This study says it's two diseases. But it still assumes that spreading alpha-synuclein pathology is the cause, and that has never been proven. The reason we would care to find subtypes is not to develop beautiful theories, but to find treatments. That we can provide data in

favor of two subtypes of Parkinson's disease tells us nothing about how to treat patients classified into each of these two types."

The commentary accompanying the paper also questioned its assumption that spreading alpha-synucleinopathy explains both the body-first and brain-first types. "It needs to be emphasized," Dr. Postuma said, "that pathology was not directly measured—rather, this study looked at indices of neuronal dysfunction."

Despite his own doubts, Dr. Postuma said that he is convinced of the validity of there being different subtypes of PD.

Dr. Postuma was senior author of a study published in 2017 in *Brain*, which would seem to provide evidence in favor of a body-first subtype. In this study, RBD was one key marker of a "diffuse malignant" subtype of PD, in which there is broad and general neurodegeneration, with more non-motor features and worse prognosis.

"The basic concept of what they found certainly does seem to be the case," he said. "If you think of the body-first subtype as being based on presence of RBD, then you can see how many studies are coming together. We know that REM sleep behavior disorder is a pretty powerful sign of a bad prognosis."

"Whether you call RBD-associated PD a 'body-first,' 'body-predominant,' or 'diffuse' subtype, vs. 'brain-first,' 'brain-predominant' or 'pure motor' subtype," he continued, "doesn't change the fact that there are different types with different outcomes and probably different patterns of spread."

Disclosures

Dr. Mestre reports receiving speaking honoraria from Abbvie; consultancy fees from CHDI Foundation/Management, Sunovion, Valeo Pharma; and fees for service on the advisory boards of Abbvie, Biogen, Roche, and Medtronic. He has received research funding from EU Joint Programme-Neurodegenerative Disease Research, OBMRI, Ontario Research Fund, CIHR, Michael J Fox Foundation (MJFF), Parkinson Canada, PDF/PSG, LesLois Foundation, PSI Foundation, and the Parkinson Research Consortium. Dr. Postuma reports grants and personal fees from Fonds de la Recherche en Sante, the Canadian Institute of Health Research, Parkinson Canada, the Weston-Garfield Foundation, the MJFF, the Webster Foundation, and personal fees from Takeda, Roche/Prothena, Teva Neurosciences, Novartis, Biogen, Boehringer Ingelheim, Theranexus, GE HealthCare, Jazz Pharmaceuticals, Abbvie, Jannsen, Curasen, and Otsuko (outside the submitted work). Dr. Espay received grant support from the NIH and the MJFF; personal compensation as a consultant/scientific advisory board member for Abbvie, Neuroderm, Neurocrine, Amneal, Adamas, Acadia, Acorda, Kyowa Kirin, Sunovion, Lundbeck, and USWorldMeds; publishing

royalties from Lippincott Williams & Wilkins, Cambridge University Press, and Springer; and honoraria from USWorldMeds, Acadia, and Sunovion.

Link Up for More Information

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Vagotomy and Subsequent Risk of Parkinson's Disease

Elisabeth Svensson, PhD,[1] Erzsébet Horváth-Puhó, PhD,[1] Reimar W. Thomsen, PhD,[1] Jens Christian Djurhuus, DMSc,[2] Lars Pedersen, PhD,[1] Per Borghammer, DMSc,[2,3] and Henrik Toft Sørensen, DMSc[1]

Objective: Parkinson's disease (PD) may be caused by an enteric neurotropic pathogen entering the brain through the vagal nerve, a process that may take over 20 years. We investigated the risk of PD in patients who underwent vagotomy and hypothesized that truncal vagotomy is associated with a protective effect, whereas superselective vagotomy has a minor effect.
Methods: We constructed cohorts of all patients in Denmark who underwent vagotomy during 1977–1995 and a matched general population cohort by linking Danish registries. We used Cox regression to compute hazard ratios (HRs) for PD and corresponding 95% confidence intervals (CIs), adjusting for potential confounders.
Results: Risk of PD was decreased in patients who underwent truncal (HR = 0.85; 95% CI = 0.56–1.27; follow-up of >20 years: HR = 0.58; 95% CI: 0.28–1.20) compared to superselective vagotomy. Risk of PD was also decreased after truncal vagotomy when compared to the general population cohort (overall adjusted HR = 0.85; 95% CI: 0.63–1.14; follow-up >20 years, adjusted HR = 0.53; 95% CI: 0.28–0.99). In patients who underwent superselective vagotomy, risk of PD was similar to the general population (HR = 1.09; 95% CI: 0.84–1.43; follow-up of >20 years: HR = 1.16; 95% CI: 0.80–1.70). Statistical precision of risk estimates was limited. Results were consistent after external adjustment for unmeasured confounding by smoking.
Interpretation: Full truncal vagotomy is associated with a decreased risk for subsequent PD, suggesting that the vagal nerve may be critically involved in the pathogenesis of PD.

ANN NEUROL 2015;78:522–529

Parkinson's disease (PD) is the second-most common neurodegenerative disorder. It primarily affects the elderly, and incidence rates are projected to double between 2005 and 2030.[1,2] Etiology of PD is assumed to be multifactorial, but is not well understood.[3] Findings are inconsistent for environmental factors associated with PD. A potential protective effect of smoking has been suggested,[3] but this association has recently been questioned.[4] There are also suggestions of a protective effect of coffee and nonsteroidal anti-inflammatory drugs and increased risk with pesticide exposure.[3,5]

The "dual hit" hypothesis for PD development posits that a neurotropic pathogen enters the brain by a nasal and/or gastric route by axonal transport through the vagal nerve.[6,7] A central tenet of the dual hit hypothesis is that after penetration of the epithelial lining, a pathogen could enter the preganglionic parasympathetic motor neurons of the vagal nerve after trans-synaptic transmission from the axon of Meissner's plexus.[6,7] There is evidence from experimental animal models that alpha-synuclein (α-Syn) forms can be transmitted to the brain from the gut.[8–10] In one study, investigators instilled rotenone into the stomach of mice and observed progressive pathological α-Syn inclusions in the enteric nervous system, the vagal nerve, and, subsequently, the brainstem.[10] Additionally, vagotomy has been shown to eliminate transport of pathological proteins from the gut to the central nervous system (CNS).[11,12]

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Vagotomy, a surgical procedure in which the vagus nerve is resected, was previously a common treatment for peptic ulcer.[13] Two primary types of vagotomy were performed: full truncal vagotomy, in which both vagal trunks were severed, and superselective vagotomy, in which only the nerves supplying the fundus and body of the stomach were resected. Thus, if the dual hit hypothesis is correct, truncal vagotomy would be expected to confer a protective effect on subsequent PD risk, whereas superselective vagotomy would be associated with a minor or no protective effect. The temporal relationship between a putative neurotropic assault on the gastrointestinal mucosa and subsequent spread through the vagus is unknown, but a nonmotor preclinical phase of more than 10 to 20 years has been suggested.[14]

In the present population-based cohort study using prospectively collected registry data, we investigated the risk of PD in patients who previously underwent vagotomy, comparing the risk in truncal vagotomy patients versus superselective vagotomy patients directly, and in relation to matched population cohorts.

Materials and Methods

We conducted this population-based registry-linkage cohort study in Denmark, which has universal tax-supported health care, including free access to hospital care. All residents are assigned a unique personal identification number, registered in the Danish Civil Registration System,[15] which permits unambiguous individual-level linkage among all Danish registries.

Vagotomy and Comparison Cohorts

We assembled a cohort of all patients who underwent vagotomy between January 1, 1977 and December 31, 1995. These patients were identified from the Danish National Patient Registry (DNPR), which has recorded all diagnoses and procedures associated with inpatient hospitalizations in Denmark since 1977 and all hospital outpatient clinic visits since 1995.[16] Diagnoses were coded according to the International Classification of Diseases (ICD), 8th edition, from 1977 to 1993, and 10th, edition since 1994.[17] All diagnostic codes used in the study are provided in the Supporting Appendix. We obtained information on whether vagotomies were truncal (including selective vagotomies requiring pyloroplasty) or superselective gastric procedures.[13]

We used the Danish Civil Registration System to assemble a matched general population comparison cohort of individuals, who had not undergone vagotomy as of the surgery date of the corresponding patient (the index date).[15] We randomly selected up to 10 persons from the general population for each patient who underwent vagotomy, matched on year of birth, gender, and index date.

PD

PD was identified by an in- or outpatient hospital clinic diagnosis in the DNPR. We excluded patients with a diagnosis of PD preceding the index date.

Possible Confounders

From the DNPR, we obtained information on comorbidities at baseline. We assessed the presence of the 19 major disease categories included in the Charlson Comorbidity Index (CCI) for each vagotomy patient and general population comparison cohort member, based on their complete hospital in- and outpatient contact history before the index date. The CCI includes a number of comorbidities that may affect the risk of both PD and peptic ulcer, through associated inflammation, increased nonsteroidal anti-inflammatory drug use, or through chronic obstructive pulmonary disease resulting from smoking. We thus obtained data on such important comorbidities as chronic pulmonary disease, rheumatoid arthritis, and other connective tissue diseases, previous cardiovascular or cerebrovascular diseases, and diabetes.[3,18–20] Based on the CCI score, we defined three comorbidity levels: low (score of 0); medium (score of 1–2); and high (score of 3+). We excluded peptic ulcer from the CCI because it is the main indication for vagotomy and likely to be present in almost all patients undergoing this procedure.[13] We also collected DNPR data—in addition to the rheumatological diseases included in the CCI—on other arthritis/arthrosis diagnoses (yes/no) (see Supporting Appendix 1 for diagnosis codes).

Follow-up

We followed all cohorts from the index date until emigration, death, December 31, 2012, or diagnosis of PD, whichever came first. General population comparison cohort members who underwent vagotomy during follow-up were censored and switched to the vagotomy cohort.

Statistical Analyses

We characterized the vagotomy and general population comparison cohorts according to gender, age group (15–39, 40–64, 65–79, or 80+ years), year of index date, and comorbidities. We used Kaplan-Meier analysis to construct cumulative incidence curves, considering death as a competing risk.

In the first analyses, we used Cox proportional hazards models to compute hazard ratios (HRs) for PD and associated 95% confidence intervals (CIs), comparing patients with truncal and superselective vagotomy. Because the power of this analysis was low, we performed

a second analysis comparing the vagotomy cohort with members of the matched general population comparison cohort, stratified by type of vagotomy (truncal or superselective). Because the proportional assumptions hazard was not fulfilled during the first 5 years of follow-up, we stratified analyses by >5 and <5 years of follow-up. Because >5-years is the biologically and etiologically interesting follow-up period, we created tables to describe characteristics of patients with more than 5 years of follow-up after the index date. This analysis was conducted with the matching dissolved, restricted to persons with follow-up time over 5 years (in order to increase precision of risk estimates) and controlling for the matching factors (age and gender) in the design.

We conducted further analyses adjusting for variables fulfilling potential confounder criteria (both associated with PD in the comparison cohort and with vagotomy). These included baseline presence of diabetes (yes/no), chronic pulmonary disease (yes/no), previous cardiovascular disease (yes/no), rheumatological disease or arthrosis (yes/no), and the remaining CCI disorders excluding peptic ulcer (CCI score of 0, 1–2, or 3+).

To test for a long preclinical phase, we selected the patients with a follow-up time >20 years since their vagotomy procedure and performed a similar analysis on this patient group.

To adjust for the potential residual or unmeasured effect of smoking, we implemented external adjustment,[21] assuming that the relative risk of PD in smokers is 0.53[22] and that the proportion of smokers in the unexposed cohort (i.e., in the general population of Denmark) was 60% in the 1970s, in accord with population surveys.[23]

This study was approved by the Danish Data Protection Agency (record no.: 1–16-02-1-08). Because this registry-based study did not involve patient contact, no separate permission from the Danish Scientific Ethical Committee was required, according to Danish legislation.

Results

Between 1977 and 1995, 14,883 patients underwent vagotomy in Denmark. Of these, 5,339 patients with truncal vagotomy had more than 5 years of follow-up after the index date, with 66,711 corresponding general population cohort members, and 5,870 patients with superselective vagotomy had more than 5 years of follow-up after the index date, with 60,500 corresponding comparison cohort members (Table 1).

As of the index date, median age among patients with truncal vagotomy cohort was 56 years and 27% were older than 65 years. Among superselective vagotomy patients, median age was 47 years, and only 10% were older than 65 years (Table 1). More members of the superselective vagotomy group were male (69% vs. 61%). Patients undergoing truncal vagotomy had a higher prevalence of comorbidity (CCI = 0: 4.0% vs. 1.6%), chronic pulmonary disease (2.4% vs. 1.4%), rheumatological disease (3.2% vs. 1.3%), and previous cardiovascular disease (4.8% vs. 1.8%).

In the first analysis, we observed 45 patients with PD among those with truncal vagotomy and 59 among those with superselective vagotomy. In the direct comparison between truncal and superselective vagotomy, patients treated by truncal vagotomy displayed an overall lower risk of PD, compared to those treated by superselective vagotomy, after adjusting for age and gender (adjusted HR = 0.85; 95% CI: 0.56–1.27) (Table 2). This risk level remained unchanged after adjustment for additional potential confounders (adjusted HR = 0.83; 95% CI: 0.56–1.25). After a follow-up period of over 20 years after the index date, the age- and sex-adjusted HR was 0.58 (95% CI: 0.28–1.20) (Table 3). No further adjustments were possible owing to nonconvergence of the model. The statistical precision of the estimates was limited, as observed from the wide associated CIs.

In the second analyses, comparing truncal and superselective vagotomy with matched comparison cohorts, there also were some notable differences between groups. Compared to the matched general population cohort, patients undergoing truncal vagotomy had a higher prevalence of diabetes (1.4% vs. 1.0%), chronic pulmonary disease (2.4% vs. 1.2%), rheumatological disease (3.2% vs. 1.9%), and previous cardiovascular disease (4.8% vs. 3.2%), whereas remaining comorbidity was similar (CCI = 0: 95.5% vs. 97.8%). Compared to the matched general population cohort, patients undergoing superselective vagotomy had a higher prevalence of chronic pulmonary disease (1.4% vs. 0.7%), rheumatological diseases (1.3% vs. 0.8%), and previous cardiovascular disease (1.8% vs. 1.4%), whereas remaining comorbidity again was similar (CCI = 0: 98.2% vs. 98.8%).

We observed an overall lower risk of PD among truncal vagotomy patients than in the general population cohort (unadjusted HR = 0.84; 95% CI: 0.63–1.14). This risk level remained unchanged after adjustment for potential confounders (adjusted HR = 0.85; 95% CI: 0.63–1.14), although statistical precision was limited (Table 2). After a follow-up period of over 20 years after the index date, incidence rate was 0.65 per 1,000 person-years (pyr) among truncal vagotomy patients and 1.28 per 1,000 pyr in their comparison cohort, corresponding to an unadjusted (and adjusted) HR of 0.53 (95% CI: 0.28–0.99; Table 3). Cumulative PD risks among patients undergoing truncal vagotomy and in the matched general population comparison cohort are shown in Figure 1.

TABLE 1. Baseline Characteristics of Danish Patients Who Underwent Truncal and Superselective Vagotomy in 1977–1995 and a Matched General Population Comparison Cohort, With Over 5 Years of Follow-up After the Surgery/Index Date

	Truncal Vagotomy				Superselective Vagotomy			
	Comparison Cohort (N = 66,711)		Truncal Vagotomy (N = 5,339)		Comparison Cohort (N = 60,500)		Superselective Vagotomy (N = 5,870)	
Sex								
Female	26,780	(40.1)	2,107	(39.5)	18,742	(31.0)	1,819	(31.0)
Male	39,931	(59.9)	3,232	(60.5)	41,758	(69.0)	4,051	(69.0)
Age, yr								
15–39	7,938	(11.9)	742	(13.9)	17,368	(28.7)	1,713	(29.2)
40–64	36,722	(55.0)	3,142	(58.8)	36,755	(60.8)	3,584	(61.1)
65–79	18,985	(28.5)	1,282	(24.0)	6,187	(10.2)	558	(9.5)
80+	3,066	(4.6)	173	(3.2)	190	(0.3)	15	(0.3)
Index date								
1977–1981	27,287	(40.9)	2,317	(43.4)	32,050	(53.0)	3,121	(53.2)
1982–1986	18,981	(28.5)	1,505	(28.2)	18,520	(30.6)	1,792	(30.5)
1987–1991	13,901	(20.8)	1,080	(20.2)	7,425	(12.3)	723	(12.3)
1992–1995	6,542	(9.8)	437	(8.2)	2,505	(4.1)	234	(4.0)
Hospital history of comorbidity (CCI score)								
0	65,225	(97.8)	5,097	(95.5)	59,790	(98.8)	5,766	(98.2)
1–2	1,377	(2.1)	214	(4.0)	665	(1.1)	94	(1.6)
3+	109	(0.2)	28	(0.5)	45	(0.1)	10	(0.2)
Diabetes								
No	66,024	(99.0)	5,263	(98.6)	60,127	(99.4)	5,835	(99.4)
Yes	687	(1.0)	76	(1.4)	373	(0.6)	35	(0.6)
Chronic pulmonary disease								
No	65,905	(98.8)	5,211	(97.6)	60,090	(99.3)	5,785	(98.6)
Yes	806	(1.2)	128	(2.4)	410	(0.7)	85	(1.4)
Any previous cardiovascular disease								
No	64,567	(96.8)	5,084	(95.2)	59,669	(98.6)	5,764	(98.2)
Yes	2,144	(3.2)	255	(4.8)	831	(1.4)	106	(1.8)
Rheumatological disease or arthrosis								
No	65,464	(98.1)	5,170	(96.8)	60,037	(99.2)	5,792	(98.7)
Yes	1,247	(1.9)	169	(3.2)	463	(0.8)	78	(1.3)
Peptic ulcer								
No	66,083	(99.1)	2,951	(55.3)	60,212	(99.5)	3,636	(61.9)
Yes	628	(0.9)	2,388	(44.7)	288	(0.5)	2,234	(38.1)

Data are number (%), unless otherwise specified.
CCI = Charlson Comorbidity Index.

TABLE 2. Incidence Rates, Hazard Ratios (HRs), and Associated 95% Confidence Intervals (CIs) for Parkinson's Disease, Comparing Patients Who Underwent Truncal Vagotomy and Superselective Vagotomy Individually, and With Matched General Population Cohort Over Time

		Incidence Rate per 1,000 Person-Years at Risk (95% CI)	Unadjusted[a] HR (95% CI)	Adjusted[b] HR (95% CI)
Superselective cohort	59	0.57 (0.43–0.73)	1	1
Truncal vagotomy cohort	45	0.65 (0.47–0.85)	0.85 (0.56–1.27)	0.83 (0.56–1.25)
Comparison cohort	860	0.86 (0.80–0.92)	1	1
Truncal vagotomy cohort	45	0.65 (0.47–0.85)	0.84 (0.63–1.14)	0.85 (0.63–1.14)
Comparison cohort	652	0.56 (0.52–0.61)	1	1
Superselective cohort	59	0.57 (0.43–0.73)	1.09 (0.84–1.43)	1.09 (0.84–1.43)

Data are number of events, unless otherwise specified.
[a]Adjusted for age and sex.
[b]Adjusted for age and sex, and for diabetes, rheumatological diseases, chronic pulmonary disease, cardiovascular disease, and other CCI diseases (excluding peptic ulcer).
CCI = Charlson Comorbidity Index.

Patients who underwent superselective vagotomy had a slightly higher risk of PD, compared to the general population cohort (unadjusted HR = 1.09; 95% CI: 0.84–1.43), again with limited statistical precision. The HR remained unchanged after adjustment (Table 2). Twenty or more years after surgery, the unadjusted HR was 1.16 (95% CI: 0.79–1.69). This finding was unchanged after adjustment (adjusted HR = 1.16; 95% CI: 0.80–1.70; Table 3). Figure 2 shows cumulative PD risks among patients who underwent superselective vagotomy and in the matched general population comparison cohort.

We externally adjusted for unmeasured confounding owing to smoking and risk of PD in the truncal vagotomy patients, compared to the general population cohort, after 20 years of follow-up, assuming that 60% of members of the population comparison cohort and 85% of patients in the vagotomy cohort smoked. This analysis yielded an adjusted relative risk (RR) of 0.66. The adjusted RR was 0.63 under the assumption that 80% of patients in the vagotomy cohort smoked and 0.61 under the assumption that 75% of patients in the vagotomy cohort smoked.

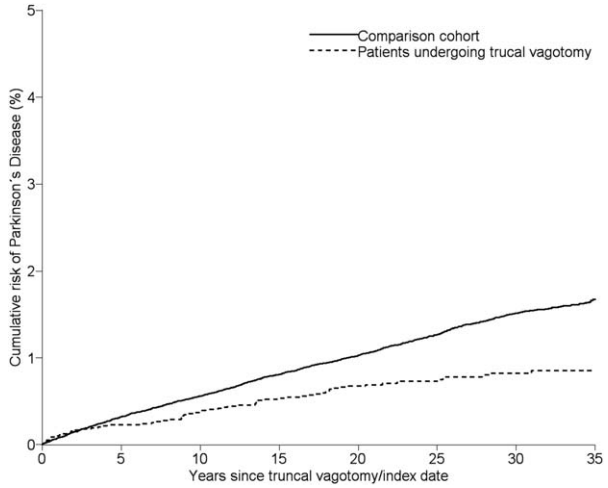

FIGURE 1: Cumulative incidence curves of Parkinson's disease for patients who underwent truncal vagotomy compared to a matched general population cohort.

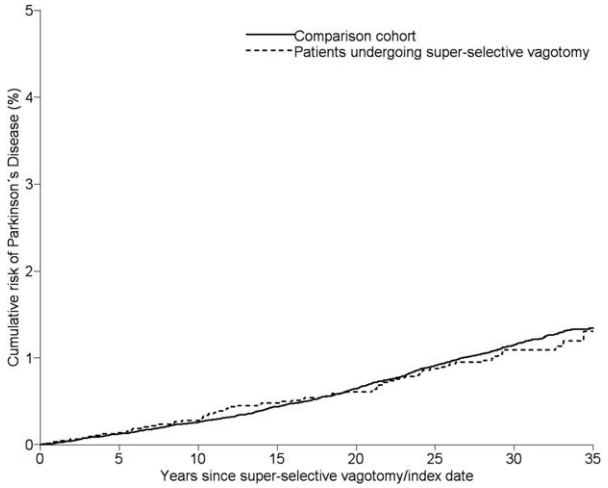

FIGURE 2: Cumulative incidence curves of Parkinson's disease for patients undergoing superselective vagotomy compared to a matched general population cohort.

TABLE 3. Incidence Rates, Hazard Ratios (HRs), and Associated 95% Confidence Intervals (CIs) for Parkinson's Disease, Comparing Patients Who Underwent Truncal Vagotomy and Superselective Vagotomy Individually, and With Matched General Population Cohort Over Time, for Patients With 20 Years Of-Follow up Postvagotomy

		Incidence Rate per 1,000 Person-Years at Risk (95% CI)	Unadjusted[a] HR (95% CI)	Adjusted[b] HR (95% CI)
Superselective cohort	29	0.96 (0.64–1.34)	1	
Truncal vagotomy cohort	10	0.65 (0.31–1.12)	0.58 (0.28–1.20)	
Comparison cohort	315	1.28 (1.15–1.43)	1	1
Truncal vagotomy cohort	10	0.65 (0.31–1.12)	0.53 (0.28–0.99)	0.53 (0.28–0.99)
Comparison cohort	319	0.87 (0.78–0.97)	1	1
Superselective cohort	29	0.96 (0.64–1.34)	1.16 (0.79–1.69)	1.16 (0.80–1.70)

Data are number of events, unless otherwise specified.
[a]Adjusted for age and sex.
[b]Adjusted for age and sex, and for diabetes, rheumatological diseases, chronic pulmonary disease, cardiovascular disease, and other CCI diseases (excluding peptic ulcer).
CCI = Charlson Comorbidity Index.

Discussion

This large-scale study found that patients who underwent truncal vagotomy, but not superselective vagotomy, and who survived 5 years postsurgery, had a clear reduction in the risk of a PD diagnosis, although the statistical precision of the estimates was limited and did not meet a statistically significant threshold. In the analyses comparing the patients undergoing truncal and superselective vagotomy to matched general population cohorts, the results were consistent, showing a significant reduction in PD among patients who underwent truncal vagotomy—especially when the follow-up period was extended to more than 20 years. These results are consistent with the long preclinical phase of PD.

It is important to realize that the seemingly higher PD incidence rate in the truncal vagotomy group (i.e., 0.65 in the truncal vagotomy group [>5–36 years] vs. 0.57 in the superselective group [>5–36 years]) was most likely owing to the differing age composition in the two groups, the median age being nearly 10 years higher in the truncal vagotomy group. With increasing follow-up time from the index date, the incidence rate in the truncal group remained stationary, whereas a marked increase in incidence rate was noted in the superselective group. When controlling for these age and sex differences, patients treated by truncal vagotomy persistently had lower risk for PD, compared to those treated by superselective vagotomy.

Our results therefore suggest that having an intact vagus nerve increases the risk of developing PD. The finding is in accord with a primary pathological process being initiated in the gastrointestinal mucosa, which then uses the vagus as a major entry point into the brain.[6,7] On the other hand, cumulative PD risk in the vagotomy group was not zero. Several explanations are possible. In some vagotomized PD patients, the putative ascending spread of misfolded proteins already may have started before the vagotomy index date, given that a prodromal phase of over 20 years is considered possible.[14] Another possibility is that the intruding pathogen reaches the CNS through alternative access points, such as the olfactory epithelium, which is strongly supported by nearly ubiquitous early involvement of the olfactory bulb and other olfactory structures.[6,7] Lewy neurites are also observed in sympathetic nerve terminals,[24–26] and distal axons of cardiac sympathetic nerves are known to degenerate before paravertebral ganglion neurons.[27,28] However, several large postmortem studies have reported that isolated α-Syn pathology in the spinal cord in the absence of concomitant cerebral pathology is extremely rare.[29,30] These findings suggest that spinal cord pathology could represent a "secondary descending feature," rather than an entry point, in the pathogenesis of PD.

Strengths of the study include the large sample size and nation-wide population-based design, in a setting with tax-funded universal health care and comprehensive

long-term follow-up, which reduced the potential for selection bias in the present study. The diagnostic accuracy of PD has been found to be high in Denmark, with 82% of diagnoses meeting strict clinical criteria for idiopathic PD in a previous validation study based on hospital diagnoses in the DNPR.[31] Still, the DNPR only records inpatient hospitalizations and hospital outpatient clinic visits for PD, and initial treatment may be given by a general practitioner or private neurologist. It is thus possible that the study only captured patients with more advanced disease. However, this potential source of error would apply equally to the comparison cohorts. Of note, the incidence observed in the general population cohort is comparable to other reports.[3,32]

The limitations pertaining to this study are related to the validity of registered diagnoses and confounding. Although surgical procedures are accurately coded,[33] no specific studies have been conducted on the validity of procedure codes for vagotomy. It is possible that a truncal vagotomy could be reported to the DNPR, when, in reality, the posterior or anterior vagal trunk was not resected. Similarly, if a superselective vagotomy was reported, but not all nerve endings were cut, this misclassification also would bias the results toward the null.

Confounding by measured comorbid disease was minimized in our study, but the lack of data on smoking history nevertheless raises some concern. Smoking is associated with increased risk of peptic ulcer[19] and, at the same time, may protect against PD[3] (although it has recently been suggested that the apparent protective effect of smoking on PD may be explained by reverse causation).[4] Controlling for chronic pulmonary diseases in our study may have removed possible confounding by smoking to some degree. Importantly, in the direct comparison of patients with truncal and superselective vagotomy, we assume similar background characteristics and thus lower confounding. Moreover, external adjustment for unmeasured confounding showed somewhat attenuated, but consistent, results.

In conclusion, full truncal vagotomy was associated with a decreased risk for subsequent PD, suggesting that the vagal nerve may be critically involved in the pathogenesis of PD; the results are consistent with a long preclinical phase. This intriguing finding suggests that an intact vagal nerve is important in the pathogenesis of PD. Given the wide-ranging implications for our understanding of PD pathogenesis, independent verification of our findings is warranted.

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Authorship

P.B. and J.C.D. conceived the idea for the study and developed this idea and the study design with H.T.S. E.S., L.A.P., R.W.T., and H.T.S. participated in the study design and E.H.P. performed the statistical analysis. All authors contributed to the drafting and revision of the current manuscript, final approval of this version, and agreed to be accountable for all aspects of the work.

Potential Conflict of Interests

Nothing to report.

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Dietary Plant Lectins Appear to Be Transported from the Gut to Gain Access to and Alter Dopaminergic Neurons of *Caenorhabditis elegans*, a Potential Etiology of Parkinson's Disease

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Lectins from dietary plants have been shown to enhance drug absorption in the gastrointestinal tract of rats, be transported trans-synaptically as shown by tracing of axonal and dendritic paths, and enhance gene delivery. Other carbohydrate-binding protein toxins are known to traverse the gut intact in dogs. Post-feeding rhodamine- or TRITC-tagged dietary lectins, the lectins were tracked from gut to dopaminergic neurons (DAergic-N) in transgenic *Caenorhabditis elegans* (*C. elegans*) [*egls1*(*Pdat-1:GFP*)] where the mutant has the green fluorescent protein (GFP) gene fused to a dopamine transport protein gene labeling DAergic-N. The lectins were supplemented along with the food organism *Escherichia coli* (OP50). Among nine tested rhodamine/TRITC-tagged lectins, four, including *Phaseolus vulgaris* erythroagglutinin (PHA-E), *Bandeiraea simplicifolia* (BS-I), *Dolichos biflorus* agglutinin (DBA), and *Arachis hypogaea* agglutinin (PNA), appeared to be transported from gut to the GFP-DAergic-N. *Griffonia Simplicifolia* and PHA-E, reduced the number of GFP-DAergic-N, suggesting a toxic activity. PHA-E, BS-I, *Pisum sativum* (PSA), and *Triticum vulgaris* agglutinin (Succinylated) reduced fluorescent intensity of GFP-DAergic-N. PHA-E, PSA, *Concanavalin A*, and *Triticum vulgaris* agglutinin decreased the size of GFP-DAergic-N, while BS-I increased neuron size. These observations suggest that dietary plant lectins are transported to and affect DAergic-N in *C. elegans*, which support Braak and Hawkes' hypothesis, suggesting one alternate potential dietary etiology of Parkinson's disease (PD). A recent Danish study showed that vagotomy resulted in 40% lower incidence of PD over 20 years. Differences in inherited sugar structures of gut and neuronal cell surfaces may make some individuals more susceptible in this conceptual disease etiology model.

Keywords: ***Caenorhabditis elegans*, dopaminergic neurons, dopamine transporter, fluorescence, plant lectins**

INTRODUCTION

Could dietary plant proteins, such as lectins, traverse the gut intact, with vesicular transfer to neurons and be transported intact along axons to affect dopaminergic neurons (DAergic-N) as one etiology of Parkinson's disease (PD)? A recent Danish study showed that patients who had vagal nerves removed 20 years ago had a 40% lower incidence of PD (1). Some reports claim that vegetarians have higher rates of PD (2, 3). This study uses *C. elegans* as a model to investigate dietary lectin transport to DAergic-N.

Plant lectins were discovered over a century ago (4). Toxicity of some lectins was first recognized, independently, by Bruylants and Vennemann (5); Warden and Waddell (6) [described by Oppenheimer (7) and Dixson (8)]. Lectins' hemagglutination properties were found by Stillmark (9), and a general recognition of antigenicity by lectins was revealed by Paul Ehrlich in 1890 (10) who won a Nobel Prize in Physiology or Medicine 1908 "*in recognition of their work on immunity*"[1]. Thereafter, lectins' "immune recognition" was used for immunological research [see Textbook of Military Medicine (11)]. In 1919, Sumner crystallized (*Canavalia ensiformis*, Concanavalin A) (12). A half century later, investigators began to determine ABO-blood subtypes due to their sugar-binding properties, and the name "lectins" was formally coined (13, 14). Recent studies report that lectins play important roles in plant defense (15) and legume–rhizobial interactions (16).

Plants contain glycoprotein-lectins ("non-immune sugar-binding proteins") in seeds, fruits, and nuts (2), and recognize and reversibly bind specific carbohydrates (17). They are involved in plant defense (15) and legume–rhizobia (16). Upon consumption by animals, they resist gut proteolytic enzymes, maintaining function under adverse gastrointestinal (GI) conditions (18, 19). They can penetrate the GI tract wall by endocytosis (20), probably by first binding a carbohydrate lectin receptor (21). Astonishingly, intact lectins can transfer trans-synaptically in an antegrade and/or retrograde fashion along nerve fibers (17, 22). Their medical importance is increasingly being recognized by being conjugated with drugs for better drug absorption from the GI tract (21, 23–25). Particularly relevant to the current studies, lectins have been utilized extensively for neuronal tracing studies [see review (22, 26)]. Ricin (*Ricinus communis*) as an extremely cytotoxic lectin has been studied extensively for its function in retrograde transport, *via* a galactose-binding β-chain-mediated endocytosis, following translocation of the enzymatically active and toxic A-chain, from the endosomes to the Golgi apparatus (27, 28). This property has been utilized for treatment of malignancies at low doses (29, 30). Lectins have also been conjugated with DNA for enhanced nervous system gene delivery (31). Most dietary plant lectins resist gut proteolytic enzymes and maintain function under usually adverse conditions for proteins (18, 19). Non-toxic lectins, such as tomato lectin and wheat germ agglutinin, are suggested to show growth factor activity in the GI tract (18). Bacteria or parasitic protozoa, through their own lectins, attach to carbohydrate receptors on epithelial cells to colonize the GI and genito-urinary tracts. Some lectins are synergistically toxic both locally and systemically to experimental animals (18). Kidney bean lectin (PHA), for example, damages intestinal epithelial cells, causes bacterial overgrowth, and induces nutritional disorders, effects which are preventable by inhibition with the specific sugars that have competitive binding capacities to lectins by sharing similar terminal structures (18, 32). Likewise, dietary saccharides or glycoconjugates, such as probiotic agents and milk oligosaccharides, may act as receptor analogs or decoys to selectively and competitively reduce lectin binding (18, 33, 34). Soybean lectin has shown potential anticarcinogenic effects (35).

Complex environmental factors play important roles for neurodevelopmental and neurodegenerative disorders, including PD (36, 37). Controversial reports suggest that a higher prevalence of PD occurs in vegetarians compared to omnivores (3, 38). In equine Parkinsonism, consuming yellow star thistles (*Centaurea solstitialis*) or Russian knapweed (*Acroptilon repens*) causes liquid necrosis in the *substantia nigra pars reticulata* and the *globus pallidus* by destroying DAergic-N, developing nigropallidal encephalomalacia (NPE), and creating histopathological features that resemble human idiopathic PD (39). These observations suggest transport of toxic substances from the horse gut to brain neurons. To date, however, in humans, epidemiology has not proven dietary lectins to have a significant impact on neuronal degenerative diseases. Signature pathologies of PD, e.g., Lewey's bodies and aggregated alpha-synuclein (α-SYN) occur in neurons of the enteric nervous system of the GI wall, in addition to the neurons of the central nervous system (CNS) (40). α-SYN also aggregates in microglia and further leads to PD though the detailed mechanism remains unclear (41, 42), while astrocytes convert neurotoxin MPTP to its active metabolite MPP^+ (43). The findings reported here support Braak and Hawkes' hypothesis that the GI tract may be a potential site of neuronal invasion by an "unknown etiologic agent," potentially responsible for causing some percentage of PD (40, 44–47). It is suggested herein that one possible etiologic agent could be dietary lectins.

Caenorhabditis elegans has a high conservation (>65%) of human disease-associated genes (48, 49). A total of eight DAergic-N in the hermaphrodite *C. elegans* (50–52) respond to signals from environmental mechano-sensory stimuli, e.g., exhausted food supply, which have offered molecular, genetic, and behavioral tools to aid human disease studies (53–57). *C. elegans* modulates locomotion behavior by using dopamine and serotonin to mediate motor circuits in chemical synapses, gap junctions, and neuromuscular junctions (58–60). Intestinal muscle cells are innervated by pharyngeal motor neurons and bioaminergic neurons *via* the preanal ganglia. Structures, sensory-motor synapses, gap junction contacts, and activities all resemble those in the mammalian GI tract (61, 62).

The features of the green fluorescent protein (GFP)-dopamine transporter (DAT) fusion protein *C. elegans* [*egIs1*(*Pdat-1:GFP*)] were evaluated by the numbers, fluorescent intensity, and sizes of GFP-DAergic-N in this study. Meanwhile, TRITC-labeled lectins were also followed post-feeding to establish the ability of lectins to bind or penetrate the GI wall or nerve cells. The question was whether dietary plant lectins can be transported

[1]http://www.nobelprize.org/nobel_prizes/medicine/laureates/1908/

to, and impair or alter apparently DAergic-N. Differences in inherited sugar structures of gut and neuronal cell surface may make some individuals more susceptible in this conceptual disease model.

MATERIALS AND METHODS

Caenorhabditis elegans (*egIs1*[*Pdat-1:GFP*]) that express GFP in the eight DAergic-N (63, 64) and the standard food *Escherichia coli* (*E. coli*) were obtained from *C. elegans* Genetics Center (CGC, MN). The *C. elegans* model does not require regulation of the Institutional Animal Care and Use Committee (IACUC).

C. elegans Culture

Caenorhabditis elegans were egg-synchronized, fed *ad libitum* with LB broth (200 µl/agar plate or 250 µl/well) containing OP50 5×10^8–5×10^{11} cfu/ml (65), grown in nematode growth media (NGM) agar plates (∅35 mm, 3 ml) and transported to new plates every other day ($n = 20$). Or, seeded in 96-well plate ($n = 10$–15/well) grown in liquid culture supplemented with fluorodeoxyuridine (FUDR, 0.6 mM/30 µl) (66). The plate was tape sealed, bagged, and covered with aluminum foil, and kept in a 20°C low temperature incubator (Revco Tech., Nashville, NC, USA) throughout the experiments.

All treatments were applied at day 3 after hatching. Four dose–responses of nine lectins were obtained for each culture condition in a dark room. Control animals were fed with OP50. Experimental groups were fed TRITC/rhodamine-conjugated lectins. The lectins were incorporated into feeding medium with OP50. Two hundred microliter treatments were added into each fresh agar dish prior to the transfer or 5 µl/well/week to liquid culture. Each group of nematodes was collected and fixed by 15–20 days for the agar dish culture as previously described (67), after the first week for the liquid culture.

Culture of *Escherichia coli* (OP50)

OP50 (10 µl) and streptomycin (10 µl/ml) were mixed with LB Broth (100 ml, see below) for 16 h at 37°C in an incubator and stored at 4°C for up to 3 months.

Select Lectins

Commercially available plant lectins conjugated to TRITC or rhodamine were from EY labs (San Mateo, CA, USA), Vector Labs (Burlingame, CA, USA), or Sigma-Aldrich (St. Louis, MO, USA). Doses of lectins (millimolar) used were comparable to those used in published work in neuronal tracing (17, 22, 26).

Average Probability of Survival Assay

All average probability of survival (APS) assays was conducted in liquid culture (96-well plate). The animals were synchronized and seeded into each well of a plate ($n = 10$–15) and OP50 was added to each well. Thirty microliters (0.6 mM) of FUDR were added to each well to sterilize the animals. Four different treatments of lectins (50 µl/treatment, $n = 6$ row) were added. The plate was then covered with aluminum foil. The whole procedure was performed in a dark room to prevent bleaching of fluorophores. The survival animals were counted every other day until all were dead.

Fluorescent Microscopy

The GFP-DAergic-N were identified by FTIC filter (480Ex/520Em) and the number of GFP-DAergic-N counted. Fluorescent intensity of GFP-DAergic-N and their average sizes (square micrometer) were determined by NIS-Elements Advanced Research (version 3.22.11) and compared among the following groups: control and lectins. Fluorescent intensity of rhodamine-lectins was determined by a TRITC filter (580Ex/620Em) to assess co-localization. The magnitude of the effect(s) of the lectin on the DAergic-N, the number, fluorescent intensity (arbitrary unit), and sizes (square micrometer) of GFP-DAergic-N were determined and compared among each group. Co-localization was initially identified with an inverted microscopy (Nikon, Eclipse Ti–S, Japan) and then confirmed at a *Z*-axle with laser scanning microscopy (Leica, TCS SP5, Germany).

Solutions and Chemicals

Standard *NGM agar plates* (g)*:* NaCl 3.0 g, Bacto-agar (Becton, MD, USA) 20 g, Bacto-peptone 2.5 g (Becton, MN, USA), Cholesterol solution 0.1% (0.005/ml 95% ethanol), and dH_2O 975ml were mixed. Additions to the autoclaved solution (M): $CaCl_2$ 1.0 1 ml, $MgSO_4$ 1.0 1 ml, KPO_4 pH6 1.0 25 ml. *LB Broth*: 25.0 g, dH_2O 1 l (autoclave). *S-basal solution* (M)*:* NaCl 0.1, KPO4 pH6 0.05, Cholesterol 0.1%, was autoclaved. *PBS* (millimolar)*:* 115 NaCl, 75 $Na_2HPO_4 \bullet 7H_2O$, and 7.5 KH_2PO_4, pH 7.4.

Statistical Analyses

Analyses were carried out using SAS/STAT® software, Version 9.4 of the SAS System for Windows (Cary, NC, USA). All results were expressed as mean ± SEM. Survival curves were displayed by binomial probabilities obtained from logistic regression models as surrogates for survival probabilities and mean lifespan was estimated via Kaplan–Meier (log rank). ANOVA models were used to analyze fluorescence intensity data. For each group, 20 animals were analyzed for agar culture and 10–15 animals were analyzed for liquid culture. Statistical significance was defined as $P < 0.05$.

RESULTS

Diets supplemented with varying concentrations of rhodamine-labeled lectins *Phaseolus vulgaris* erythroagglutinin (PHA-E), *Bandeiraea simplicifolia* (BS-I), or *Dolichos biflorus* agglutinin (DBA) in agar dish, or TRITC-conjugated *Arachis hypogaea* agglutinin (PNA) in liquid culture were fed to *C. elegans,* and subsequently detected by fluorescence microscopy associated with GFP-DAergic-N (**Table 1**). The only explanation for this observation is that rhodamine- or TRITC-labeled lectins traveled in some manner from the gut to the neurons. We observed that some lectins had the following effects: (a) reducing the number of DAergic-N, (b) decreasing fluorescent intensity of GFP-expressing neurons (less GFP-DAT), or (c) altering neuron size. GSL-I, Con A, *Pisum sativum* (PSA), WGA, or S-WGA were not detected as transported to neurons, but nevertheless, some had significant effects on the neuron measurements, possibly indicating that undetectable amounts of these lectins caused the effects,

TABLE 1 | Lectins detected in the neurons by co-localization.

Lectins	Dose (mM)	GFP #	GFP intensity	GFP size
Phaseolus vulgaris (PHA-E)-rhodamine	2.0×10^{-4}	$P > 0.05$	$P < 0.01$	$P > 0.05$
	6.0×10^{-4} ↓	$P > 0.05$ ↓	$P < 0.01$ ↓	$P > 0.05$ ↓
	2.0×10^{-3}	$P < 0.01$	$P < 0.01$	$P < 0.01$
Bandeiraea simplicifolia (BS-I)-TRITC	5.3×10^{-4} ↓	$P > 0.05$ ↓	$P < 0.01$ ↑	$P < 0.01$
	1.8×10^{-3}	$P > 0.05$	$P < 0.01$	$P < 0.01$
Dolichos biflorus (DBA)-rhodamine	1.0×10^{-3} ↔	$P > 0.05$ ↔	$P > 0.05$ ↔	$P > 0.05$
	3.3×10^{-3}			
Arachis hypogaea agglutinin (PNA)-TRITC	1.8×10^{-5}	$P > 0.05$	$P < 0.01$	$P > 0.05$
	5.4×10^{-5} ↓	$P > 0.05$ ↓	$P > 0.05$ ↓	$P > 0.05$ ↓
	1.8×10^{-4}	$P < 0.01$	$P < 0.01$	$P < 0.01$

↓ *Decreasing trend.*
↑ *Increasing trend.*
↔ *No significant alternation.*

or that some unexplained secondary effect of the lectins caused pathological effects.

Lectins Co-Localized with the GFP-DAergic Neurons

Phaseolus vulgaris erythroagglutinin-rhodamine co-localized with GFP-DAergic-N within 2 weeks after feeding (**Figure 1**). The number of GFP-DAergic-N was reduced in a dose-dependent manner ($P < 0.01$, **Figure 1D**). PHA-E-rhodamine co-localized to a subgroup of GFP-DAergic-N (**Figure 1B**). The fluorescence intensity of GFP-DAergic-N was decreased dose-dependently ($P < 0.01$, **Figure 1E**), suggesting a diminution of the GFP-DAT. The average size of GFP-DAergic-N was also reduced at the highest dose ($P < 0.01$, **Figure 1F**). PHA-E-rhodamine fluorescence image size was inversely proportional to the number, average intensity, and average size of the GFP-DAergic-N. The APS was increased dose-dependently (**Figure 1G**). The mean lifespan was increased at a medial dose (5.4×10^{-5} mM) from 17 to 23 days (39%, $P < 0.05$, **Figure 1H**).

Bandeiraea simplicifolia–TRITC (Sigma-Aldrich) co-localized with GFP-DAergic-N (**Figure 2**). The number of the GFP-DAergic-N was reduced in a dose-dependent trend ($P > 0.05$, **Figure 2D**). The fluorescence intensity of GFP-DAT protein in DAergic-N was dose-dependently reduced ($P < 0.01$, **Figure 2E**). The size of GFP-DAergic-N was elevated ($P < 0.01$, **Figure 2F**). The APS was dose-dependently decreased at all doses (**Figure 2G**). Mean lifespan was decreased at the highest dose (1.8×10^{-4} mM) from 17 to 10 days (−37%, $P < 0.05$, **Figure 2H**).

Griffonia Simplicifolia I (GSL-I)-rhodamine (Vector) did not show fluorescence co-localization, and did not affect the number or size of GFP-DAergic-N (**Figures 3A,C**). The fluorescence intensity of the neuron was increased at all doses (**Figure 3B**).

Dolichos biflorus agglutinin-rhodamine co-localized with GFP-DAergic-N within 3 days after feeding. In aged nematodes, 50% of neurons co-localized with DBA-rhodamine at high doses (**Figure 4**). The number (**Figure 4G**), fluorescence intensity (**Figure 4H**), or size (**Figure 4I**) of DAergic-N were not altered ($P > 0.05$). In 2-day-old animals, the number of GFP-DAergic-N was similar to aged animals and 65–80% co-localized with DBA-rhodamine (**Figures 4E,G**). The size of GFP-DAergic-N was reduced in a dose-dependent trend at higher doses of DBA-rhodamine ($P > 0.05$) (data not shown). The fluorescence intensity and size of GFP-DAergic-N in 2-day-old animals were similar to aged animals (**Figure 4E,F**). The APS was increased at lower doses of DBA, and decreased at higher doses (**Figure 4J**). Mean lifespan was reduced at the highest dose (1.8×10^{-4} mM) from 19 to 11 days (−43%, $P < 0.05$, **Figure 4K**). Although some experiments were done adding lectin-specific inhibitory sugars to the medium for mitigation of the lectin effects, very high concentrations, up to 200 mM were needed to show any effects, and there were concerns about pleiotropic effects of these sugars fed at high amounts. For example, the animals died in presence of GalNAc (50 mM) within 2 days, possibly because of uridine diphosphate (UDP) depletion (68). Another example of problems with dosing live animals with dietary sugars to offset the lectin effects was that the mean lifespan was reduced by galactose (200 mM) from 19 to 12 days (−36%, $P < 0.05$, **Figure 5H**). Use of high concentrations of sugars on whole animals had their own effects. Therefore, we have not included data herein using lectin-inhibitory sugars.

Arachis hypogaea agglutinin (PNA)-TRITC co-localized with GFP-GAergic neurons after 1-week treatment (1.8×10^{-5}, 5.4×10^{-5}, 1.8×10^{-4} mM, **Figure 5**). The size and intensity of GFP-GAergic neurons was not altered. The number of GFP-GAergic neurons was increased at low dose (1.8×10^{-5} mM, $P < 0.05$) and reduced at higher doses. APS was dose-dependently reduced at all doses (**Figure 5G**). Mean lifespan was reduced by higher doses (5.4×10^{-5} mM and 1.8×10^{-4} mM) from 19 to 15 and 14 days (−24 and −27%, $P < 0.05$, **Figure 5H**), with an increase at the highest dose (1.8×10^{-4} mM) from 12 to 16 days (33%, $P < 0.05$).

Lectins Altered the GFP-DAergic Neurons Without Co-Localization

Lectins that alter number, GFP-intensity, or size of neurons without observed co-localization are given in **Table 2**.

Pisum sativum-rhodamine feeding did not reduce the number of GFP-DAergic-N ($P > 0.05$, **Figure 6A**). However, the

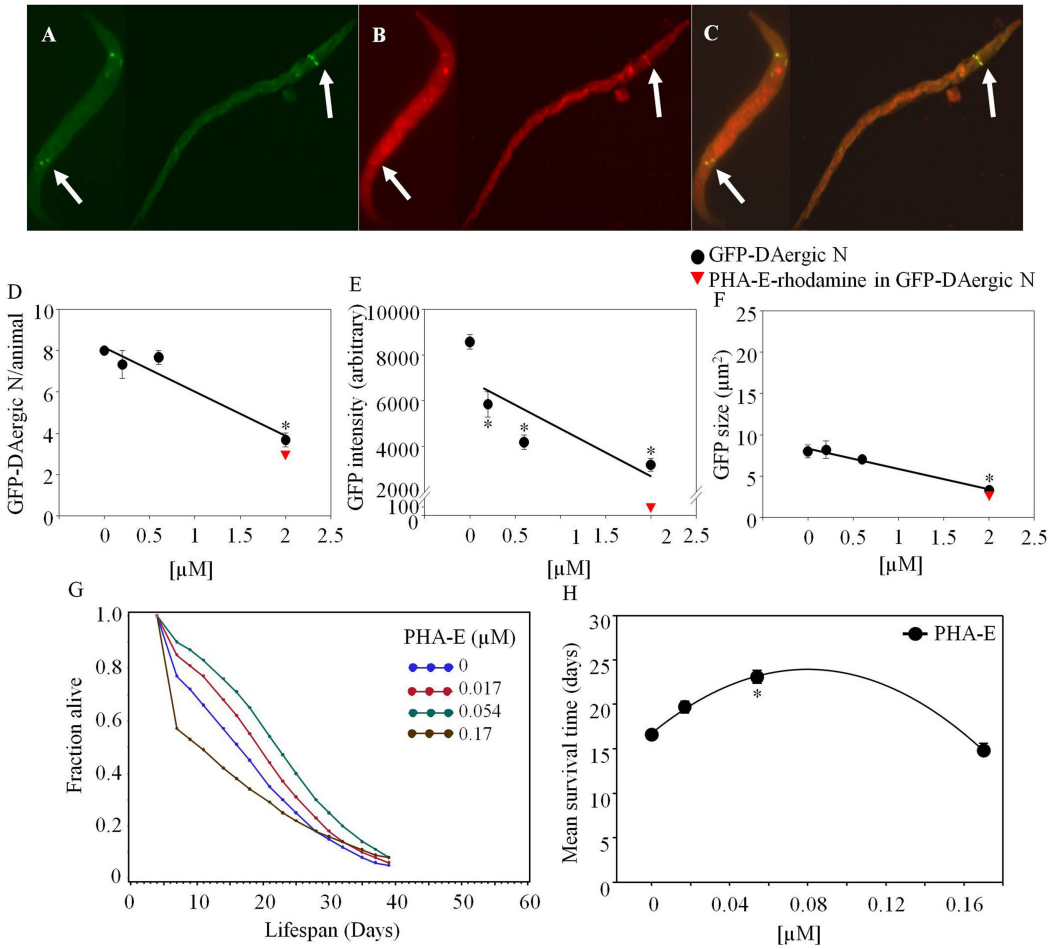

FIGURE 1 | *Phaseolus vulgaris* (PHA-E)-rhodamine co-localized and diminished GFP-DAergic neurons in *C. elegans* post feeding. (A) GFP-DAergic neurons. **(B)** PHA-E-rhodamine in several GFP-DAergic neurons. **(C)** Merged **(A,B)** showing co-localization of the PHA-E-rhodamine with GFP-DAergic neurons. **(D)** Dose-dependent reduction of the number of GFP-DAergic neurons per animal: 4.4 ± 0.3 (2×10^{-3} mM, $n = 3$, $P < 0.01$). **(E)** Dose-dependent reduction of fluorescence intensity per GFP-DAergic neurons: 5,837 ± 556 (2×10^{-4}–2×10^{-3}mM, $n = 3$, $P < 0.01$). **(F)** Size reduction of GFP-DAergic neurons: 3.3 ± 0.4 μm² (2×10^{-3} mM, $n = 3$, $P < 0.01$). **(G)** The APS was increased dose-dependently. **(H)** Mean survival time of each group. *indicates statistical significance.

fluorescence intensity ($P < 0.001$) and size ($P > 0.05$) of GFP-DAT in DAergic-N were dose-dependently decreased with PSA feeding ($P = 0.8$, **Figures 6B,C**). In liquid culture, the number of the GFP-DAergic-N was not altered (**Figure 6D**), the intensity was diminished at high dose (4.3×10^{-4} mM, $P < 0.05$, **Figure 6E**), and the size was reduced at low dose (4.3×10^{-5} mM, $P < 0.05$, **Figure 6F**). The APS was increased at all doses (**Figure 6G**). Mean lifespan was increased by lower doses (4.3×10^{-5} mM and 4.3×10^{-4} mM) from 22 to 27 days (22 and 23%, $P < 0.05$, **Figure 6H**).

Concanavalin A (Con A)-TRITC appeared to have a mild effect on the GFP-DAergic-N reducing the number of GFP-DAergic-N in a dose-dependent trend (**Figure 7A**). The fluorescence intensity of GFP-DAT protein image in DAergic-N was dose-dependently increased by Con A feeding ($P < 0.05$, **Figure 7B**). The apparent size of GFP-DAergic-N was significantly reduced ($P < 0.05$, **Figure 7C**). Con A-TRITC did not alter the number of GFP-DAergic-N in liquid culture ($P > 0.05$, **Figure 7D**), increased the intensity at high dose (1.9×10^{-4} mM, $P < 0.05$, **Figure 7E**), and decreased the size of neuron at middle dose (5.7×10^{-5} mM, $P < 0.05$, **Figure 7F**). The APS was increased at all doses (**Figure 7G**). Con A did not affect the mean lifespan (**Figure 7H**).

Triticum vulgaris (WGA)-rhodamine or WGA-TRITC was not detected as transported (**Figure 8**). The intensity of DAergic-N was increased ($P < 0.05$, **Figure 8A**), while the size of the neurons was decreased ($P < 0.05$) at the highest dose (4.6×10^{-4} mM, **Figure 8B**). The APS was increased at all doses (**Figure 8D**). Mean lifespan was increased at the highest dose (4.6×10^{-4} mM) from 20 to 24 days (22%, $P < 0.05$, **Figure 8E**).

Triticum vulgaris (Succinylated) S-WGA-TRITC feeding had a similar effect as WGA-rhodamine. The number of DAergic-N did not show a significant change (**Figure 9A**), however, fluorescent intensity of the GFP-DAergic-N was decreased ($P < 0.05$,

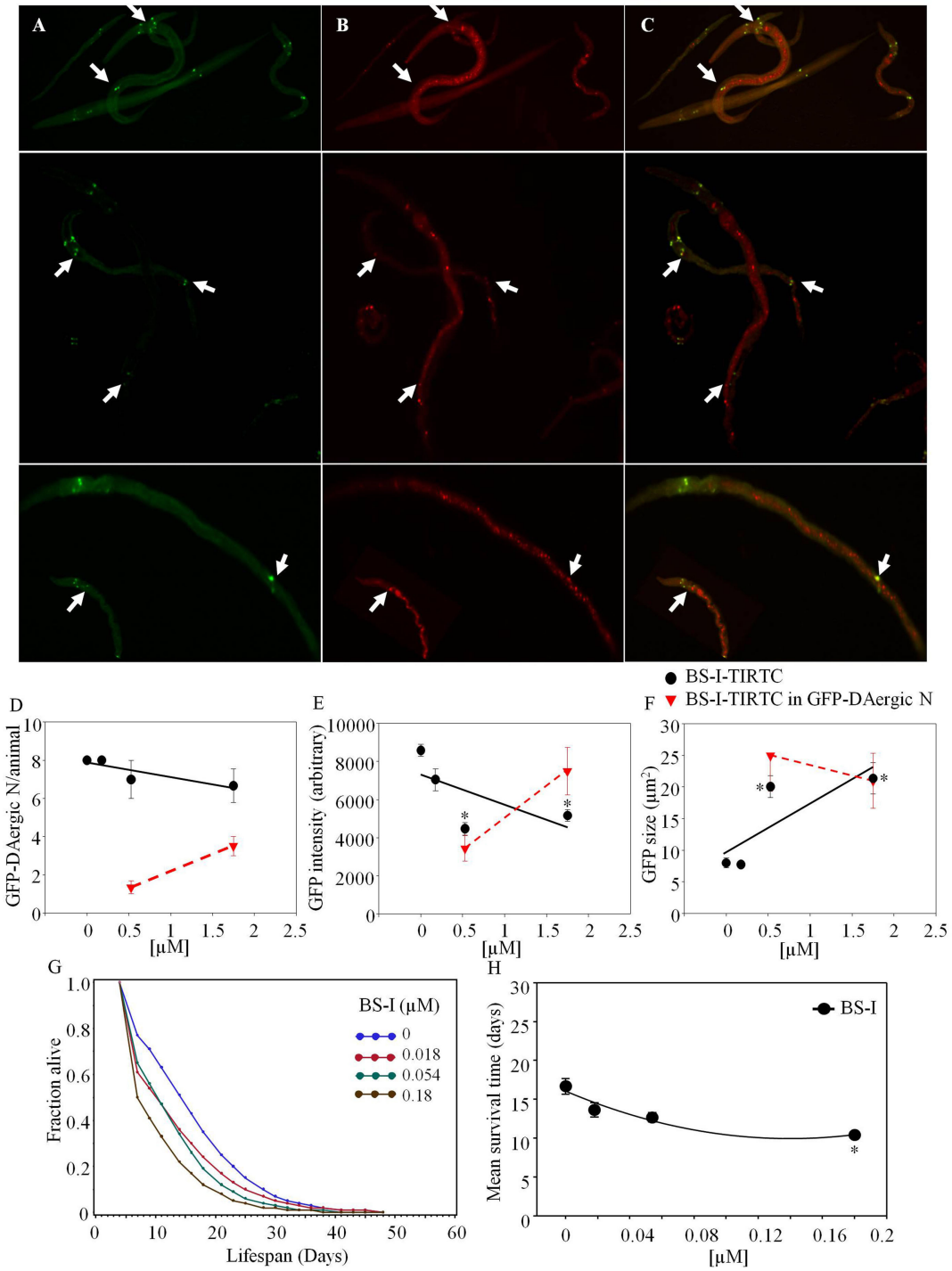

FIGURE 2 | ***Bandeiraea simplicifolia*** **(BS-I)-rhodamine co-localized with GFP-DAergic neurons: (A) GFP-DAergic neurons**. **(B)** BS-I-rhodamine in GFP-DAergic neurons (5.26×10^{-4} or 1.75×10^{-3} mM). **(C)** Merged **(A,B)** showing co-localization of the BS-I-rhodamine with GFP-DAergic neurons. **(D)** Dose-dependent reduction of GFP-DAergic neurons per animal: 7.0 ± 1.0 (1.75×10^{-4} mM) or 6.7 (1.75×10^{-3} mM) ($n = 3$, $P > 0.05$). **(E)** Reduction in fluorescence intensity per GFP-DAergic neuron (up to 60%): 4,468 ± 332 or 5,166 ± 300 (5.3×10^{-4} or 1.75×10^{-3}mM, $n = 3$, $P < 0.01$). **(F)** The size of GFP-DAergic neurons was elevated dose-dependently from 8.0 ± 0.8 to 20.1 ± 1.7 μm² (5.26×10^{-4}) or 21.4 ± 2.5 μm² (1.57×10^{-4}) ($n = 3$, $P < 0.01$). **(G)** The APS was dose-dependently decreased at all doses. **(H)** Mean survival time of each group. *indicates statistical significance.

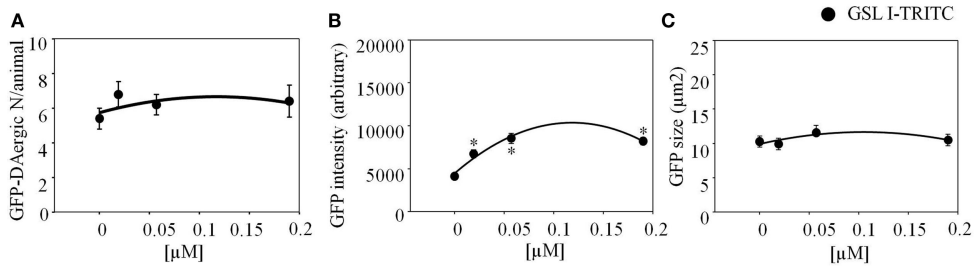

FIGURE 3 | ***Griffonia simplicifolia* I (GSL-I)-rhodamine appeared to affect the GFP-DAergic neurons**. **(A)** GSL-I did not affect the number of the GFP DAergic neuron. **(B)** GSL-I increased fluorescence intensity of the neuron at all doses ($P < 0.05$). **(C)** GSL-I did not affect neuron size. *indicates statistical significance.

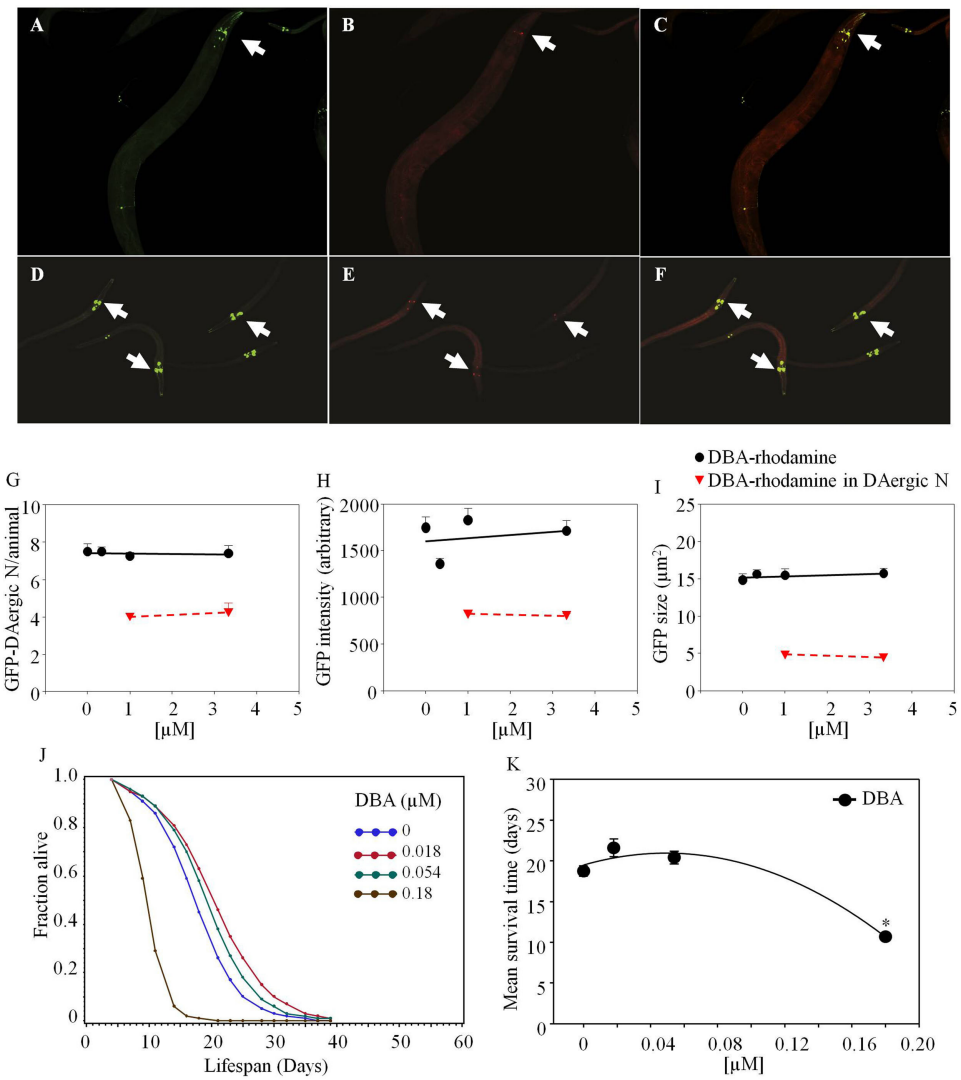

FIGURE 4 | ***Dolichos biflorus* agglutinin (DBA)-rhodamine in *C. elegans* co-localized with GFP-DAergic neurons of aged and young nematodes (L3)**. GFP-DAergic neurons in aged animals **(A)** or young animals **(D)**. DBA-rhodamine in GFP-DAergic neurons in aged animals (1.0×10^{-3} or 3.33×10^{-3} mM) **(B)** or young animals **(E)**. Merged **(A,B)** showing co-localization of the DBA-rhodamine with GFP-DAergic neurons in aged animals **(C)** or young animals **(F)**. **(G)** The number of DAergic neurons was not altered (7.5, $n = 5$, $P > 0.2$) and 50% of them were co-localized with DBA-rb (1.0×10^{-3} or 3.33×10^{-3} mM, $n = 3$) in the aged animals. **(H)** The fluorescence intensity per GFP-DAergic neuron. **(I)** The size of the GFP-DAergic neurons was not changed. These observations were similarly seen in young animals. **(J)** The APS was increased at lower doses of DBA, and decreased at higher doses. **(K)** Mean survival time of each group. *indicates statistical significance.

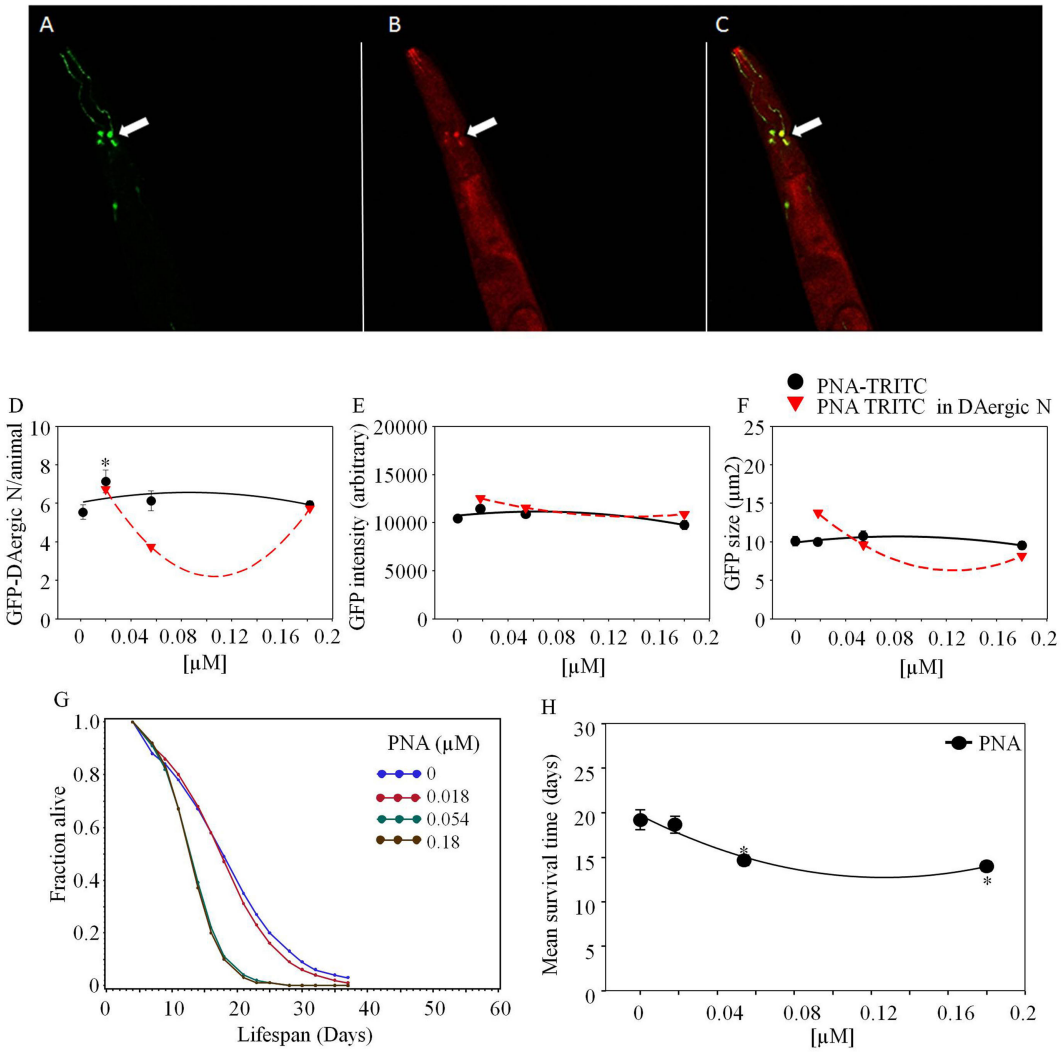

FIGURE 5 | *Arachis hypogaea* agglutinin (PNA)-TRITC post-feeding *C. elegans* co-localized with GFP-DAergic neurons (Leica, TCS SP5, Germany). **(A)** GFP-DAergic neurons (green), **(B)** PNA-TRITC in the neuron (red), **(C)** Co-localization of the GFP-DAergic neurons in merged **(A,B)** (yellow). **(D)** Number of GFP-DAergic neurons was increased at low dose ($P < 0.05$). **(E)** The intensity of GFP-DAergic neurons was not altered. **(F)** The size of GFP-DAergic neurons was not altered. **(G)** APS was dose-dependently reduced at all doses. **(H)** Mean survival time of each group. *indicates statistical significance.

Figure 9B). The size of GFP-DAergic-N was slightly reduced in a dose-dependent trend ($P = 0.4$, **Figure 9C**). In these cases, it is not clear whether the lectin has some undefined indirect effect or that there is a direct effect by undetectable amounts of lectin. S-WGA-TRITC feeding *C. elegans* did not show co-localization in liquid culture. The number of the GFP-DAergic-N was decreased at lower doses (4.6×10^{-5} mM and 1.4×10^{-4} mM, $P < 0.05$, **Figure 9D**). The fluorescent intensity of GFP-DAergic-N was increased at all doses ($P < 0.05$, **Figure 9E**). The size of GFP-DAergic-N was decreased at all doses ($P < 0.05$, **Figure 9F**). The APS was increased at a low dose, and decreased does-dependently at higher doses (**Figure 9G**). The mean lifespan was increased at a low dose (4.6×10^{-5} mM) from 21 to 23 days (9%, $P < 0.05$), and decreased at the highest dose (4.6×10^{-4} mM) to 12 days (−43%, $P < 0.05$, **Figure 9H**).

DISCUSSION

Nine examined plant dietary lectins were conjugated to TRITC or rhodamine. Lectins were tested in the *in vivo C. elegans* (*egIs1*[*Pdat-1:GFP*]) model. An elevated GFP-DAT that is expressed under control of the DAT gene promoter shows enhanced DAT expression and trafficking by the promoter, transcription factor, and nuclear receptor (69).

Carbohydrate-binding protein toxins are known to survive and traverse the gut intact, as an acutely toxic substance and can induce serious life-threatening illness in humans and animals. Distance pathogenicity of botulinum toxin, as well as cholera toxin, impairs the CNS (70). The present study was aimed at a new, surprising property of lectins based upon the hypothesis that lectins may be transported directly by gut absorption to local

TABLE 2 | Lectins alter number, GFP-intensity, or size of DAergic neurons without observed co-localization.

Fluorescence lectins	Dose (mM)	GFP #	GFP intensity	GFP size
Griffonia simplicifolia I (GSL-I)-rhodamine	5.2×10^{-4}			
	1.7×10^{-3}	↔ $P > 0.05$	↑ $P < 0.05$	↔ $P > 0.05$
	5.2×10^{-3}			
Pisum sativum (PSA)-rhodamine (agar)	8.7×10^{-4}	↔ $P > 0.05$	↓ $P < 0.001$	↓ $P > 0.05$
	8.7×10^{-3}			
Pisum sativum (PSA)-rhodamine (liquid)	4.3×10^{-5}		↔ $P > 0.05$	↓ $P > 0.05$
	1.3×10^{-4}	↔ $P > 0.05$	↔ $P > 0.05$	↔ $P > 0.05$
	4.3×10^{-4}		↓ $P > 0.05$	↔ $P > 0.05$
Concanavalin A (Con A)-TRITC (agar)	3.8×10^{-4}		↔ $P > 0.05$	
	1.2×10^{-3}	↔ $P > 0.05$	↑ $P < 0.05$	↓ $P > 0.05$
	3.8×10^{-3}			
Concanavalin A (Con A)-TRITC (liquid)	1.9×10^{-5}		↔ $P > 0.05$	↔ $P > 0.05$
	5.7×10^{-5}	↔ $P > 0.05$	↔ $P > 0.05$	↓ $P > 0.05$
	1.9×10^{-4}		↑ $P < 0.05$	↔ $P > 0.05$
Triticum vulgaris (WGA)-TRITC	4.6×10^{-5}		↔ $P > 0.05$	
	1.4×10^{-4}	↔ $P > 0.05$	↔ $P > 0.05$	↓ $P < 0.05$
	4.6×10^{-4}		↑ $P < 0.05$	
Triticum vulgaris (Succinylated) S-WGA-TRITC (agar)	1.1×10^{-3}			
	1.1×10^{-2}	↔ $P > 0.05$	↓ $P < 0.05$	↔ $P > 0.05$
Triticum vulgaris (Succinylated) S-WGA-TRITC (liquid)	4.6×10^{-5}	↓ $P < 0.05$		
	1.4×10^{-4}	↓ $P < 0.05$	↑ $P < 0.05$	↓ $P < 0.05$
	4.6×10^{-4}	↔ $P > 0.05$		

↓ *Decreasing trend.*
↑ *Increasing trend.*
↔ *No significant alternation.*

neurons and transported axonally to distal neurons where they have an anatomical and potentially a physiological pathophysiological effect. Fluorescent intensity and co-localization of lectins was observed to suggest transport to GFP-DAergic-N. Number and area changes of GFP-dopamine receptor fluorescence in DAergic-N were observed to be an effect of the lectins. For the fact that multiple system atrophy features the accumulation of α-SYN in glial cytoplasmic inclusions (71–73), involvement of other pathways such as indirect effects by interaction of lectins with glial cells that affected DAergic-N may be possible. Lectins have been used for histochemistry and neuronal tracing only, but were not previously associated with neuronal toxicity (26, 74, 75).

The Occurrence and Intensity of Individual Fluorescently Labeled Lectins in GFP-DAergic-N Detected by Co-Localization

Four lectins (PHA-E, BS-I, PNA, or DBA) appeared to co-localize with a subgroup of GFP-DAergic-N, while some other lectins had effects where co-localization was not observed. Because some effects were seen in neurons where lectins were not detected, this may be due to undetectable amount of transported lectin, or some unexplained indirect effect. Thus, the number and GFP-DAT image of neurons were also evaluated, even when the fed lectin was not detected in the neurons. The lack of observation of fluorescence in the neurons using other fed lectins (which, however, seemed to affect these neurons) may be due to a variety of characteristics. For instance, a critical window for the lectin to be detectable may have been missed, the lectin may have been partially degraded, losing the fluorophore, but still retaining neuron-effective activity, or, more likely, undetectable levels of the lectin have observable activities. Future studies with ELISA, other immunocytochemical studies or radiolabeling may confirm the transport of small amounts of these specific lectins where an effect is observed without fluorescence co-localization.

Lectin-Caused Differences in the Number of GFP-DAergic Neurons

Four lectins [GSL-I (BSL I), PHA-E, Con A, and PSA] reduced the number of DAergic-N, with GSL-I having the greatest effect. Toxicity of some lectins and newly discovered side effects of ingestion of PHA and WGA lectins in human and animals have been observed, mitigated by sucrose feeding (76–78). In the present study, PHA-E-rhodamine appeared to be transported intracellularly, probably by axonal transport after ingestion, to *C. elegans* GFP-DAergic-N causing a reduction in their number. This reduction was inversely proportional to the size of the observed image of PHA-E-rhodamine fluorescence co-localized with the neurons. Thus, the greatest area of PHA-E-rhodamine co-stains with the lowest observed number of GFP-DAergic-N. In addition, both fluorescence intensity and size of the GFP-DAergic-N were significantly reduced, suggesting a possible toxic effect of cytoplasmic PHA-E. This observation is in agreement with other studies showing that PHA can damage intestinal epithelial cells (32, 78), which was prevented or reversed by a PHA-E inhibitor sucrose (78). Interestingly, PHA-E did not show a significant

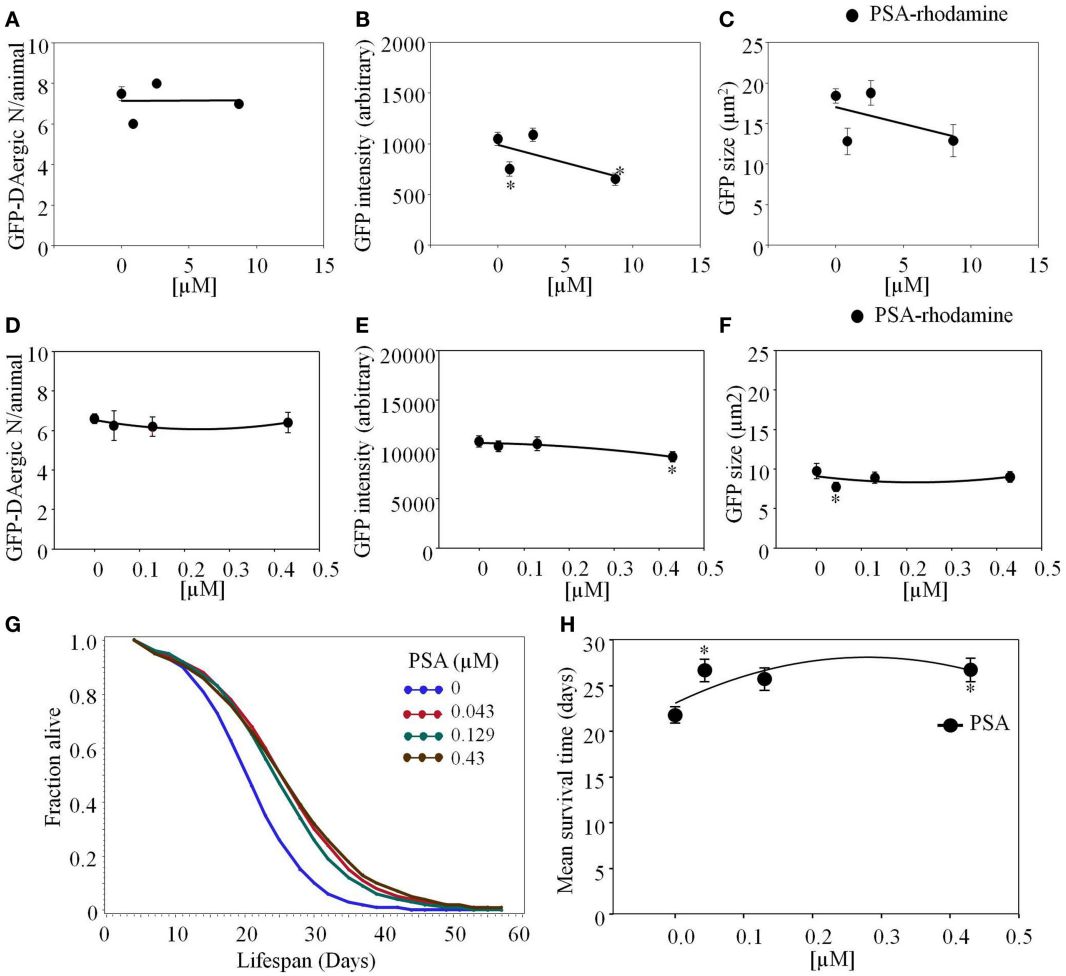

FIGURE 6 | ***Pisum sativum*** **(PSA)-rhodamine affected the GFP-DAergic neurons in *C. elegans*.** **(A)** The number of GFP-DAergic neurons per animal was not reduced overall (8.70×10^{-4}–8.7×10^{-3} mM, $n = 6$, $P > 0.05$). **(B)** The fluorescence intensity of GFP-DAergic neurons was decreased at high dose (651 ± 61, 8.7×10^{-3} mM). **(C)** The size of GFP-DAergic neurons appeared to be reduced at high dose from 18.4 ± 0.9 μm² (control) to 12.9 μm² (8.70×10^{-4} mM, $n = 3$, $P > 0.05$) in a dose-dependent trend. Direct co-localization of PSA-rhodamine with GFP-DAergic neurons was not detected. **(D)** PSA alone did not affect the number of the neurons. **(E)** PSA decreased the intensity of DAergic neurons at the highest dose ($P < 0.05$). **(F)** PSA decreased the size of the DAergic neurons at lowest dose ($P < 0.05$), which was mitigated at the higher dose ($P > 0.05$). **(G)** The APS was increased at all doses. **(H)** Mean survival time of each group. *indicates statistical significance.

decrease in the number and size of GFP-neurons in *C. elegans* but demonstrated decreased expression of GFP-DAT fluorescence intensity. PSA, in some studies, has been shown to be essentially non-toxic in mice both *in vivo* and *in vitro* (79). However, short-term toxicity measurements in these studies do not include more subtle, possible long-term effects of neuronal damage.

Lectin Feeding Effects on the Fluorescent Intensity of GFP-DAergic Neurons

Three lectins (PHA-E, PSA, or S-WGA) reduced GFP-DAT fluorescence in DAergic-N suggesting damage to DAT, while BS-I, GSL-I, or Con A induced an increase indicating a promotion of DAT in the DAergic-N. WGA slightly reduced the fluorescent intensity of GFP-DAT in DAergic-N, while DBA was inactive. Although WGA did not affect the number of GFP-DAergic-N in the present study, and in other laboratories, in the *in vivo* rat gut lumen, reduced expression of heat shock proteins resulting in lowered protection and greater permeability of epithelial cells. WGA also increases thrombin in human platelets, and escalates adipogenesis in mesenchymal cells of the mouse limb bud *in vitro* by unknown mechanisms (25, 77, 80, 81).

In liquid culture, individual lectins affect the number and intensity of GFP-DAergic-N in a different manner. GSL-I, Con A, WGA, and PSA mildly affected the number of GFP-DAergic-N. The intensity of GFP-DAergic-N was increased by GSL-I, Con A, and WGA, while decreased by PSA. The number of GFP-DAergic-N was reduced, while the intensity of GFP-DAergic-N was increased by S-WGA in lower doses. PNA did not affect the intensity of the GFP-DAergic-N, while increased the number of neurons at lower doses.

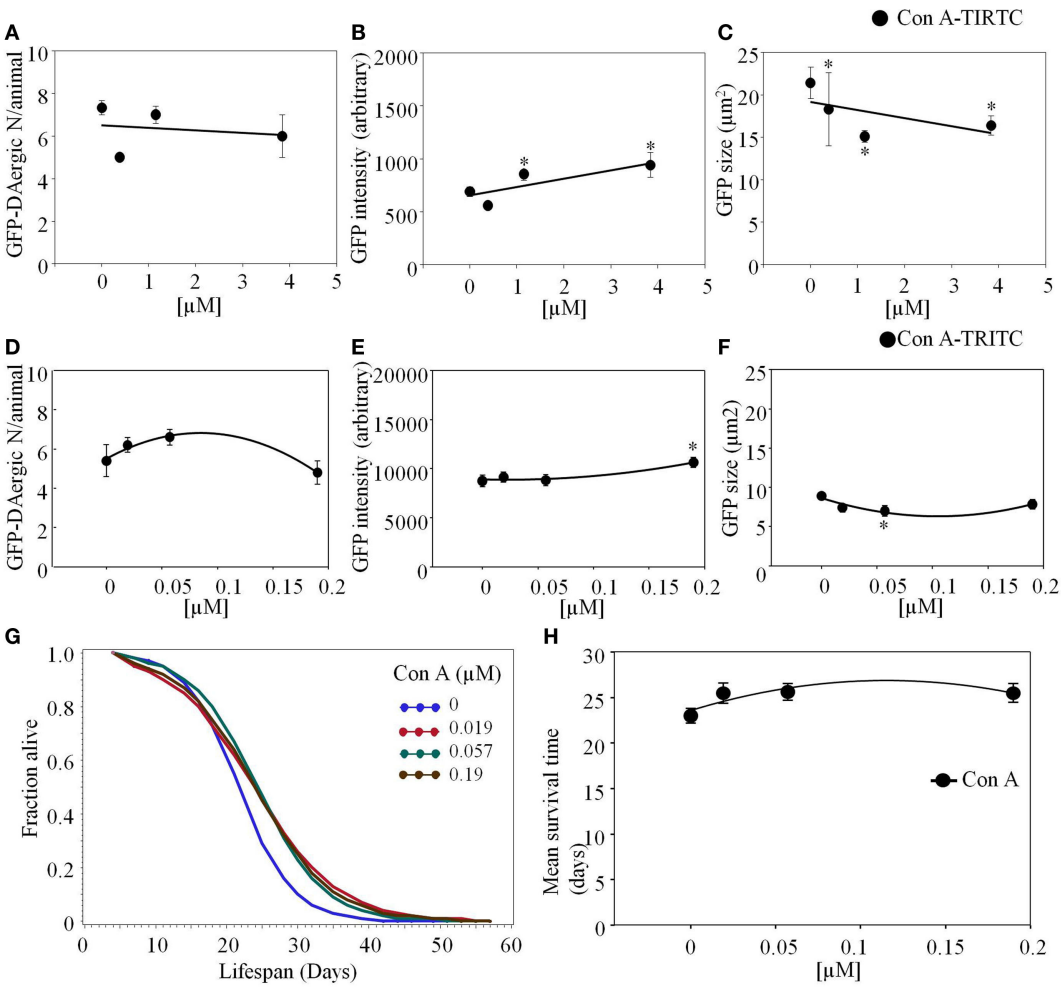

FIGURE 7 | *Concanavalin A* (Con A)-TRITC in *C. elegans* appeared to have a mild effect on the GFP-DAergic neurons. **(A)** The number of GFP-DAergic neurons per animal was slightly reduced at the high dose (7 ± 0.4, $P > 0.05$). **(B)** The fluorescence intensity per GFP-DAergic neuron was dose-dependently increased from 691 ± 45 (control, $n = 3$) to 942 ± 118 (3.84×10^{-3} mM, $n = 4$, $P < 0.05$). **(C)** The size of GFP-DAergic neurons was significantly reduced from 21.4 ± 1.8 μm² (control) to 15.1 ± 0.7 μm² (1.15×10^{-3} mM, $n = 4$, $P < 0.05$). Direct co-localization of Con A-TRITC with GFP-DAergic neurons was not detected. In liquid culture, Con A-TRITC in *C. elegans* appeared to have a mild effect on the GFP-DAergic neurons. **(D)** The number of the GFP-DAergic neuron was not affected ($P > 0.05$). **(E)** The fluorescent intensity of the DAergic neuron was increased at the highest dose ($P < 0.05$). **(F)** The area of GFP-DAergic neurons was decreased at middle dose ($P < 0.05$). **(G)** The APS was increased at all doses. **(H)** Mean survival time of each group. *indicates statistical significance.

Sizes of GFP-DAergic Neurons

PHA-E or Con A significantly diminished the size of GFP-DAergic-N, while BS-I, PSA, or S-WGA slightly reduced the size. Whether these effects signify damage to the neurons is not known. Increased neuron size of a subgroup of GFP-DAergic-N, however, was also observed with DBA or WGA, which may have promoted DAT expression, however, whether decrease or increase in the apparent size of neurons has a physiological effect, or indicates that lectin-mediated damage is not yet known.

The effect of lectins on the number of GFP-DAergic-N appeared in the following order PHA-E > GSL-I > BS-I > Con A > PSA > S-WGA. An elevation effect of lectins seemed to be in the order of DBA > BS-I > WGA > GSL-I, and ConA. It is now well known that O-linked β-*N*-acetylglucosamine (*O*-GlcNAc) is a common epitope on cytoplasmic and some nuclear proteins sharing common features with protein phosphorylation [see review (82)]. Although very difficult to detect due to sub-stoichiometric amounts, *O*-GlcNAc occurs exclusively within the nuclear and cytoplasmic compartments of the cell and responds to external signaling, such as mitogen or antigen activation, by altering Ser- and Thr-phosphoprotein profiles (83–84). The protein modification is modulated by *O*-GlcNAcase and *O*-GlcNAc transferase, and even glucose levels modulate the *O*-GlcNAc cycling rate. Characteristics of half-life in *O*-GlcNAc cycling (≤1 min) have been linked closely to diabetes, cardiovascular disease, neurodegenerative disorders, and cancer. Many protein O-GlcNAc modifications occur in nucleocytoplasmic compartments across plant and animal species, including humans [see review (82)]. WGA and other GlcNAc binding lectins, if present in the cytoplasm, may affect this balance (our hypothesis). PNA

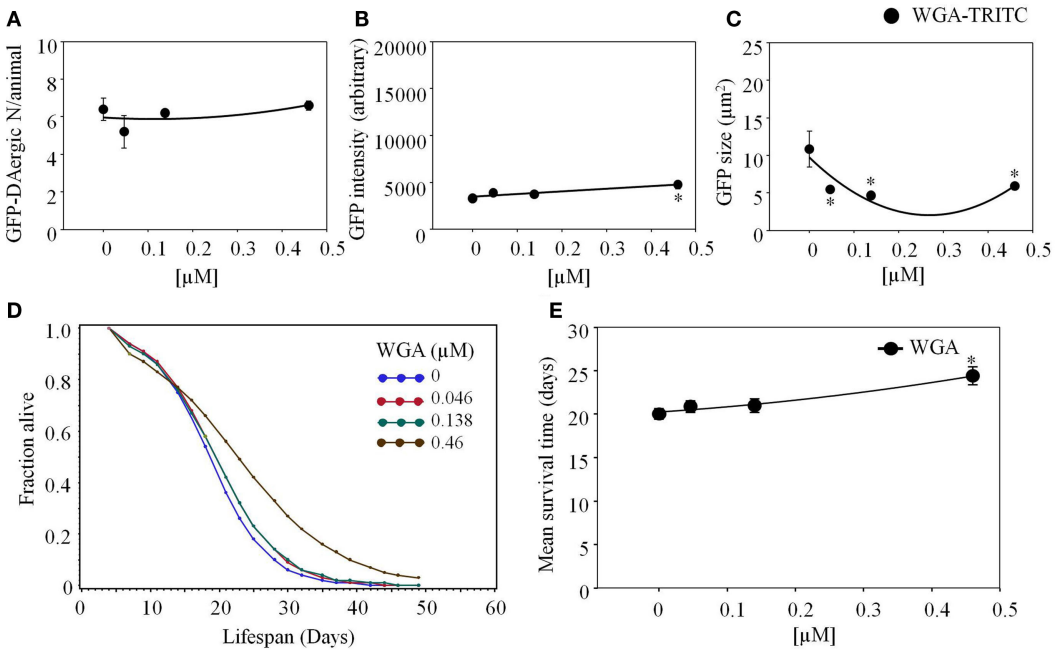

FIGURE 8 | ***Triticum vulgaris* (WGA)-rhodamine affected the intensity and area of DAergic neurons in liquid culture**. **(A)** WGA did not affect the number of DAergic neurons **(B)** WGA increased the intensity of the GFP-DAergic neurons. **(C)** The area of the DAergic neurons was reduced at all doses ($P < 0.05$). **(D)** The APS was increased at all doses. **(E)** Mean survival time of each group. *indicates statistical significance.

strengthens extracellular matrixes by promoting production of proteoglycan in mouse chondrocytes *in vitro* (81). Similar to PNA, Con A has also been reported to strengthen extracellular matrixes in mouse chondrocytes *in vitro* (81). In our study, Con A altered GFP-DAergic-N by reducing the area of GFP-DAT fluorescence.

Some specific beneficial activities of a variety of lectins have been reported (81, 85). In our studies, DBA was observed in the GFP-DAergic-N that had the effect of increasing the observed area of the GFP-labeled DA transporter. This increase may suggest enhanced DAT expression and trafficking, where GFP is expressed under the DAT promoter (69, 86). A major adverse effect of DBA lectin has not been reported elsewhere. In fact, DBA significantly facilitates cartilagenesis and osteogenesis in mouse limb bud mesenchymal cells *in vitro* (81).

BS-I and GSL-I displayed similar activities. Significantly, BSI I dose-dependently reduced the intensity of GFP-DAergic-N. Functionally, BS-I containing 5-Hydroxytryptophan (5-HTP), a catecholamine neurotransmitter, only binds to a subgroup of small dorsal root ganglion neurons, which are of the C-nociceptor type (C-fiber nociceptive or unresponsive) neurons. These C-nociceptor neurons mediate visceral pain and express receptors to BS-I isolectin-B4 (IB4) (87–89). BS-I may increase thrombin in human platelets (80). One possible mechanism that might be related is over-excitation of neurons caused by excessive glutamate neurotransmission being neurotoxic, which may cause neuronal death (90). Indeed, glutamatergic stimulation may indirectly induce DAergic neuronal death *via* unbalanced calcium homeostasis and oxidative stress, *in vitro* or *in vivo* (91). Some of these systems could be altered by either sugar-binding or other unexplored properties of lectins.

In liquid culture, PSA, Con A, WGA and S-WGA decreased the area of GFP-DAergic-N. GSL-I and PNA did not show significant change.

Elevated Fluorescent Intensity and Size of DAergic Neurons

These alterations may reflect a relationship with the insulin receptor and DAT. Glucose provides a vital energy source for brain and clearly modulates neuronal function (92, 93). In *C. elegans*, hyperglycemia reduces APS, related to human diabetes. These relationships in our study, however, may represent some signaling interaction of glycemia/insulinemia and DAT. As with other catecholamine neurotransmitters, inhibitory neurotransmitters are inversely proportional to glycemia, and DA kinetics is sensitive to hypoglycemia in a complex manner (94). In rodents, insulin receptors and DAT are densely present in *substantia nigra*, insulin may increase DAT mRNA expression, and glycemic index is inversely associated with the risk of PD (95, 96).

Lectins Affect Average Probability of Survival of *C. elegans*

Lectins can be categorized into three groups based on their effects on APS of *C. elegans*. (1) PHA-E, DBA, and S-WGA showed J-shaped effects on APS that was increased with lower doses, while decreased at higher doses. (2) PSA, Con A, and WGA demonstrated augmented effects on APS that was increased at all

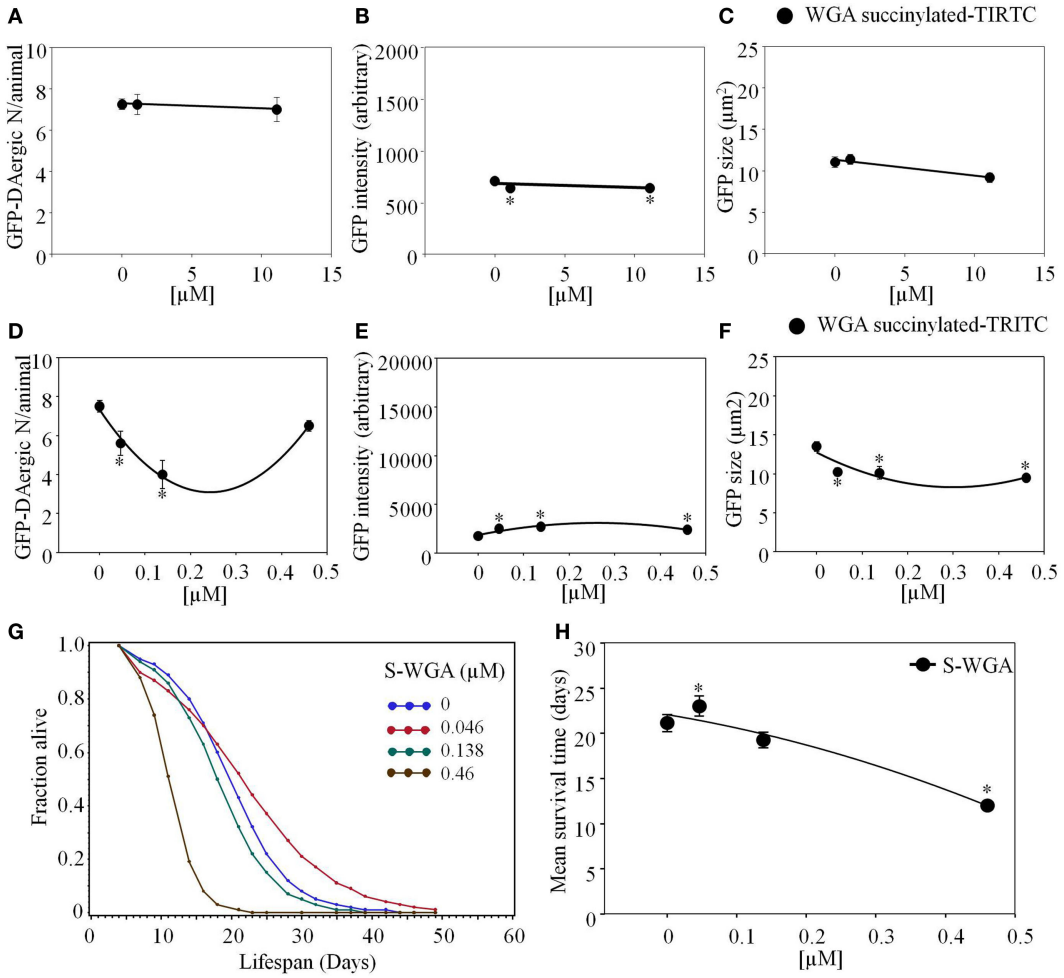

FIGURE 9 | ***Triticum vulgaris*** **(Succinylated) S-WGA-TRITC affected *C. elegans* GFP-DAergic neurons**. **(A)** The number of GFP-DAergic neurons per animal was similar to the effect of WGA-TRITC (7.3–7.0, $n = 4$, $P > 0.5$). **(B)** The fluorescence intensity was reduced from 735 ± 26 (control) to 667 ± 25 (1.11×10^{-2} and 11.12×10^{-3}, $n = 4$, $P < 0.05$). **(C)** The size of GFP-DAergic neurons were slightly reduced at the high dose only to 9.2 ± 0.6 μm² (11.12×10^{-2}, $n = 4$, $P = 0.4$). Direct co-localization of WGA-TRITC with GFP-DAergic neurons was not detected. S-WGA-TRITC affected the GFP-DAergic neurons in liquid culture. **(D)** The number of the GFP-DAergic neurons was decrease at low doses. **(E)** The fluorescence intensity of the GFP-DAergic neurons was increased. **(F)** The size of the GFP-DAergic neurons was decreased. **(G)** The APS was increased at a low dose, and decreased does-dependently at higher doses. **(H)** Mean survival time of each group. * indicates statistical significance.

doses in a dose-dependent manner. (3) GSL-I and PNA decreased the APS dose-dependently.

Neurotoxins such as botulinum toxin or ricin bind to specific receptors on cell membrane, to be internalized, and exert toxic effects (97, 98). Similarly, lectins bind to carbohydrate ligands of targeted cells to be functional, producing effects. Specific sugars may competitively bind and inhibit lectins uptake (99). Structurally similar lectins might share similar binding receptors and compete for each other's binding sites. The complex GI environment, including nutrients and cellular environment, might alter the absorption of certain lectins and their potential biological consequences (100).

In a recent Danish report, patients who had vagal nerves removed 20 years earlier had 40% lower incidence of PD than control populations. If dietary proteins are one potential etiology for PD, by transport to neurons from the gut, as hypothesized here, removal of the vagal nerve would have prevented or reduced this etiology pathway. Symptoms of motor impairment are typical in PD patients, and dysfunction of aspects of the autonomic nervous system are often underrated, such as GI motility (101), rapid eye movement (102), and so on. The current study indicates potential transport of some dietary plant lectins from the GI tract to the DAergic-N in *C. elegans*, with direct or indirect effects on these neurons and diverse effects on APS. This observation may be related to the Braak and Hawkes' hypothesized unknown etiologic agent for PD or related, for example, to damaged DAergic-N that have been found in PD (40, 47). If related, the process may be gradual, may be additive, related to the frequency of consumption of certain lectins, and may be determined by the association of lectins with other factors. Certainly, there is potential for inputs

from individual genetic susceptibility, varying sugar structures profiles in different cell membranes, the receptivity to endocytosis, a disorder or leakage of the GI lining, and dietary content. Our observations are a tantalizing possible explanation for why dietary plants have been linked to a risk of developing PD.

AUTHOR CONTRIBUTIONS

JZ designed the study; RL and JK gave advice regarding the design and conduct of the study; JZ, MW, WW, BA, and MK conducted data acquisition and data analyses; JZ, MW, WW, JK, and RL participated in drafting the manuscript; all of the authors contributed to review and revision of the submission.

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100 years of Lewy pathology

Michel Goedert, Maria Grazia Spillantini, Kelly Del Tredici and Heiko Braak

Abstract | In 1817, James Parkinson described the symptoms of the shaking palsy, a disease that was subsequently defined in greater detail, and named after Parkinson, by Jean-Martin Charcot. Parkinson expected that the publication of his monograph would lead to a rapid elucidation of the anatomical substrate of the shaking palsy; in the event, this process took almost a century. In 1912, Fritz Heinrich Lewy identified the protein aggregates that define Parkinson disease (PD) in some brain regions outside the substantia nigra. In 1919, Konstantin Nikolaevich Tretiakoff found similar aggregates in the substantia nigra and named them after Lewy. In the 1990s, α-synuclein was identified as the main constituent of the Lewy pathology, and its aggregation was shown to be central to PD, dementia with Lewy bodies, and multiple system atrophy. In 2003, a staging scheme for idiopathic PD was introduced, according to which α-synuclein pathology originates in the dorsal motor nucleus of the vagal nerve and progresses from there to other brain regions, including the substantia nigra. In this article, we review the relevance of Lewy's discovery 100 years ago for the current understanding of PD and related disorders.

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Introduction

In 1912, Fritz Jakob Heinrich Lewy (1885–1950) described the cellular inclusions that are characteristic of Parkinson disease (PD; originally known as the shaking palsy).[1] Konstantin Nikolaevich Tretiakoff (1892–1956) named them after Lewy ('corps de Lewy',[2] or Lewy bodies) in 1919. Besides describing abnormal inclusions in nerve cell bodies, Lewy also reported their presence in nerve cell processes (later called Lewy neurites[3]).

The centenary of Lewy's discovery gives us an opportunity to review his contributions in the light of what we know about the aetiology and pathogenesis of PD and related disorders. For example, it is now clear that the formation of Lewy pathology is central to the neurodegenerative process, but for many years the significance of the inclusions described by Lewy was unknown. This changed in 1997, when two findings brought the little-studied protein α-synuclein to the fore.[4,5] First, a missense mutation in *SNCA*, the α-synuclein gene, was found to cause a rare, familial form of PD. Second, Lewy bodies and Lewy neurites of idiopathic PD were shown to be immunoreactive for α-synuclein. Three different missense mutations in *SNCA*, as well as various genomic duplications and triplications, have been described in patients with dominantly inherited PD. Moreover, genome-wide association studies have shown that sequence variation in *SNCA* is an important risk factor for idiopathic PD.[6,7]

Lewy bodies and Lewy neurites were long known to be found outside the substantia nigra in patients with PD, but the temporal sequence of their emergence was unclear. In 2003, this issue was addressed by the introduction of a staging scheme based on the distribution of α-synuclein inclusions over time.[8]

In this article, we present an overview of Lewy's life, including the events that led up to the discovery of the inclusion bodies that now bear his name (Figure 1). We then discuss the central role of Lewy pathology in PD and other neurodegenerative disorders, and the research that has elucidated the mechanisms through which α-synuclein aggregation causes neuronal dysfunction and death.

Historical overview

Lewy was born on 28th January 1885 in Berlin, Germany, where his father worked as a physician.[9,10] He studied medicine at the Universities of Berlin and Zurich, Switzerland, and obtained his medical degree in Berlin in 1910. From 1908–1910, Lewy was based at the Institute of Physiology of the University of Breslau, Germany (now Wroclaw, Poland). From 1910–1912, he worked with Alois Alzheimer (1864–1915) at the Royal Psychiatric Clinic of the University of Munich, Germany (Figure 2). In 1912, Alzheimer was appointed to the Chair of Psychiatry and the Directorship of the Psychiatric Institute at the University of Breslau. Lewy moved with him back to Breslau, to take charge of the anatomical laboratory.

During World War I, Lewy served as medical officer of the German army in France, Russia and Turkey. In 1919, he became staff neurologist at the Charité Hospital in Berlin, where he was appointed to an Associate Professorship in Neurology and Internal Medicine in 1923. From 1928, Lewy was busy establishing a neurological institute in Berlin. At the time, neurology was

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M. Goedert declares associations with the following companies: Eli Lilly, GlaxoSmithKline, Hoffmann-La Roche. See the article online for full details of the relationships. The other authors declare no competing interests.

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Key points

- 100 years ago, Fritz Heinrich Lewy used light microscopy to describe the nerve cell inclusions that are characteristic of Parkinson disease (PD)
- The Lewy pathology consists of the protein α-synuclein in an insoluble form
- Missense and gene dosage mutations in *SNCA*, the α-synuclein gene, cause inherited cases of PD and dementia with Lewy bodies
- In PD, α-synuclein pathology is widespread in the CNS and PNS
- α-Synuclein pathology originates in a small number of nerve cells, from which it spreads in a prion-like fashion
- Clinically, the development of the pathological changes of PD is reflected by the presence of nonmotor and motor symptoms

still subsumed under psychiatry in Prussia, and Lewy's plan was for a new building consisting of a clinic with 100–150 beds and several research departments, including a neuropathology laboratory led by Max Bielschowsky (1869–1940). Lewy also wanted the Institute to become an integral part of Berlin University. He was able to move into the former clinic of the AEG (Allgemeine Elektrizitätswerke), but a University affiliation was not forthcoming, due in large part to strong opposition from the medical faculty of the Charité, in particular the psychiatrist Karl Bonhoeffer.

The Institute of Neurology opened its doors on 1st July 1932, but it remained under Lewy's directorship for only a year. On 30th January 1933, Adolf Hitler became Reich Chancellor, and on 7th April 1933 the so-called 'Reich Law for the Restoration of a Professional Civil Service' was passed by the Nazis. This law led to the summary dismissal of most 'non-Aryan' civil servants. On 2nd August 1933, Lewy was informed that he had been dismissed from his position on racial grounds, with retroactive effect to 1st July 1933. By the beginning of the academic year 1933–1934, approximately one-third of the Professors of Berlin University had lost their positions. Lewy's Institute was incorporated into the Charité in April 1934, and was destroyed during World War II.

During the summer of 1933, at the age of 48 years, Lewy left Germany.[11] He spent a year in the UK, where he worked on the effects of lead on the human body at the Chloride Electric Storage Company in Manchester, before emigrating to the USA. He became a Rockefeller Fellow and visiting Professor of Neurophysiology at the Hospital of the University of Pennsylvania in Philadelphia, PA in 1934. He changed his name from Fritz Heinrich Lewy (he had dropped his middle name in 1912) to Frederic Henry Lewey when he became an American citizen in 1940. During World War II, Lewey served in the US Army Medical Corps, where he was neurologist to the Surgeon General's Office. In 1947, he became Professor of Neuroanatomy and Associate Professor in Neuropathology at the University of Pennsylvania. During these years, he continued to work on basal ganglia and developed an interest in peripheral nerve injuries. Lewey died suddenly on 5th October 1950, at 65 years of age.

Lewy and Parkinson disease

Lewy initially examined the brains of 25 individuals with PD from the Städtisches Siechenhaus der Stadt Berlin and published his findings in Volume 3 of the *Handbuch der Neurologie* in 1912.[1] He then examined a further 60 brains obtained from the same institution, using more-sophisticated histological techniques. This work was presented at the annual meeting of the German Association of Psychiatrists and Neurologists in 1913.[12] Lewy described the characteristic inclusions in the dorsal motor nucleus of the vagus nerve, the basal nucleus of Meynert, the globus pallidus, the lateral nucleus of the thalamus, and the periventricular nucleus of the thalamus. He noticed similarities—but also some differences—with inclusions that Gonzalo Rodriguez Lafora (1886–1971) had described in 1911 in patients with progressive myoclonic epilepsy.[13] The inclusions described by Lewy were eosinophilic, and were insoluble in alcohol, chloroform and benzene, consistent with the presence of a major protein component. Lafora bodies are made of hyperphosphorylated forms of insoluble glycogen.

In 1919, Tretiakoff reported the presence of Lewy bodies in the substantia nigra in PD.[2] He also showed degeneration of the substantia nigra and postulated a connection between nerve cell loss, rigidity and tremor. This discovery followed earlier work by Paul Blocq (1860–1896) and Georges Marinesco (1863–1938), who had reported a case of parkinsonian tremor caused by a tumour of the substantia nigra.[14] In 1923, Lewy published a monograph of 673 pages on the shaking palsy (Figure 3).[15] He confirmed Tretiakoff's findings in only 11 out of 50 cases of PD, and he suspected that parkinsonism originated in the globus pallidus. In 1938, however, Rolf Hassler (1914–1984) confirmed Tretiakoff's observation that degeneration of the substantia nigra was the cause of parkinsonism.[16] He also demonstrated the focal distribution of pathology, with the most pronounced nerve cell loss being found in the caudal and ventrolateral parts of the substantia nigra. The fact that nerve cells in the ventrolateral part of the pars compacta of the substantia nigra are severely affected in PD is now well-established. These cells project mainly to the dorsal putamen, which is the most severely dopamine-depleted region of the striatum in PD.

Prior to Hassler's publication, Lewy had revisited the issue of inclusion bodies in a talk given at the first International Congress of Neurology in 1931, where he emphasized the similarities between the Negri bodies of rabies and the inclusions of the shaking palsy.[17] This is interesting in the light of recent work suggesting that the pathological inclusions of PD may spread through the brain via a prion-like mechanism.[18] In 1942, Lewy reviewed the history of research into basal ganglia diseases, but failed to attach much importance to either the inclusion bodies he had discovered, or the later findings of Tretiakoff and Hassler.[19]

Lewy body Parkinson disease

PD is the second most common neurodegenerative disorder of the human brain, after Alzheimer disease.[20] PD is not known to affect any other vertebrates besides humans and, provided that it is not arrested by death from other causes, it progresses relentlessly for decades.[21] Unlike Alzheimer disease, the pathological process of idiopathic

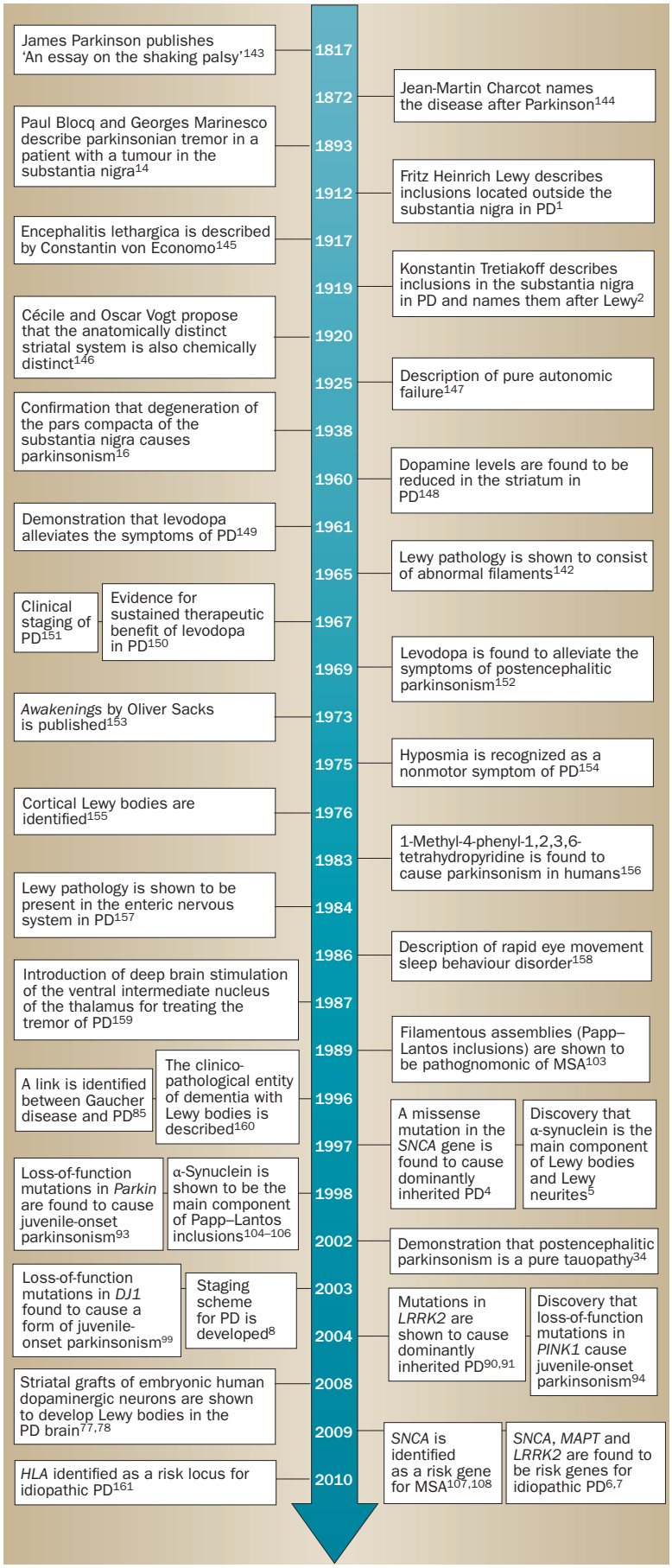


Figure 1 | 100 years of Lewy pathology: timeline of discoveries. Abbreviations: PD, Parkinson disease; MSA, multiple system atrophy; *SNCA*, α-synuclein gene; *LRRK2*, leucine-rich repeat kinase 2 gene; *MAPT*, microtubule-associated protein tau gene; *PINK1*, PTEN-induced kinase-1 gene.

PD develops not only in the CNS, but also in the PNS and enteric nervous system.[22]

Definition as a synucleinopathy

In 1997, a missense mutation (Ala53Thr) in *SNCA* was shown to cause a dominantly inherited form of PD with Lewy pathology.[4] Two additional missense mutations (Ala30Pro and Glu46Lys) were subsequently identified in families with PD or dementia with Lewy bodies (DLB).[23,24] All three mutations are located in the amino-terminal repeat region of α-synuclein, which consists of seven imperfect 11-amino-acid repeats with the consensus sequence KTKEGV (Figure 4).[25] In the presence of negatively charged lipids, the natively unfolded α-synuclein folds into amphipathic α-helices through its amino-terminal repeats.

Overexpression of wild-type α-synuclein has also been identified as a cause of PD in families with heterozygous triplications or duplications of the region of chromosome 4 that comprises *SNCA*, with disease penetrance being highest for triplication cases (Figure 4).[26–28] Moreover, genome-wide association studies identified sequence variation in the regulatory region of *SNCA* as the most important genetic risk factor for idiopathic PD,[6,7] in confirmation of previous findings.[29]

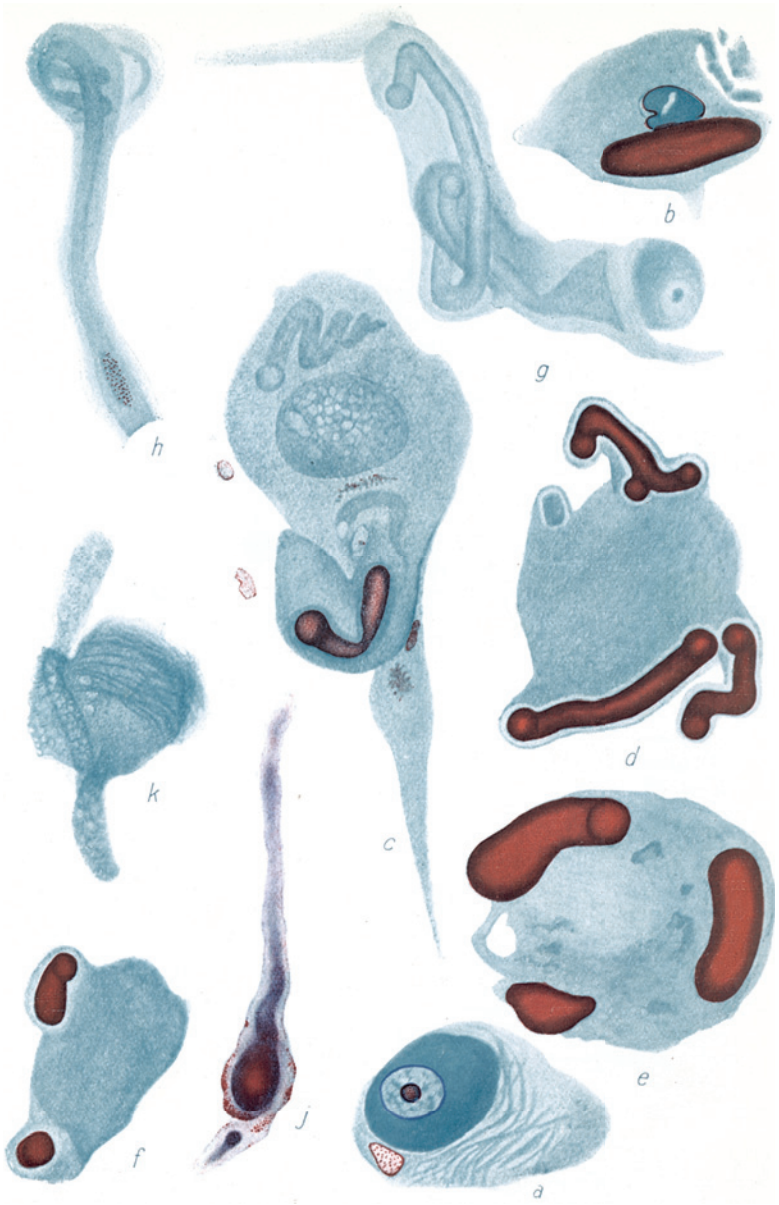
In 1997, Lewy bodies and Lewy neurites from cases of idiopathic PD were shown to be immunoreactive for α-synuclein.[5] Abundant α-synuclein inclusions are also characteristic of the diseases caused by *SNCA* mutations.[30] These findings established the central importance of α-synuclein aggregation for all cases of Lewy body PD. α-Synuclein-positive aggregates appear in neurites before they appear in nerve cell bodies, and may contain oligomeric assemblies that increase the production of reactive oxygen species.[31,32]

Lewy pathology is also the defining feature of several rarer diseases, including pure autonomic failure, in which Lewy bodies and Lewy neurites are mostly restricted to the PNS.[25] In incidental Lewy body disease, a condition that is characteristic of 5–10% of individuals over the age of 60 years and may be a preclinical form of PD,[33] small numbers of Lewy bodies and Lewy neurites are present in the absence of clinical symptoms. By contrast, abundant filamentous tau aggregates, in the absence of α-synuclein inclusions, are typical of postencephalitic parkinsonism.[34]

Lewy pathology

Electron microscopy revealed that Lewy bodies and Lewy neurites are made of unbranched α-synuclein filaments, with a length of 200–600 nm and a width of 5–10 nm.[35] The core of the filament extends over 70 amino acids and overlaps with the repeat region of α-synuclein. Like other amyloids, these filaments have a cross-β structure.[36] On

Figure 2 | Members of Alois Alzheimer's research group at the Royal Psychiatric Clinic of the University of Munich, Germany in 1910. Back row: Fritz Jakob Heinrich Lewy (circled) is on the far right, Alois Alzheimer is third from the right.



the basis of a model derived from solid-state nuclear magnetic resonance, the core of the α-synuclein filament comprises five β-strands reminiscent of a five-layered β-sandwich.[37] Hyperphosphorylation of Ser129 by G-protein-coupled receptor kinases is the main post-translational modification of filamentous α-synuclein,[38,39] and the α-synuclein filaments become ubiquitinated after assembly.[25]

Although Lewy bodies have been the most widely studied pathological feature of PD, aggregated α-synuclein also appears in a particulate form in nerve cell bodies (Figure 5a–c).[40] Some nerve cells develop multiple Lewy bodies. Pale bodies form occasionally in neuromelanin-containing cells and are probably precursors of Lewy bodies (Figure 5c).

Two types of Lewy bodies have been described: a brainstem type and a cortical type. The brainstem type has an acidophilic and argyrophilic core, and a pale-staining halo; the latter is strongly immunoreactive for α-synuclein. The cortical type is less well-defined and lacks a halo. Spindle-like or thread-like Lewy neurites (Figure 5d–f) occur in axons and dendrites of affected neurons.[3] Lewy plaques consist of a core of aggregated extracellular amyloid-β (Aβ) that is surrounded by dystrophic α-synuclein-immunoreactive neurites (Figure 5g). Cortical deposits of Aβ are required for the formation of Lewy plaques.[22]

Dementia is common in PD, especially in advanced cases.[21] A diagnosis of PD dementia (PDD) is made when cognitive impairment develops in a patient with long-standing idiopathic PD, whereas dementia develops within a year of the appearance of parkinsonian signs in cases of DLB.[41] PDD and DLB show similar neuropathological profiles, including the presence of widespread cortical α-synuclein-positive Lewy pathology. Many cases also have Alzheimer-type plaques and tangles.[42] Conversely, a substantial number of individuals with Alzheimer disease develop Lewy pathology, especially in the amygdala.[43] Some individuals with *SNCA* mutations develop both PD and DLB.[24,26]

Nerve cells can survive for decades in the presence of multiple Lewy bodies and Lewy neurites, raising the question of whether α-synuclein aggregates are harmless, neuroprotective[44] or detrimental to nerve cell function.[45–47] In the CNS, they form along the entire neuraxis, including the spinal cord (Figure 6a).[48,49] α-Synuclein aggregates are also found in the ganglia of Meissner's and Auerbach's plexuses in the gastrointestinal tract (Figure 6c,d),[50,51] as well as in sympathetic ganglia (Figure 6b) and the sympathetic trunk,[49,52] the adrenal medulla,[53] the submandibular gland,[54] and the heart,[55,56] including the cardiac conduction system.[57] Consequently, specific neurotransmitter systems are

◀ **Figure 3** | Abnormal nerve cell bodies and processes in the dorsal motor nucleus of the vagal nerve in Parkinson disease. Some filamentous inclusions appear as elongated eosinophilic bodies (red). With kind permission of Springer Science+Business Media © Lewy, F. H. *Die Lehre vom Tonus und der Bewegung*. (Springer-Verlag, Berlin, 1923).[15]

insufficient for identifying neurons that are prone to develop Lewy pathology, and PD can no longer be viewed as a monosystemic disease that predominantly affects the nigrostriatal dopaminergic system. Instead, PD is a multisystem disorder that affects many different regions of the nervous system (Figures 5 and 6).[58–62]

Disease staging

Idiopathic PD constitutes over 90% of PD cases. Extensive studies of normal and diseased human brains have shown that α-synuclein inclusions emerge in a predictable order in different parts of the brain, making it possible to distinguish six stages of α-synuclein deposition (Figures 7 and 8).[8,22]

The first α-synuclein-positive structures in the brain usually occur in the olfactory bulb and/or the dorsal motor nucleus of the glossopharyngeal and vagal nerves (stage 1). In stage 2, Lewy pathology develops in the medulla oblongata and the pontine tegmentum. By stage 3, pathology has reached the amygdala and the substantia nigra. Generally, at some point during this stage, the motor symptoms of PD (bradykinesia, with at least one of the three features of rigidity, rest tremor or gait disturbance) begin to appear. The pathology worsens and the α-synuclein inclusions reach the temporal cortex (stage 4). During stages 5 and 6, Lewy bodies and Lewy neurites appear in the neocortex, accounting for many of the cognitive problems associated with advanced PD.

Other groups have confirmed the accuracy of this staging scheme.[63–65] Exceptions have been reported in 10–20% of cases, which have included an amygdala plus olfactory variant[66] and an amygdala variant.[67] Discrepant results[68,69] may be attributable to section thickness[8] or variable protocols for processing and analysing tissues,[64,70] and also to the fact that not all brain regions included in the staging system[8] are assessed routinely by all laboratories, making it difficult to compare results.

α-Synuclein deposits may form early in the enteric nervous system—which is connected to the brain via the the vagal nerve—and in the PNS.[71] The mechanism through which the disease process spreads remains unclear: it could begin in the gut and move retrogradely to the brain via the vagal nerve; it could start in the vagal dorsal motor nucleus and move from there to the spinal cord and gut in an anterograde fashion;[48] or it could begin in the periphery at multiple autonomic sites and subsequently be transmitted to the spinal cord.[49,72] The distribution of Lewy pathology in the gut parallels the input from the vagal dorsal motor nucleus;[73] this occurs in the absence of myenteric ganglion cell loss, indicating that the contribution of cell dysfunction to the pathological process underlying PD should not be underestimated. Accumulation of α-synuclein has been described in some nerve cell bodies and processes in Meissner's plexus of the large intestine several years before the appearance of the first motor symptoms of PD.[74] The presence of α-synuclein inclusions in the large intestine may, therefore, be a useful biomarker of PD.

The staging system described by Braak *et al.*[8] has been expanded in an attempt to incorporate not only the α-synuclein deposits in postganglionic neurons of part of the enteric nervous system,[71] but also those in the coeliac and superior cervical ganglia and in the spinal cord.[48,49,54,72] Spinal cord lesions are first seen during stage 2 in sympathetic and sacral parasympathetic preganglionic nerve cells and, during stage 3, in the motor neurons of Onuf's nucleus and the ventral horn, as well as in layer 1 nociceptive neurons of the dorsal horn.[48]

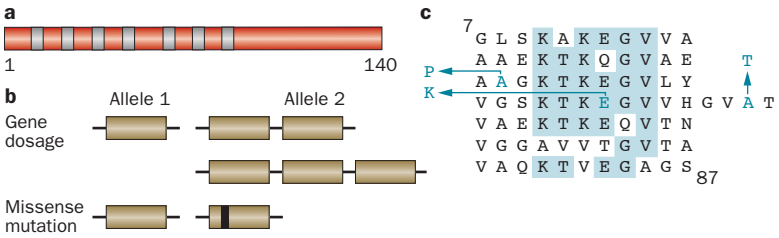


Figure 4 | Human α-synuclein and its disease-causing mutations. **a** | Diagram of the 140-amino-acid human α-synuclein protein. The core regions of the amino-terminal repeats are shown as blue bars. **b** | An increase in gene dosage (duplication or triplication) of the chromosomal region containing *SNCA* or missense mutations in *SNCA* cause dominantly inherited forms of Parkinson disease and dementia with Lewy bodies. **c** | The seven repeats (residues 7–87) of human α-synuclein are shown, with the disease-causing missense mutations (Ala30Pro, Glu46Lys and Ala53Thr) indicated in blue text. Amino acids that are identical in at least five of the seven repeats are shaded in blue. Abbreviation: *SNCA*, α-synuclein gene.

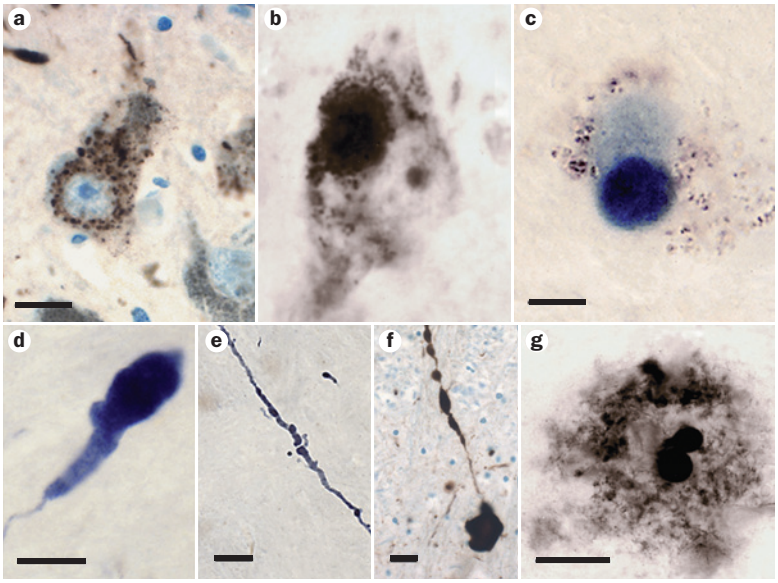


Figure 5 | Synuclein-immunoreactive Lewy pathology in the Parkinson disease brain. **a** | Particulate aggregates (punctate inclusions) in dopaminergic nerve cells of the substantia nigra, which probably precede Lewy body formation. **b** | Mossy cell with Lewy body in sector CA4 of Ammon's horn of the hippocampus. **c** | Lewy bodies in dopaminergic nerve cells of the substantia nigra. Pale body in the background (pale blue area) and Lewy body (dark blue) in a neuromelanin-containing cell in the foreground. **d** | Club-shaped, **e** | filiform and **f** | varicose Lewy neurites. **g** | Lewy plaque consisting of an extracellular Aβ core that is surrounded by a perimeter of α-synuclein-immunoreactive dystrophic neurites. Sections are immunostained for α-synuclein, with the addition of Campbell–Switzer silver staining for Aβ in part g. Scale bars, 20 μm. Abbreviation: Aβ, amyloid-β. With kind permission of Springer Science+Business Media © Braak, H. & Del Tredici, K. Neuroanatomy and pathology of sporadic Parkinson's disease. *Adv. Anat. Embryol. Cell Biol.* **201**, 1–119 (2009).[22]

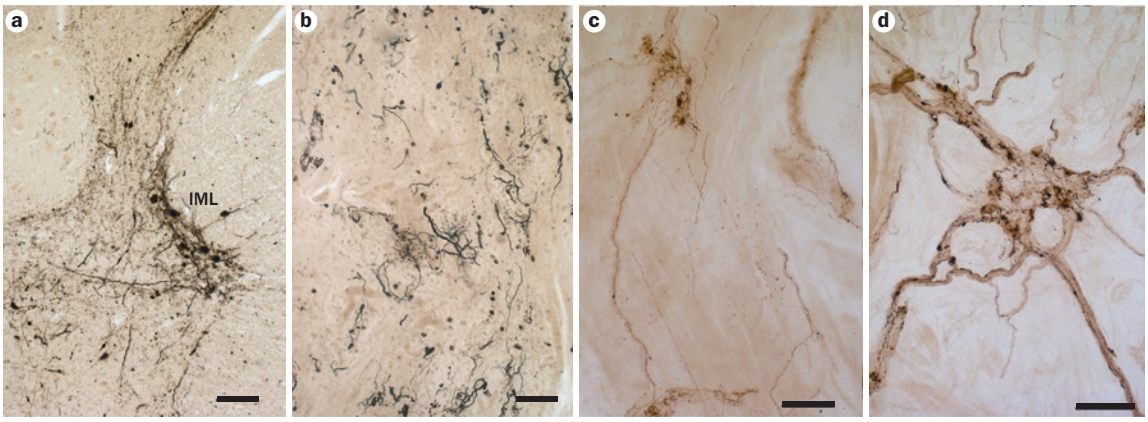


Figure 6 | Synuclein-immunoreactive Lewy pathology in the PD spinal cord, coeliac ganglion and gastrointestinal tract. **a** | IML with affected preganglionic sympathetic neurons. The dorsal nucleus (pale round area at upper right) is virtually uninvolved. **b** | Lewy bodies and Lewy neurites are widespread in nerve cells of the coeliac ganglion (postganglionic sympathetic neurons), shown here from a case at stage 6 of PD pathology. Scale bars in a and b, 200 μm. **c** | Auerbach's plexus of the stomach from an asymptomatic individual at stage 3 of PD pathology. Aggregates are seen in axons of the fibre bundles that connect individual ganglia. **d** | At stage 6 of PD pathology, heavy involvement of the enteric nervous system is a major reason why many patients experience gastrointestinal dysfunction. Scale bars in c and d, 500 μm. Abbreviations: IML, interomediolateral column; PD, Parkinson disease. With kind permission of Springer Science+Business Media © Braak, H. & Del Tredici, K. Neuroanatomy and pathology of sporadic Parkinson's disease. *Adv. Anat. Embryol. Cell Biol.* **201**, 1–119 (2009).[22]

Together with previous findings, this report indicates that the disease process within the CNS does not originate in the spinal cord.[63,75]

The staging scheme is consistent with the fact that most PD patients have nonmotor symptoms that appear before motor dysfunction. Autonomic dysfunction, hyposmia, depression and rapid eye movement sleep behaviour disorder can precede the motor symptoms by many years.[76] These symptoms are consistent with the distribution of Lewy bodies and Lewy neurites in the brain during the early pathological stages.[60,76] Incidental Lewy body disease may be at one end of the Lewy body disease spectrum, with DLB at the other end, and with Lewy body dysphagia, pure autonomic failure and PD in between.

The presence of Lewy bodies in human fetal brain cells a decade or more following their transplantation into the striatum of patients with PD is consistent with the spreading of α-synuclein inclusions from the host brain to the grafted cells,[77,78] although the microenvironment of the graft may also play a role.[79] In the grafts, up to 5% of dopaminergic neurons contained Lewy bodies, similar to the proportion of Lewy body-bearing neurons in the substantia nigra of patients with PD.[80,81] It has been suggested that nerve cells with Lewy bodies might die within 6 months of inclusion formation, with Lewy bodies and nerve cell loss ultimately reaching a steady state.[81]

Recent work has led to the development of a unifying mechanism of neurodegeneration.[18,82] According to this view, protein aggregation is a relatively common event, with cells efficiently removing early aggregates in the vast majority of cases. Following a rare successful aggregation event, the prion-like replication and intercellular transfer of pathology may provide a mechanism for the rapid propagation of protein inclusions. A stochastic misfolding event may be the primary cause, with the subsequent influence of more-deterministic processes. The early misfolding of a given disease protein is not entirely random, however, in that it tends to develop in a predictable manner within a given brain region. The causal relationships between α-synuclein aggregation, spreading and neurodegeneration are unknown. In particular, the relative contributions of Lewy bodies and Lewy neurites remain to be established.[83]

Other forms of Parkinson disease

Following the discovery of *SNCA*, additional PD-associated genetic loci were identified, raising the question of whether multiple forms of PD exist or whether a single pathway can account for all cases. As discussed above, the aggregation of α-synuclein is central to at least one form of PD, and its relevance has been reinforced with the demonstration of a progressive and spreading disease, encompassing widespread pathology and a long presymptomatic phase.[84] It seems unlikely that the same pathogenic process is central to forms of clinical PD that lack Lewy bodies and Lewy neurites at autopsy.

Homozygous mutations in the gene encoding the enzyme glucocerebrosidase (*GBA*), resulting in the lysosomal accumulation of glucocerebroside, cause Gaucher disease; some patients with this condition develop PD. Heterozygous *GBA* mutation carriers (without Gaucher disease) also have an increased risk of developing PD.[85,86] Among individuals with Gaucher disease, the probability of developing PD before the age of 80 years is 9–12%, compared with 2.6% in the general population.[87] Moreover, patients with PD are over five times more likely to carry *GBA* mutations than are healthy controls. Patients with PD and *GBA* mutations exhibit an earlier age of onset and more-severe nonmotor symptoms, including autonomic dysfunction, neuropsychiatric symptoms and

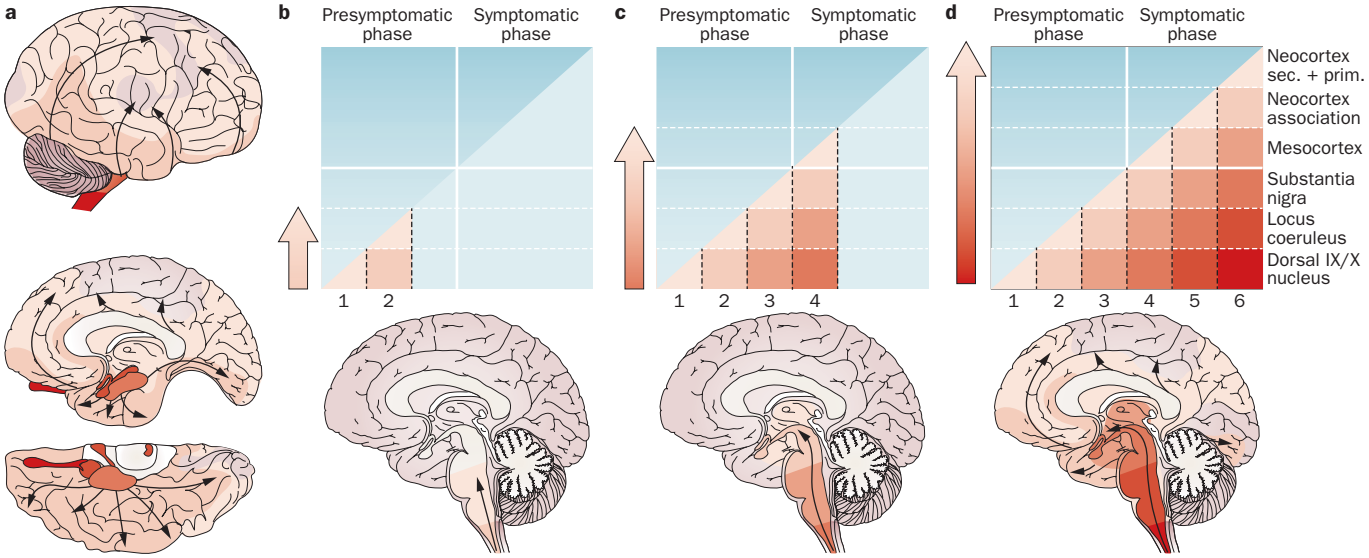


Figure 7 | Six stages of PD pathology. Cases with α-synuclein inclusions fall into one of six groups according to the brain regions involved. Progression between groups involves additional brain areas and worsening of pathology in previously affected brain regions. **a** | Rostrocaudal progression of the pathological process (arrows). Variable red shading reflects the ascending disease process and increasing severity of pathology. **b** | Stage 1: lesions occur in the olfactory bulb, the anterior olfactory nucleus and/or the dorsal motor nuclei of the vagal and glossopharyngeal nerves in the brainstem. Stage 2: lesions are observed in the pontine tegmentum (locus coeruleus, magnocellular nucleus of the reticular formation, and lower raphe nuclei). **c** | Stages 3 and 4: lesions reach the pedunculopontine nucleus, the cholinergic magnocellular nuclei of the basal forebrain, the pars compacta of the substantia nigra (stage 3), the hypothalamus, portions of the thalamus and, as the first cortical region, the anteromedial temporal mesocortex (stage 4). First clinical symptoms of PD appear during stage 3 or early stage 4. **d** | Stages 5 and 6: lesions reach neocortical high-order association areas (stage 5), followed by first-order association areas and primary fields (stage 6). Abbreviation: PD, Parkinson disease.

dementia, than do PD patients without *GBA* mutations. At autopsy, all cases with *GBA* mutations and PD exhibit abundant Lewy bodies and Lewy neurites, many of which also contain glucocerebrosidase.[88] Current models suggest that *GBA* mutations enhance, but do not initiate, the aggregation of α-synuclein.[89] This work has established a link between lysosomal dysfunction, α-synuclein aggregation and PD.

Heterozygous mutations in the leucine-rich repeat kinase 2 (*LRRK2*) gene are a common cause of PD.[90,91] The Gly2019Ser mutation in *LRRK2* is estimated to account for up 1% of idiopathic PD cases and 4% of familial PD cases. Although the physiological substrates of LRRK2 are not known, the Gly2019Ser mutation in the kinase domain is believed to increase its kinase activity. Disease penetrance in individuals with this mutation is age-dependent and ranges from 30–74%.

The neuropathology resulting from the presence of *LRRK2* mutations can be variable.[30,92] The majority of Gly2019Ser carriers with PD have typical Lewy bodies and Lewy neurites. However, some individuals with Gly2019Ser or other mutations in *LRRK2* develop a progressive supranuclear palsy-like syndrome with filamentous tau inclusions, and a third group with *LRRK2* mutations exhibits dopaminergic nerve cell death in the substantia nigra in the apparent absence of filamentous deposits.

Defects in mitochondrial damage repair cause recessive forms of juvenile-onset parkinsonism.[93,94] These forms of disease progress slowly, and the patients experience symptomatic improvements after sleep. The associated proteins Parkin (an E3 ubiquitin ligase) and PINK1 (a mitochondrial protein kinase) function in the same pathway.[95,96] Following the depolarization of mitochondria, PINK1 is stabilized and activated. It then recruits, phosphorylates and activates Parkin on the surface of mitochondria,[97] which results in the ubiquitination of a number of target proteins and the removal of defective mitochondria by autophagy.[98] In juvenile parkinsonism, this pathway is defective because of homozygous and compound heterozygous loss-of-function mutations in *Parkin* or *PINK1*.

Loss-of-function mutations in the *DJ1* gene also cause juvenile parkinsonism.[99] DJ1 may function in the same pathway as Parkin and PINK1, since it has been shown to translocate to mitochondria and to protect against oxidative stress.[100]

Most cases of juvenile parkinsonism with *Parkin* mutations lack α-synuclein inclusions. Of the nine cases examined, six lacked Lewy bodies, two had typical Lewy bodies, and one had Lewy body-like inclusions in the pedunculopontine nucleus and the anterior horn of the lumbar spinal cord.[30] All but the two cases with Lewy body pathology had more-severe nerve cell loss in the substantia nigra than in the locus coeruleus, in contrast to the typical pattern in idiopathic PD.[101] Only one autopsy case with a compound heterozygous mutation in *PINK1* has been reported.[102] Lewy body pathology

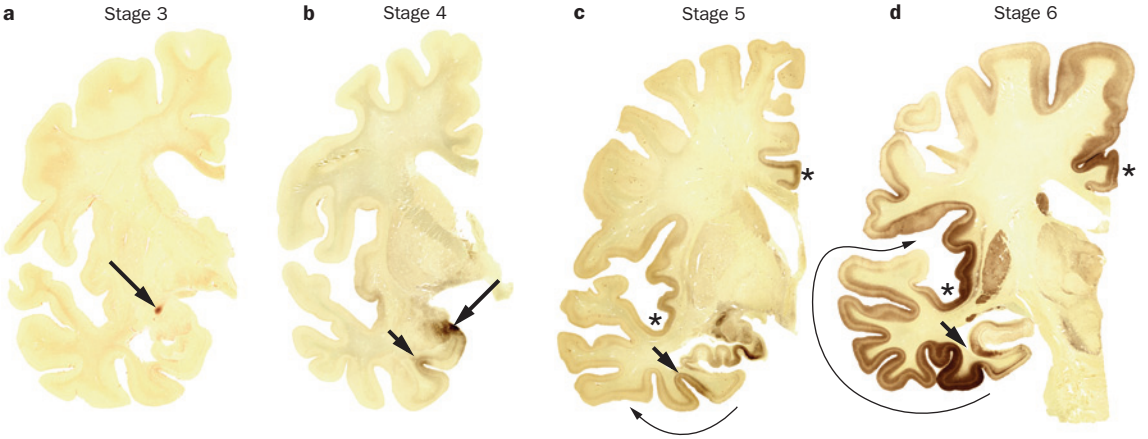


Figure 8 | Stages 3–6 of Parkinson disease pathology. **a** | Stage 3: α-synuclein staining in the central subnucleus of the amygdala (arrow). **b** | Stage 4: the amygdala is more severely affected (long arrow) and α-synuclein staining is also present in the anteromedial temporal transition zone between allocortex and neocortex (short arrow). **c** | Stage 5: a thick network of Lewy neurites is present in the superficial layers of the anteromedial temporal cortex, with Lewy bodies in the projection neurons of the deep layers (short arrow). The disease process encroaches on the insular and cingulate cortices (asterisks). From here, α-synuclein inclusions progress to high-order association fields of the neocortex. Immunoreactivity tapers off as it approaches the secondary and primary fields of the temporal cortex (long arrow). **d** | Stage 6: areas of the insular, cingulate (asterisks) and temporal mesocortex (short arrow) are strongly immunoreactive. Cortical staining increases in severity and extent. The disease process reaches secondary and, in advanced cases, primary neocortical fields, as indicated by staining of Heschl's gyrus (long arrow). Permission obtained from John Wiley and Sons © Braak, H. *et al. Mov. Disord.* **21**, 2042–2051 (2006).[162]

and nerve cell loss were present in the substantia nigra, but not in the locus coeruleus. The brainstem reticular formation and the nucleus basalis of Meynert were also affected. Information from additional autopsy cases is required to establish whether a mechanistic link exists between reduced turnover of defective mitochondria and α-synuclein aggregation.

Multiple system atrophy

Glial cytoplasmic inclusions (GCIs, or Papp–Lantos inclusions) consist of abnormal filaments and are the defining neuropathological feature of multiple system atrophy (MSA), an atypical parkinsonian movement disorder.[103] GCIs are found mostly in the cytoplasm and, to a lesser extent, in the nucleus of oligodendrocytes. The inclusions are also present in some nerve cells. The substantia nigra, striatum, locus coeruleus, pontine nuclei, inferior olives, cerebellum and spinal cord are predominantly affected, and nerve cell loss and gliosis are widespread. The filamentous inclusions of MSA are made of α-synuclein[104–106], but filament morphologies differ between MSA and Lewy body diseases, suggesting that distinct conformers of assembled α-synuclein can give rise to different neurodegenerative diseases.[105] Sequence variation in *SNCA* is a risk factor for MSA, which is largely a sporadic disease.[107,108]

Animal models of synucleinopathies

Loss of function of α-synuclein is probably not pathogenic: α-synuclein-knockout mice do not develop neurodegeneration,[109,110] and mice with knockouts of all three synucleins (besides α-synuclein, vertebrates also express β-synuclein and γ-synuclein; only α-synuclein is found in the disease inclusions) do not exhibit any nerve cell loss.[111] Together with the fact that even a modest overexpression of α-synuclein is detrimental in humans, this identifies a reduction in the level of soluble α-synuclein as a promising approach for the development of mechanism-based therapies for PD and related diseases.[112,113]

Since the identification of the central role of α-synuclein aggregation in PD, DLB and MSA, the human diseases have been modelled in animals.[114] In mice transgenic for human mutant Glu46Lys or Ala53Thr α-synuclein, abundant α-synuclein filaments formed in the brain and spinal cord.[115,116] Surprisingly, in the Glu46Lys line, numerous inclusions consisting of filamentous hyperphosphorylated tau were present alongside α-synuclein inclusions.[116] The formation of α-synuclein inclusions correlated with the development of a movement disorder. In a mouse line transgenic for wild-type human α-synuclein, dephosphorylation of α-synuclein at Ser129 by protein phosphatase 2A protected against neurotoxicity.[117] In these and other models, a major difference with PD was the absence of significant pathology and neurodegeneration in dopaminergic nerve cells of the substantia nigra. This problem has been partly addressed through the production of transgenic mouse lines expressing carboxy-terminally truncated human α-synuclein under the control of the rat tyrosine hydroxylase promoter.[118,119] These mice developed α-synuclein aggregates, a striatal dopamine deficiency and reduced locomotion. However, a transgenic mouse line that fully recapitulates the behavioural phenotype, neuropathology and pathophysiology of PD remains to be produced.

One report described a neurotoxin model of α-synuclein pathology in the rat, which was generated through chronic intravenous administration of the pesticide rotenone, a high-affinity inhibitor of mitochondrial complex I of the respiratory chain.[120] Some rats developed inclusions that were immunoreactive

for α-synuclein and ubiquitin, and showed progressive degeneration of nigrostriatal neurons. The rats exhibited bradykinesia, postural instability and resting tremor. The inhibition of complex I was partial, suggesting that reactive oxygen species can link mitochondrial dysfunction and α-synuclein aggregation. Intragastric administration of rotenone has been reported to cause accumulation of α-synuclein in the enteric nervous system, the dorsal motor nucleus of the vagal nerve, the intermediolateral nucleus of the spinal cord, and the substantia nigra.[121]

Adeno-associated and lentiviral vectors have been used to express human wild-type and mutant α-synuclein in the rodent and primate substantia nigra,[122,123] leading to the formation of Lewy body-like inclusions and the degeneration of many nerve cells. In this system, aggregation of α-synuclein promoted the progressive degeneration of nigral dopaminergic neurons.[124,125]

Expression of human α-synuclein in *Drosophila melanogaster* resulted in the formation of filamentous Lewy body-like inclusions, age-dependent loss of some dopaminergic neurons, and locomotor deficits.[126] Aggregation of α-synuclein was necessary for neurodegeneration, and these effects were modulated by chaperones.[127,128] It is not clear which molecular species caused neurodegeneration, although a prevalent idea is that oligomeric species of α-synuclein are the most neurotoxic. Overexpression of human α-synuclein in *Caenorhabditis elegans* also resulted in dopaminergic nerve cell loss and motor deficits.[129]

Genome-wide screens have identified proteins involved in vesicle transport, lipid metabolism and protein degradation as modifiers of α-synuclein toxicity, indicating that lipid binding and vesicle transport are important for early toxic events.[130] Small organic compounds that inhibit the aggregation of α-synuclein *in vitro* have been identified,[131] but it remains to be seen whether they are beneficial in models of synucleinopathy.

Experimental evidence supports the intercellular transfer of α-synuclein and the seeding of aggregation. Internalized filaments made from recombinantly expressed α-synuclein induced the aggregation of endogenous α-synuclein in mouse primary hippocampal neurons, resulting in synaptic dysfunction and nerve cell death.[132] Moreover, human α-synuclein has been shown to transit from host cells to neurons grafted into the striatum.[133–135] Furthermore, injection of brain lysates from symptomatic mice transgenic for human mutant Ala53Thr α-synuclein into the cerebral cortex and striatum of asymptomatic transgenic mice accelerated the initiation of disease, even in brain regions that were at a distance from the injection sites.[136,137] The effects of brain lysates could be replicated by filaments made from recombinantly expressed α-synuclein. These findings indicate that immunotherapy with α-synuclein antibodies, which is likely to reduce the intercellular transfer of aggregates, may turn out to be an effective mechanism-based therapy for the synucleinopathies.[138,139]

Induced pluripotent stem cell (iPSC)-derived neurons from *SNCA* mutation carriers are likely to occupy an important place between humans and model organisms in future.[140,141] The application of iPSC technology to the modelling of diseases with a long latency and caused by a gain of toxic function mechanism, such as PD, may be challenging. In principle, the earliest pathogenic changes that lead to disease can be studied in these model systems. However, their interpretation may only be meaningful if end-stage pathology also develops over time.

Conclusions

Specific protein aggregates constitute the defining pathological characteristics of the most common neurodegenerative diseases. 100 years ago, Lewy used light microscopy and PD tissue sections to describe the inclusions that were subsequently named after him.[1,2] In the 1960s, electron microscopy showed that these inclusions are made of abnormal filaments.[142] In the 1990s, α-synuclein was identified as the main component of the Lewy pathology filaments.[5,35] A causal connection is believed to exist between inclusion body formation and the degenerative process.[4–7] As a result of these efforts, Lewy's name is better known now than during his lifetime. Prevention of the formation of the pathological inclusions that he first described is a major goal for the years to come.

Review criteria

For the historical parts of the Review, the PubMed database (all years) was searched using, for example, the terms: "dementia with Lewy bodies", "genetics of Parkinson's disease", "Lewy" ("Lewy body", "Lewy neurite", "Lewy pathology"), "multiple system atrophy", "Parkinson's disease" and "synucleins". Selected full-length papers and books available in English were used and articles from the reference lists of these items were used as further leads. Where deemed appropriate, papers and books written in German and French were also consulted. For the remaining parts of the review, literature was obtained from the PubMed database (all years). The authors attempted to achieve a judicious balance between original studies and timely reviews.

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Ingestion of subthreshold doses of environmental toxins induces ascending Parkinsonism in the rat

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Increasing evidence suggests that environmental neurotoxicants or misfolded α-synuclein generated by such neurotoxicants are transported from the gastrointestinal tract to the central nervous system via the vagus nerve, triggering degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and causing Parkinson's disease (PD). We tested the hypothesis that gastric co-administration of subthreshold doses of lectins and paraquat can recreate the pathology and behavioral manifestations of PD in rats. A solution containing paraquat + lectin was administered daily for 7 days via gastric gavage, followed by testing for Parkinsonian behavior and gastric dysmotility. At the end of the experiment, brainstem and midbrain tissues were analyzed for the presence of misfolded α-synuclein and neuronal loss in the SNpc and in the dorsal motor nucleus of the vagus (DMV). Misfolded α-synuclein was found in DMV and SNpc neurons. A significant decrease in tyrosine hydroxylase positive dopaminergic neurons was noted in the SNpc, conversely there was no apparent loss of cholinergic neurons of the DMV. Nigrovagally-evoked gastric motility was impaired in treated rats prior to the onset of parkinsonism, the motor deficits of which were improved by L-dopa treatment. Vagotomy prevented the development of parkinsonian symptoms and constrained the appearance of misfolded α-synuclein to myenteric neurons. These data demonstrate that co-administration of subthreshold doses of paraquat and lectin induces progressive, L-dopa-responsive parkinsonism that is preceded by gastric dysmotility. This novel preclinical model of environmentally triggered PD provides functional support for Braak's staging hypothesis of idiopathic PD.

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INTRODUCTION

While the etiology of Parkinson's disease (PD) is unknown, both genetic and environmental factors have been theorized to play a role in its pathogenesis. In the search for environmental triggers for the development of idiopathic PD, Braak's group hypothesized that an ingested "unknown pathogen" enters the gastrointestinal (GI) tract, and is itself either transported retrogradely via the vagus nerve to the dorsal motor nucleus of the vagus (DMV) within the brainstem, or induces retrogradely spreading neural dysfunction.[1] As a consequence of DMV involvement, the finely-tuned vagal modulation of GI motility is disrupted.[2] Autonomic dysfunction, including delayed gastric emptying and reduced gastric motility, can occur long before the onset of the classical motor symptoms of PD.[3–5] The recent discovery of an anatomical connection between the DMV and the substantia nigra pars compacta (SNpc) (i.e., the nigro–vagal pathway), as well its demonstrated importance in the control of gastric tone and motility,[6] might be the pathway by which the "unknown pathogen" travels or induces the impairment of the neurocircuit from the DMV to SNpc. Disruption of this nigro-vagal circuit may, therefore, explain the prodromal gastric dysmotility observed in PD patients.

In many studies, different pesticides, e.g., rotenone, herbicides, e.g., paraquat, and toxins, e.g., MPTP and 6-OHDA, have been administered by various routes, including oral administration, in order to model idiopathic PD.[7–12] Although very useful as animal models, environmentally induced idiopathic PD in humans is unlikely to result from either single or multiple exposures to high doses of an agent over a short period of time. Rather, individuals are exposed to a myriad of environmental toxins over the course of their lifetime. A more likely scenario, therefore, involves repeated exposures to low doses of toxins, or a combination of toxins, whose pathogenicity may be enhanced by external factors, including diet.[13,14] Epidemiological studies, for example, have demonstrated that the drinking of well water, as well as long-term exposure to pesticides/herbicides and heavy metals, are all associated with an increased incidence of idiopathic PD.[14–19]

Paraquat is a widely used herbicide and, given the strong positive correlation between its use and the incidence of idiopathic PD (reviewed in ref. 20), paraquat administration (up to 100 mg/kg p.o. or i.p., either alone or in combination with the fungicide, maleb), once or twice a week for three–six consecutive weeks is used commonly to induce experimental idiopathic PD.[10] In this model, parkinsonian symptoms are observed typically after at least 4 weeks.[10,21] Other investigators have raised issues, however, with contradictory studies that have not consistently shown loss of dopaminergic neurons in SNpc or replicated reliable parkinsonism following oral paraquat administration, while other reports have failed to show strong evidence for paraquat contamination of food.[22–25]

Dietary factors such as lectins have been implicated in the pathogenesis of idiopathic PD-like pathology in C. elegans.[20] Lectins are ubiquitous carbohydrate-binding proteins that are present worldwide in the human diet.[26] Lectins can penetrate the

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GI tract, either by endocytosis, via a breakdown in gut barrier function, or via a lectin receptor (saccharide)-mediated mechanism, and can be transported retrogradely within neurons.[27–30] While lectins are environmentally pervasive, dietary lectins in properly cooked food are harmless and generally thought to pose no health risk.[26] The consumption of raw uncooked vegetables, grains, and eggs that are rich in lectins, however, can potentially enhance the toxicity of pesticides and herbicides resulting in higher prevalence of idiopathic PD.[17] By virtue of their membrane permeability, lectins have been developed as a chaperones for drugs, but have also been shown to transport viruses and toxin (s),[31,32] including those that may be responsible for α-synuclein inclusions in PD.[33] As such, a lectin-mediated insult is likely to be gradual, and may be influenced by association with other macro/micronutrients or ingested chemicals. It is possible, therefore, that dietary lectins contribute to the transport from the GI tract to the central nervous system (CNS) of pathogens that induce degeneration of dopaminergic neurons and Lewy body-like protein aggregation, i.e., the histological hallmark of idiopathic PD.[34] Thus, lectins may represent a key environmental factor in the development of this disease.

In the present study, therefore, we tested the hypothesis that gastric administration of subthreshold doses of paraquat and lectins induces an ascending pattern of α-synuclein aggregation in the vagus nerve and DMV and consequent gastric dysmotility, followed by degeneration of SNpc neurons and motor features of idiopathic PD.

RESULTS

Incubation of α-synuclein with subthreshold concentrations of lectin and paraquat accelerates the rate of fibril formation in vitro

In vitro incubation of α-synuclein alone ($n = 6$) induced fibril aggregation with a half-time ($t_{1/2}$) of 25 ± 1.9 h. Incubation of α-synuclein in the presence of either paraquat (100 μM; $n = 7$) or lectin (0.0025% w/v; $n = 4$) accelerated the rate of fibrillation ($t_{1/2}$ = 19 ± 0.8 and 18 ± 1.1 h for paraquat and lectin, respectively, $p <$ 0.05 vs. α-synuclein alone). The $t_{1/2}$ fibrillation was accelerated further upon incubation of α-synuclein with a solution containing both paraquat and lectin ($t_{1/2} = 16 \pm 1.1$ h, $p < 0.05$ vs. paraquat or lectin alone; $n = 6$; Fig. 1).

These data suggest that, in combination, paraquat and lectin act co-operatively to accelerate the rate of α-synuclein fibrillation in vitro, compared to either paraquat or lectin alone.

Misfolded α-synuclein is present in myenteric neurons of the GI tract, in the DMV, and in SNpc of paraquat + lectin treated animals

In control rats, ^{129}Ser α-synuclein-immunoreactivity (-IR) was not detected in myenteric neurons of the stomach (Fig. 2a), small (Fig. 2b) or large intestine (Fig. 2c). Two to 4 weeks after the end of the treatment with paraquat + lectin, however, expression of ^{129}Ser α-synuclein-IR was observed in myenteric neurons through the whole extent of the GI tract, including rats that received vagotomy prior to the treatment (Fig. 2d-l). At either 2–4 weeks after the end of the treatment, ^{129}Ser α-synuclein-IR was also observed in choline acetyltransferase (ChAT)- and tyrosine hydroxylase (TH)-positive neurons of the DMV, the A2 area, and the SNpc (Fig. 3d-i) of treated rats, but not in control animals (Fig. 3a-c) or in animals treated with lectin of paraquat alone. In animals ($n = 9$) that underwent complete subdiaphragmatic vagotomy prior to paraquat + lectin administration, ^{129}Ser α-synuclein-IR was not observed in in vagal neurons of the dorsal vagal complex (DVC) or in SNpc (Fig. 3j-l).

These results provide further support to the observation that a histological hallmark of idiopathic PD is observed in enteric neurons, as well as in key CNS nuclei following treatment with paraquat + lectin, but following subdiaphragmatic vagotomy, ^{129}Ser α-synuclein-immunoreactivity is limited to myenteric neurons.

Paraquat and lectin treated animals have impaired motor functions

Prior to treatment with paraquat + lectin, the baseline score for the vibrissae test was 9.6 ± 0.1 successful forelimb placement/10 trials ($n = 12$). Two weeks after the end of the treatment, the score decreased significantly to 6.1 ± 0.7 successful forelimb placement/10 trials ($n = 12$; $p < 0.05$). The impaired motor performance showed no further deterioration, with the score 4 weeks after the end of the treatment being 6.2 ± 0.9 successful forelimb placement/10 trials ($n = 11$; $p < 0.05$ vs. baseline). A significant amelioration of Parkinsonism was evident after four doses of L-dopa, with an increase in vibrissae test scores to 8.3 ± 0.65 successful forelimb placement/10 trials ($p < 0.05$ vs. paraquat + lectin; $p > 0.05$ vs. baseline), supporting the hypothesis that exposure to subthreshold doses of paraquat + lectin induces ongoing nigrostriatal dopaminergic degeneration that is reversibly ameliorated with L-dopa treatment (Fig. 4a).

A similar, but lesser impairment in motor behavior was observed with the stepping test. The baseline stepping test score was decreased significantly to 76.4 ± 4.7% ($n = 9$; $p < 0.05$ vs. baseline), 2 weeks after the end of the treatment with paraquat + lectin. The motor performance continued to deteriorate by

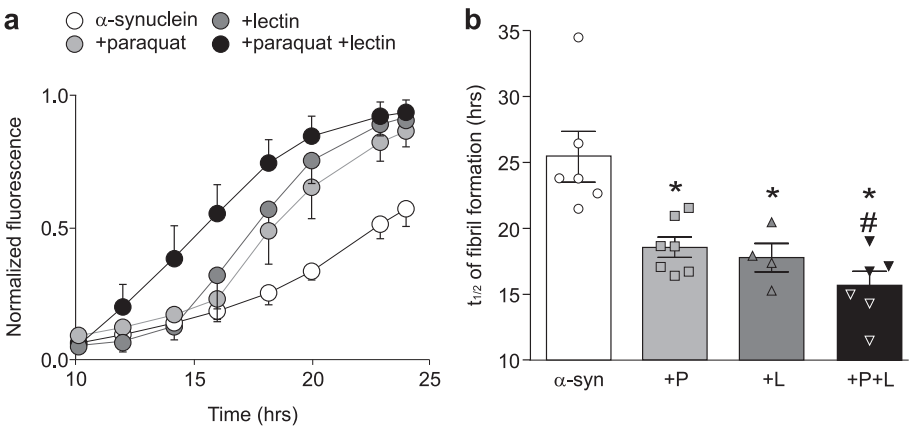

Fig. 1 Incubation with paraquat + lectin increases the rate of α-synuclein fibrillation. **a** Time course of α-synuclein fibrillation in the presence of α-synuclein alone (white, α-syn, $n = 6$), paraquat (light gray, P, $n = 7$), lectin (dark gray, L, $n = 4$) or a combination of paraquat + lectin (black, P + L, $n = 6$). **b** Graphic summary of fibrillation $t_{1/2}$ for α-synuclein. $^{*}p < 0.05$ vs. α-synuclein alone; $^{\#}p < 0.05$ vs. paraquat or lectin

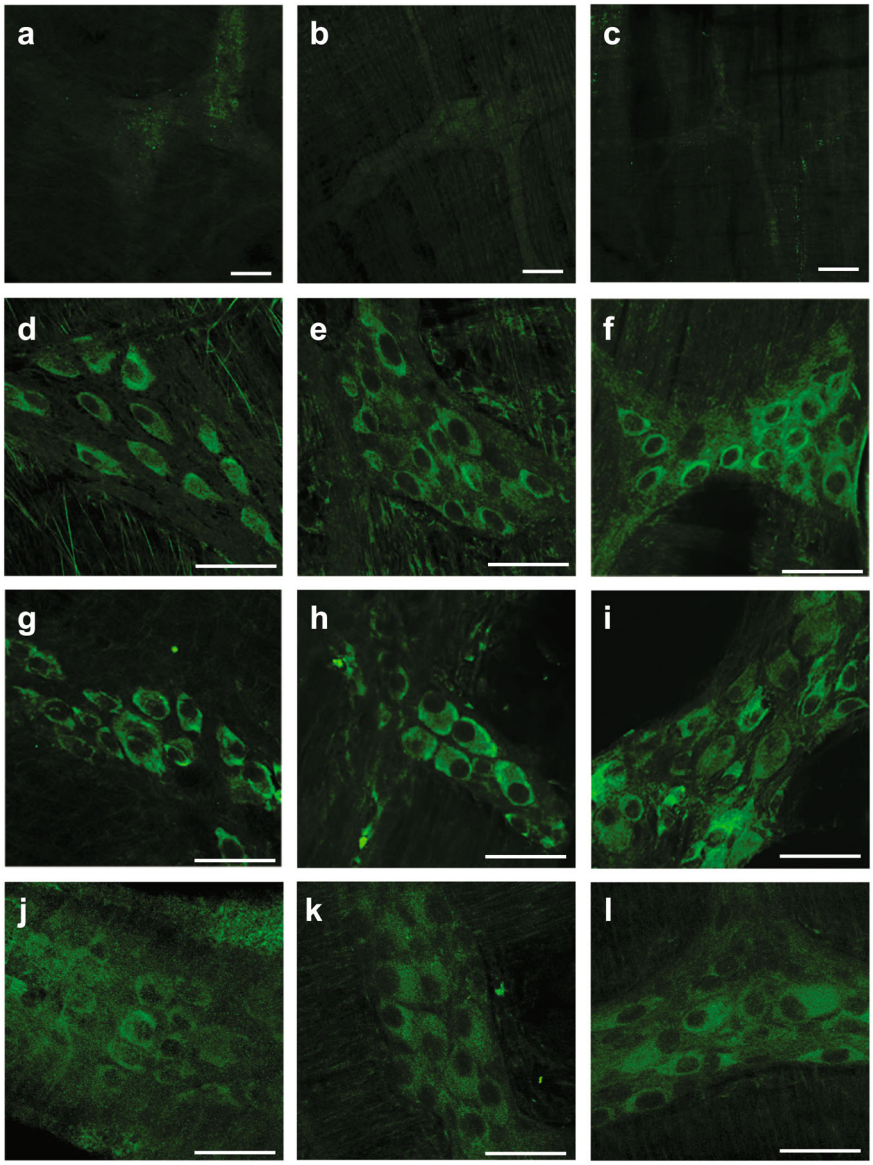

Fig. 2 Paraquat + lectin treatment promotes α-synuclein misfolding in myenteric neurons of the GI tract. Representative micrographs showing ^{129}Ser α-synuclein in the myenteric plexus isolated from the stomach **a**, **d**, **g**, **j**, the small intestine **b**, **e**, **h**, **k**, and the large intestine **c, f, i, l** from control animals (top row), animals sacrificed two (second row) or four (third row) weeks after the end of the gavage with paraquat + lectin, or animals that received subdiaphragmatic vagotomy prior to the paraquat + lectin treatment (bottom row). Calibration bars: 50 μm

4 weeks after the end of the treatment to 66.4 ± 4.7% ($p < 0.05$ vs. own baseline). Administration of L-dopa did not show a significant amelioration of the stepping impairment, likely determined by the minor impairment observed in this partial forced motor test (Fig. 4b).

As expected, from the extent of the toxin-induced bilateral lesion of the nigrostriatal pathways (see below), these rats did not exhibit any rotational behavior either spontaneously or following L-dopa administration. These brief treatments did not induce any drug-related dyskinesias.

In contrast, the motor performances remained at baseline levels when rats were gavaged with lectin or paraquat alone (10 ± 0 vibrissae test, and 92.2 ± 4 and 85 ± 3% stepping test, $n = 5$ for both groups; $p > 0.05$ vs. P + L).

Similarly, rats that underwent subdiaphragmatic vagotomy prior to the paraquat + lectin treatment did not show motor impairment two weeks after the end of the gavage in either tests (vibrissae: 9.8 ± 0.1, and stepping: 96 ± 1.7% of baseline; $n = 9$; $p > 0.05$ vs. own baseline). At 4 weeks, their motor score was significantly higher than that of nonvagotomized rats at the same time point (vibrissae: 10 ± 0, and stepping: 86.6 ± 1.6% of baseline; $n = 9$; $p < 0.05$ vs. nonvagotomized rats at 4 weeks; Fig. 4).

These data indicate that paraquat + lectin treatment induces parkinsonism that is relieved by L-dopa treatment and it is prevented by subdiaphragmatic vagotomy prior to the treatment.

Paraquat and lectin-treated animals have a decreased number of TH-positive neurons in the SNpc

Stereological estimates showed a significant loss of TH-positive neurons in the SNpc four weeks after the end of the treatment ($n = 3$, 4 for control and P + L, respectively; $p < 0.05$). Nissl staining showed a comparable decline in SNpc neuronal number, suggesting a loss of neurons, rather than a temporary down regulation of TH expression (Fig. 5). Conversely, the number of

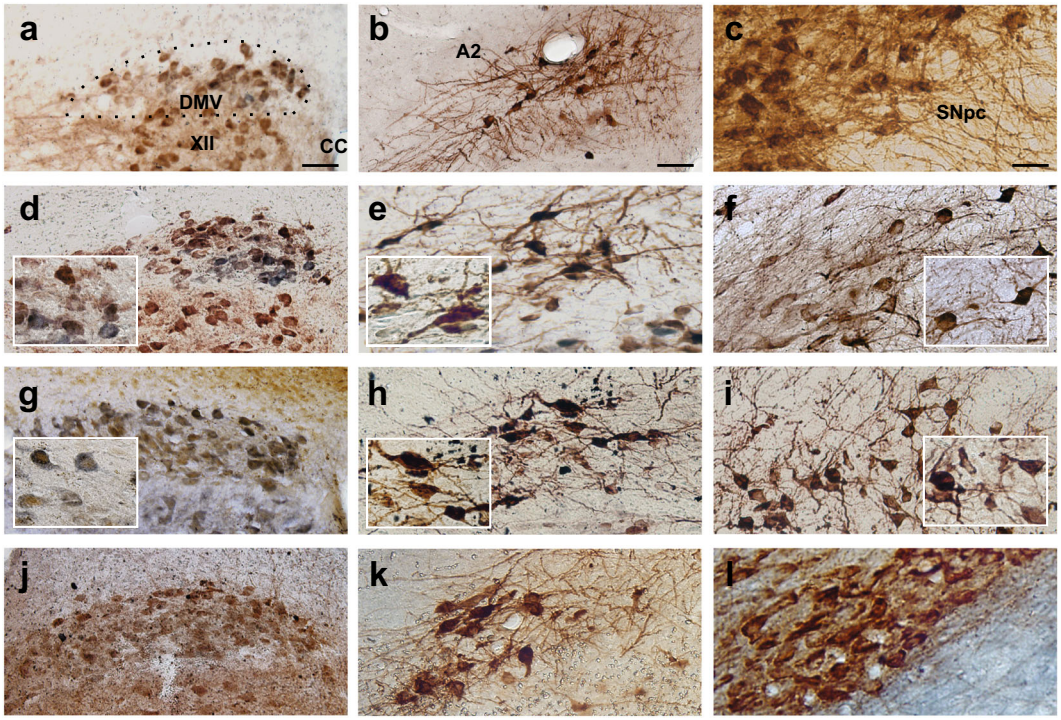

Fig. 3 Misfolded α-synuclein is present in the DMV, the A2 area, and the SNpc after treatment with paraquat and lectin. Representative micrographs showing the co-localization of ChAT- or TH-immunoreactivity and ^{129}Ser α-synuclein-immunoreactivity. Representative micrographs showing the same rostro-caudal level of the DMV in control **a**, 2 **d**, and 4 **g** weeks after the last gavage with paraquat and lectin, or in animals that received subdiaphragmatic vagotomy prior to the treatment **j**. ChAT-immunoreactivity (brown, **a**, **d**, and **j**; blue in **g**) and ^{129}Ser α -synuclein (blue in **a**, **d**, and **j**; brown in **g**); calibration bar: 75 µm. Representative micrographs showing the A2 area in control **b**, 2 **e**, and 4 **h** weeks after the last gavage with paraquat + lectin, or in animals that received subdiaphragmatic vagotomy prior to the treatment **k**. TH-IR (brown) and ^{129}Ser α -synuclein (blue); calibration bar: 75 µm. Representative micrographs showing the same area of the SNpc in control **c**, 2 **f**, and 4 **i** weeks after the last gavage with paraquat + lectin, or in animals that received subdiaphragmatic vagotomy prior to the treatment **l**. TH-IR (brown) and ^{129}Ser α -synuclein (blue); calibration bar: 50 µm. Insets are higher magnifications of their respective panels

ChAT-IR neurons in the DMV was unchanged, i.e., 1900 ± 368 and 2123 ± 126 neurons in control and paraquat + lectin treated rats, respectively ($p > 0.05$). Rats treated with paraquat or lectin alone did not show any decline in SNpc neuronal number ($n = 5$ for both groups; $p > 0.05$ vs. P + L).

These data indicate that animals treated with subthreshold doses of paraquat + lectin induces a significant, bilateral nigros-triatal degeneration of dopaminergic neurons of SNpc, but not of cholinergic DMV neurons.

The Nigro–Vagal pathway that controls gastric motility is impaired following treatment with paraquat and lectin

We confirmed our previous findings[6,35] showing an increase in gastric tone and motility following microinjection of N-methyl-D-aspartate (NMDA, 5 nmoles/200 nl) into the SNpc of control rats. This NMDA-induced gastroexcitation, observed in both the antrum and corpus, was diminished markedly in rats tested either 2 or 4 weeks after treatment with paraquat + lectin. Data for paraquat + lectin are summarized in Fig. 6 (antrum) and Table 1 (corpus).

Similarly, we confirmed previous reports[36] showing that DVC microinjection of thyrotropin-releasing hormone (TRH, 0.1–3 pmoles/60 nl) increased gastric tone and motility in control rats in a dose-dependent manner. Following paraquat + lectin treatment, however, the TRH-induced increase in tone and motility was reduced significantly. Data are summarized in Fig. 6 (antrum) and Table 1 (corpus).

Treatment with either paraquat ($n = 5$) or lectin ($n = 5$) alone, did not alter significantly any of the gastric responses to NMDA or TRH microinjection.

To evaluate if the attenuated increase in gastric tone and motility observed in treated animals in response to central microinjection of either NMDA or TRH was due to an impairment of the gastric smooth muscle functionality, bethanecol was administered (dose: 10 µg/kg i.v.). The bethanecol-induced increases in gastric tone and motility were comparable among all groups, indicating that paraquat + lectin treatment did not compromise gastric smooth muscle. Data are summarized in Table 2.

Together, these data support the hypothesis that gastric administration of subthreshold doses of paraquat + lectin induces an impairment of the nigro–vagal pathway without compromising gastric smooth muscle.

DISCUSSION

In the present study, we demonstrated that co-administration of subthreshold doses of lectin + paraquat produce (i) consistent pathological hallmarks of α-synuclein aggregation in enteric, brainstem, and midbrain neurons, (ii) stable parkinsonism associated with modest, but significant, degeneration of SNpc dopaminergic neurons, and (iii) motor parkinsonism is reversibly treatable with L-dopa. We also demonstrated a sequential progression of α-synuclein aggregation, with inclusions in the DMV preceding those in the SNpc. This temporal pattern of central dysfunction was mirrored functionally, with dysregulated gastric responses to stimulation of either the nigro–vagal pathway or the DVC preceding the development of motor parkinsonism. Finally, animals that received subdiaphragmatic vagotomy prior to

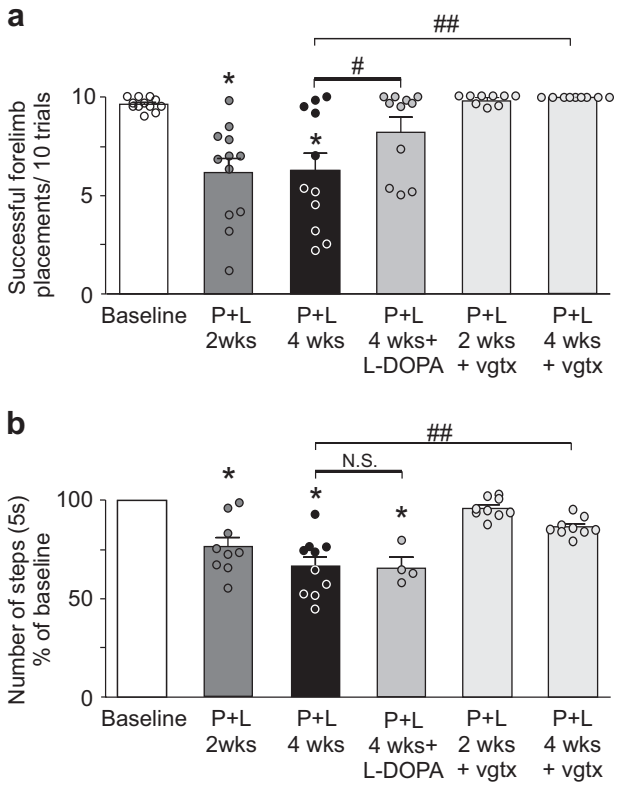

Fig. 4 Paraquat and lectin treatment impairs motor activity. Graphic summary showing the motor performance of rats examined with the vibrissae **a** and stepping **b** tests following treatment with paraquat + lectin. Note that the motor activity was significantly reduced two weeks after the end of treatment and persisted thereafter. L-dopa pretreatment induced a significant improvement of motor performance assessed with the vibrissae test ($n = 12$). Animals that received subdiaphragmatic vagotomy ($n = 9$) prior to the treatment did not show any motor impairment. $^{*}p < 0.05$ vs. baseline

paraquat + lectin administration did not show motor parkinsonism, and the accumulation of misfolded α-synuclein was confined to myenteric neurons, further supporting a vagally mediated progression of the synucleopathy.

The temporal pattern and progression of parkinsonism described in our current animal model replicates faithfully the predictions of a temporally distinct pattern for idiopathic PD[1,37,38] as outlined by Braak's staging hypothesis, i.e., a spread of synucleopathy that starts with the ingestion of an unknown pathogen, which enters myenteric neurons of the enteric nervous system, then travels to the CNS via retrograde transport through the vagus nerve, affecting the DMV (without causing neuronal loss) and the fine vagal modulation of GI motility first and, later, higher areas including the dopaminergic neurons of the SNpc, thus impairing motor functions.

Herein, we have shown that subdiaphragmatic vagotomy restricted misfolded α-synuclein to gastric myenteric neurons following paraquat + lectin administration, with no progressive synucleinopathy being observed in either DMV or SNpc, or Parkinsonism, thus supporting a ENS–vagal route of the pathology. Support for the involvement of the vagus nerve in different phases of PD is found in studies showing that patients who received truncal vagotomy, thereby severing the myenteric neuron–vagal–DMV connection, showed a clear reduction in the incidence of PD.[39] Moreover, a potential vagal pathology in PD is reinforced by findings that the electrogastromyography patterns of PD patients are similar to those of vagotomized patients.[40,41]

Despite multiple studies demonstrating that misfolded α-synuclein can spread and propagate in a prion-like fashion, it is important to note that it is likely that other factors such as unique histological features, mitochondrial stress, and cytosolic calcium levels are responsible for the regional distribution of idiopathic PD pathology.[42] Therefore, it is possible that a combination of the spread of pathogenic α-synuclein together with endogenous factors renders neurons susceptible to damage.

As with most paradigms, the experimental models of PD used currently have many advantages and disadvantages.[43,44] A plethora of studies have shown that exposure to paraquat is correlated positively with parkinsonism in humans.[14,45,46] Indeed, several studies have reported that systemic chronic administration of high doses of paraquat in experimental animals induce some of the hallmark parkinsonian disturbances.[13,14,23,25,45,47] The ability of paraquat itself to cause idiopathic PD has, however, been called into question due to the lack of reproducibility of the pathology using oral dosing models, the high doses used in most animal studies, the lack of paraquat residues in food, and scientific fraud in some reported studies (reviewed in recent letter by Cook et al.[22]).[7–11,24,48] Other toxin models that have stable behavioral outcomes, such as the 6-OHDA induced model in rats, require intracranial administration of the toxin, while systemically administered toxins like rotenone, MPTP, and paraquat do not replicate the route of entry of environmental toxins.[10,49] Moreover, these models induce severe Parkinsonism over a relatively short-time period, which does not reflect the temporal pattern and progressive nature of idiopathic PD. Additionally, models that overexpress α-synuclein, either via genetic manipulation or via recombinant vector administration,[50] either do not replicate the natural course of the disease or fail to replicate the full repertoire of motor deficits.[43]

In contrast, our model replicates the likely route of environmental agents that instigate idiopathic PD pathology, namely ingestion and enteric entry, follows a temporally defined pattern inducing brainstem disruption that precedes the nigral pathology that results in bradykinesia, i.e., the behavioral hallmark of idiopathic PD. The extent of the behavioral deficits observed in our model are milder and more variable, which also simulates the natural course of early idiopathic PD. Our data also provide a putative reason for the variability noted in previous oral paraquat toxicity models in that co-administration of lectin is required to induce a more consistent pathology. Furthermore, this raises the possibility that consumption of raw lectin-rich food in rural communities where chronic environmental exposure to toxins such as paraquat is endemic, provides an underlying mechanistic basis for the pathogenesis of idiopathic PD.[13–18,47] Ingestion of such a diet, therefore, by virtue of the chemical properties of lectins[27–30] may facilitate the absorption and/or transport of toxins in susceptible individuals.[31,32] At the doses and administration route, i.e., oral gavage, used in the present study, however, lectins or paraquat, when administered alone, did not induce any significant effect on the parameters analyzed, i.e., motor performance, gastric emptying, tone and motility, and immunohistochemical properties.

Our data suggest that impairment of both the recently described nigro-vagal,[6] as well as the TRH-activated vagal efferent pathway[36] occur in the absence of functional disruption of the gastric smooth muscle itself. Such gastric dysfunctions were observed prior to impairment of motor control and in advance of SNpc neuronal degeneration, mimicking the prodromal GI issues observed in many parkinsonian patients.

There has also been considerable debate on the mechanism(s) through which paraquat may enter the CNS across the blood–brain barrier.[51] Results from our study suggest that the presence of lectins is also required to induce α-synuclein

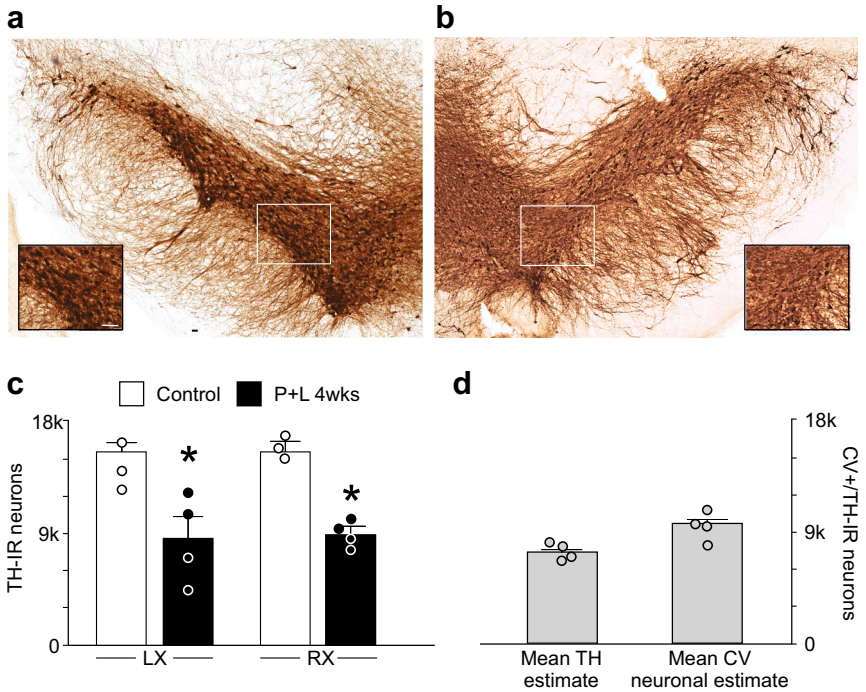

Fig. 5 Treatment with paraquat + lectin induces loss of TH-positive neurons in the SNpc. Representative images of TH-positive neurons in SNpc of control **a** and treated animals **b**. Insets are higher magnifications of the boxed areas in the respective panels. Calibration bar: 100 µm. Graphic summary of TH-IR neuronal number in both the left **c** and right **d** SNpc of control (white) and P + L treated (black) rats. Note that a significant loss of neurons was detected in SNpc of animals 4 weeks after the last gavage of paraquat + lectin ($n = 3$ for controls and 4 for P + L 4 weeks, respectively; $^{*}p < 0.05$ vs. control). **d** Graphic summary showing that the mean estimate number of TH-IR neurons is not significantly different from the mean estimate number of CV-positive neurons in the SNpc of P + L treated animals ($n = 4$)

aggregation in the gut as well as its spread into the CNS via the vagus nerve, and subsequently into the SNpc via the newly discovered nigro–vagal pathway.[6] Further support for this novel mechanism of action is provided by the recent description of α-synuclein accumulation in a subpopulation of enteroendocrine cells that exhibit neuron-like properties and have direct connections to enteric and/or extrinsic nerves.[52] Indeed, the suggestion has been raised that α-synuclein itself could act as a lectin.

In conclusion, our study shows that the ability of orally administered subthreshold doses of paraquat in the presence of lectins to trigger parkinsonian pathology. Although we did not characterize the mechanistic features of this model, the range of neurodegenerative and pathophysiological changes induced by co-administration of paraquat + lectin reproduces many of the cardinal features of the human disease, including, parkinsonism that is responsive to L-dopa therapy, neurocircuit dysfunction, induction of neuronal α-synucleinopathy, neurodegeneration leading to the loss of SNpc dopaminergic neurons while sparing the DMV, as well as prodromal gastric motility disturbances that were observed in absence of smooth muscle impairment. The appearance of gastric dysfunction and Parkinsonism, its prevention by subdiaphragmatic vagotomy, and the distinct sequence of pathological and degenerative changes described herein, make for an attractive experimental model that will help identify triggering factors essential to idiopathic PD etiology, as well as being of use in the discovery of biomarkers and testing of new therapeutics at a stage where interventions would be disease-modifying rather than symptom-alleviating.

METHODS

All procedures were conducted in accordance with the National Institutes for Health guidelines, with the approval of the Penn State University-College of Medicine Institutional Animal Care and Use Committee and according to journal policies and regulations on animal experimentation.

In vitro α-synuclein fibril formation

To examine the effect of paraquat and lectin on the kinetics of fibril formation, an in vitro fibrillation assay was used, as described previously.[53] Briefly, solutions containing: (i) purified recombinant α-synuclein alone (35 µM in 50 mM Tris-HCl buffer, pH 7.5); (ii) lectin from *Pisum sativum* (0.0025%); (iii) paraquat (100 µM); or (iv) a combination of lectin and paraquat, were incubated at 37 °C with constant shaking at 300 rpm for ~40 h. Each sample was plated in triplicate on a 96-well plate, and 20 µM Thioflavine T, a fluorescent dye that binds to fibrillary structures, was added. The fluorescence (excitation at 450 nm and emission at 485 nm) was measured at different time points using a fluorescence plate reader (Spectramax Gemini EM, Molecular Devices, Sunnyvale, CA) interfaced with Softmax® pro 6.3.1 software (Molecular Devices). The relative fluorescence units were averaged and plotted as a function of time; the resulting plot was interpolated, normalized and fitted to a sigmoidal curve using GraphPad Prism® software (GraphPad Software, LaJolla, CA, USA).

Animals and treatment

Male Sprague–Dawley rats were housed in an AAALAC accredited Animal Care Facility maintained at 24 °C on a 12:12 h light/dark cycle. Food and water were provided ad libitum. Rats were gavaged daily, for seven consecutive days, with 1% sucrose (control; $n = 12$) or (i) 1% sucrose and 0.05% lectin from *P. sativum* + paraquat (1 mg/kg, P + L; $n = 20$), (ii) 1% sucrose and 0.05% lectin (L; $n = 5$), or (iii) 1% sucrose and paraquat (1 mg/kg, P; $n = 5$). To promote absorption, gastric emptying was delayed by injection of cholecystokinin (3 µg/kg i.p.) 15 min prior to each gavage. Rats were allowed to recover 2 ($n = 9$) or 4 ($n = 11$) weeks before experimental procedures were carried out. A group of rats received injections of L-dopa (4 mg/kg) and benserazide (15 mg/kg, i.p. diluted in ascorbate saline; $n = 11$) twice a day for 2 days, after the third week of recovery. Rats treated with lectins at doses up to 0.2% were observed for up to 12 weeks.

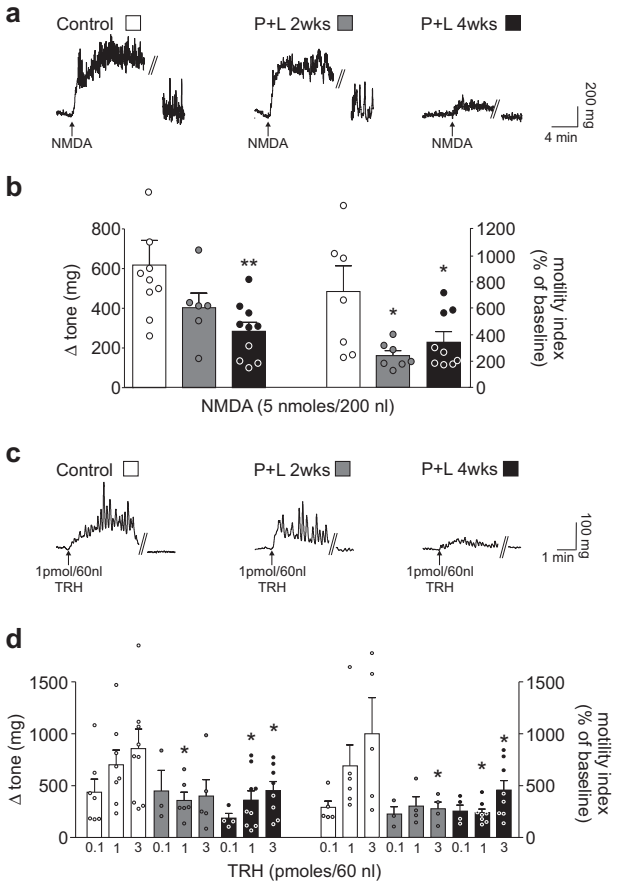

Fig. 6 Treatment with paraquat and lectin induces impairment of the nigro–vagal pathway. **a** Representative recordings from the gastric antrum showing that the increase of tone and motility following microinjection of NMDA in the left SNpc was reduced progressively at 2 and 4 weeks after the last gavage treatment. **b** Bottom panel: graphic summaries showing that 2 or 4 weeks after the paraquat and lectin treatment, microinjection of NMDA in the left SNpc increased gastric antrum tone ($n = 9, 6, 10$ for control, P + L 2 weeks and P + L 4 weeks, respectively) and motility ($n = 7, 7, 9$ for control, P + L 2 weeks and P + L 4 weeks, respectively) to a significantly lesser extent than in controls ($^{*}p < 0.05$ vs. control). **c** Representative recordings from the gastric antrum showing that the increase of tone and motility following microinjection of TRH (1 pmole/60 nl) in the DMV was reduced progressively at 2 and 4 weeks after the last gavage treatment. **d** Graphic summaries showing that, 2 or 4 weeks after the paraquat and lectin treatment, microinjection of TRH (0.1–3 pmoles/60 nl) in the DMV increased gastric antrum tone and motility to a significantly lesser extent than in controls ($^{*}p < 0.05$ vs. control)

A group of rats was anesthetized with isoflurane (2.5% in 100% O_2) and an abdominal laparotomy was performed to expose and severe both posterior and anterior subdiaphragmatic vagal branches, as described previously.[6,54] The efficacy of the vagotomy was assessed with i.p. administration of 0.2 mg/kg fluorogold.

Tissue collection

At the conclusion of the behavioral or gastric experiments (see below), rats were euthanized under deep general anesthesia, rapid sternal thoracotomy and transcardiac perfusion with 200 ml of heparinized saline followed by 200 ml of 4% paraformaldehyde (PFA) in PBS. Brains were removed and postfixed in 4% PFA and 20% sucrose for 24–48 h at 4 °C, and then transferred in a solution containing PBS, 0.08% Na azide, and sucrose. The brains were sliced in 50µm-thick coronal sections using a freezing microtome using either a 1:4 or 1:8 systematic random sampling routine and preserved as floating sections prior to further processing.

Immunohistochemistry

Detailed methodology has been described previously.[55,56] Primary antibodies were (i) rabbit-α-^{129}Ser α-synuclein (Abcam, Cambridge, UK; 1:1000); (ii) goat-α-ChAT (Chemicon, Temecula, CA; 1:5000); (iii) mouse-α-TH (Immunostar, Hudson, WI; 1:10000) or rabbit α-TH (Pel-Freez biological, Rogers, AR; 1:200). For immunoperoxidase staining, secondary antibodies were biotinylated donkey immunoglobulins (IgGs) for multiple labeling (Jackson ImmunoResearch Laboratories, West Grove PA) diluted 1:1000; the detection complex was ExtrAvidin-horseradish peroxidase (ExtrAvidin-HRP; 1:1500). For immunofluorescence staining, secondary antibodies were donkey immunoglobulins Alexa Fluor 488 or 568 (ThermoScientific, Waltham,MA; 1:1000).

Both primary and secondary antibodies were incubated at room temperature on a shaker for 3 days or overnight, respectively. Brain slices were rinsed in PBS, mounted on gelatin-coated slides, air-dried overnight, dehydrated in alcohol, cleared in xylene, coverslipped with DePeX (Electron Microscopy Sciences, Hatfield, PA, USA).

Behavioral testing

A well-established rodent behavioral battery of tests[57,58] was used to identify the parkinsonian phenotype in treated rats, as described previously.[59] Briefly, these consist of the (1) vibrissae-evoked forelimb placement test (*Vibrissae test*), a forced reflex test in which the tester restrains three limbs and allows stimulation of the ipsilateral vibrissae to evoke a reflex ipsilateral forelimb placement on a firm surface. This test is repeated 10 × 3 at each testing session. (2) Stepping test,[58,59] a partial-forced reflex test in which the experimenter holds the testing rat, restraining both hind limbs and one forelimb at a time, with the free forelimb touching a flat surface. The rat is moved sideways along the surface at a rate of 90 cm/5 s in the direction of the testing forelimb. The test is repeated 3× separately for both forelimbs. Results are expressed as percentage of baseline. (3) Post L-dopa/benserazide treatments twice daily for 3 days and behavioral assessment using the vibrissae test at 1 and 2 hours post-treatment.

These motor behavioral tests were used to assess the parkinsonian phenotype prior to treatment (baseline), every week thereafter, as well as on the day of the gastric motility test. To avoid any pharmacological interaction, the last L-dopa treatment was conducted at least 1 week prior to the gastric motility studies

Stereology

In each animal, an entire series of brain sections (1:4 or 1:8), containing the whole SNpc or the whole DVC, were stained using cresyl violet (CV) to identify key anatomical structures and structural integrity. Brain slices in representative groups were stained for TH as described below. TH-positive neurons in the SNpc were quantified using the Stereo Investigator software suite from MBF bioscience with a 100x magnification using a Olympus BX53 microscope (Olympus, Tokyo, Japan) fitted with a digital CCD camera (Hamamatsu, Hamamatsu City, Japan) and a motorized stage (Prior Scientific, Rockland, MA, USA). The total numbers of cells were estimated using the optical fractionator,[60] the coefficient of error was calculated according to Gundersen et al.,[61] and values ≤0.05 were accepted as significant. TH stained sections were counterstained with CV to assess neuronal loss, as opposed to TH down-regulation, and estimated independently using design based stereology as detailed above.

Gastric studies

Gastric tone and motility recordings were performed as described previously.[6,54] Briefly, animals were fasted overnight (water ad libitum) before being anaesthetized deeply with Na-thiobutabarbital (Inactin;® 100–150 mg/kg i.p). After intubation with a tracheal catheter, a midline laparotomy was performed and two custom-made 6 × 8 mm strain gauges (AT Engineering, Hershey, PA) were sutured to the serosal surface of the anterior gastric corpus and antrum in alignment with the circular smooth muscle. Leads were exteriorized, prior to suturing the abdominal laparotomy; the jugular vein was catheterized to permit systemic administration of bethanechol (10 µg/kg), a muscarinic agonist that does not cross the blood-brain barrier and excites the smooth muscle directly supramaximally. Animals were then placed in a stereotaxic frame and were

Table 1. Tone and motility response in gastric corpus following NMDA and TRH microinjections indicate a compromised nigrovagal innervation

	NMDA 5 nmol/200 nl in SNpc		TRH 0.1 pmol/60 nl in DMV		TRH 1 pmol/60 nl in DMV		TRH 3 pmol/60 nl in DMV	
	TONE (mg)	MOTILITY (% of baseline)	TONE (mg)	MOTILITY (% of baseline)	TONE (mg)	MOTILITY (% of baseline)	TONE (mg)	MOTILITY (% of baseline)
Control	410 ± 51.9	553 ± 135	593 ± 133	369 ± 120.2	419 ± 81.7	803 ± 139.7	439 ± 77.5	1020 ± 351.9
P + L 2 weeks	211 ± 63.8*	307 ± 49.7	136 ± 91.5*	247 ± 75	776 ± 272	270 ± 20*	433 ± 228	326 ± 90.9
P + L 4 weeks	255 ± 37*	289 ± 38.2*	161 ± 42.3*	229 ± 61.9	353 ± 75.9	332 ± 66.7*	522 ± 121	536 ± 98.8

*$p < 0.05$ vs. control.
Values expressed as mean ± S.E.M.

Table 2. The gastric motility response to bethanecol i.v. administration shows no alteration of smooth muscle functionality

	Bethanecol i.v. (10 µg/kg)		Bethanecol i.v. (10 µg/kg)	
	Antrum		Corpus	
	Tone (mg)	Motility (% of baseline)	Tone (mg)	Motility (% of baseline)
Control	142 ± 85.0	214 ± 47.6	138 ± 79.4	210 ± 5.0
P + L 4 weeks	166 ± 49.14	160 ± 18.4	161 ± 40.8	194 ± 22.5

Values expressed as mean ± S.E.M.; $p > 0.05$

instrumented for measuring the effects of microinjections in SNpc and DVC on gastric tone and motility as described previously.[6]

The ionotropic glutamate receptor agonist, NMDA, (5 nmoles/200 nl) was microinjected into the SNpc (in mm, rostro-caudal (RC): −5.0 to 5.6 from bregma; medio-lateral (ML): 1.6–2.4 from midline; dorso-ventral (DV): −7.6 to 7.8 from the surface of the dura mater). To assess the effects of direct activation of vagal efferent motoneurons, TRH (0.1–3 pmol/60 nl) was microinjected into the left DVC (in mm, RC: 0.0–0.6 from calamus scriptorius; ML: 0.2–0.4 from midline; DV: 0.5–0.65 from the brainstem surface). All drugs were dissolved in isotonic phosphate buffered saline (PBS, in mM: 115 NaCl, 75 Na_2HPO_4, 7.5 KH_2PO_4; pH = 7.4).

Strain gauges signals were acquired with a Wheatstone bridge, filtered (low pass filter cutoff = 0.5 Hz; AT Engineering), amplified (EXP CLSG-2; QuantaMetrics, Newton, PA, USA) and recorded on a computer using Axotape® 10 software (Molecular Devices, San Jose, CA). Gastric tone and motility were recorded for 2–5 min before and 15–20 min after drug application; the drug-induced effects on tone and motility were calculated through average value of the calibration measures as described previously.[6,35] Since variations in size of the animal and in the strain gauge placement may lead to slight differences in responses between individual animals, each animal served as its own control, and motility data were measured as percentage changes over baseline (=100%).

Gastric motility was calculated using the following formula, as described previously:[6]

$$\text{Motility index percent} = [(N1 \times 1) + (N2 \times 2) + (N3 \times 4) + (N4 \times 8)/t] \times 100.$$

Where N = number of peaks in a particular force range and t = interval time in which the gastric motility is measured. $N1$ = 20–59 mg, $N2$ = 60–100 mg, $N3$ = 101–200 mg, $N4 \geq 201$ mg.

Materials

Unless indicated otherwise, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Statistical analysis

Data are reported as mean ± SEM and in all instances significance was set at $p < 0.05$.

Data were evaluated using one-way ANOVA followed by post-hoc Tukey's multiple comparison test or one sided, paired *t*-test using GraphPad® software (Graph Pad Prism).

DATA AVAILABILITY

The data are maintained by Dr. R. Alberto Travagli, department of Neural and Behavioral Sciences Penn State College of Medicine, and can be made available for review upon request.

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AUTHOR CONTRIBUTIONS

L.A., C.B., F.H.C., L.K., M.P.S., V.K., and R.A.T. performed experiments; L.A., C.B., M.P.S., V. K., T.S., and R.A.T. analyzed data; L.A., C.B., V.K., T.S., and R.A.T. interpreted results of experiments; L.A., C.B., M.P.S., V.K., T.S., and R.A.T. prepared figures: L.A., C.B., V.K., M.P. S., T.S., and R.A.T. drafted and edited manuscript: all approved final version of manuscript.

ADDITIONAL INFORMATION

Competing interests: The authors declare no competing interests.

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