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COMMENTARY LETTER

Alpha-synuclein: prion or prion-like?

Rehana K. Leak[1] · Matthew P. Frosch[2] · Thomas G. Beach[3] · Glenda M. Halliday[4]

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Misfolded alpha-synuclein is a corruptive seed, but is it infectious?

Alpha-synuclein is a natively unfolded or intrinsically disordered protein, but it can also assume amphipathic alpha-helical shapes in the presence of negatively charged lipids. In Lewy body disorders, alpha-synuclein monomers aggregate into oligomers, protofibrils, and fibrils, forming part of the hallmark amyloid inclusions known as Lewy bodies and Lewy neurites. Proteins exist in biological settings in multiple conformations, often with varied biological functions, and the transitions between conformations include structures that seed the nucleated growth of aggregates [10]. After the kinetic barrier to aggregation of natively monomeric proteins is (rarely) overcome, the global free-energy minimum favors the precipitation of hydrophobically packed protein masses [15]. Once this low-energy state is acquired, it may not be energetically feasible to proteolyze and resolve the protein mass, and therefore, "the aggregate state always wins," in the words of Guest et al. [15].

The thermodynamic principles outlined above would favor the retention of aggregated forms of alpha-synuclein in Lewy inclusions, and the aggregated alpha-synuclein could then provoke the polymerization of neighboring, native alpha-synuclein molecules into higher-order species, perhaps by imprinting its beta-sheet template onto monomers—not unlike the scrapie-associated prion protein (PrP^{Sc}). PrP^{Sc} (scrapie) is responsible for inherited and acquired forms of spongiform encephalopathy in cattle (bovine spongiform encephalopathy), sheep (scrapie), deer and elk (chronic wasting disease), and humans (Kuru, Creutzfeldt–Jakob disease, variant Creutzfeldt–Jakob disease, fatal familial insomnia, Gerstmann–Sträussler–Scheinker syndrome). The term prion, coined by Stanley Prusiner, is a portmanteau of two unambiguous words, "protein" and "infection." Therefore, a prion particle is, by definition, the underlying cause of an infectious disease, rather than a secondary consequence of disease processes or a generalized stress response, and it is purely proteinaceous in nature.

In an influential series of histopathological investigations, Heiko Braak, Kelly Del Tredici et al. laid the groundwork for the speculation that misfolded versions of alpha-synuclein act as "prion-like" molecules, traveling transneuronally across brain circuitry in a stereotypic fashion, leaving in their wake a "falling row of dominoes" and exposing six stages of Parkinson's disease [4–6, 11]. The provocative "Braak hypothesis," as it came to be known, spawned a large body of work addressing new concepts that (1) the olfactory and gastrointestinal mucosae serve as peripheral entry points for an unnoticed invading agent, prion-like or otherwise, and (2) the alpha-synucleinopathy is then transcellularly transmitted deep into the brain, through a preestablished set of neural circuits, and is responsible for the onset of disease in Lewy body disorders.

If we assume that alpha-synuclein is not prion-like, but a bona fide prion, it then follows that a misfolding event in alpha-synuclein might be the cause of Lewy body disorders (including in patients without an inherited form of disease), rather than being a consequence of the disease process. This postulate differs in important ways from the notion that alpha-synuclein aggregation is an innocent bystander of disease or a natural protective mechanism [33, 49]. According to the latter concepts, misfolded alpha-synuclein molecules are tucked away in somal Lewy inclusions, shunted from the synapse, axon, and critical organelles. This mechanism mirrors a garbage dump full of nonrecyclable waste, built in a sheltered location that does not contaminate human living spaces. A parallel argument was dubbed the "airbag

✉ Rehana K. Leak
leakr@duq.edu

[1] Pharmaceutical Sciences Division, Graduate School of Pharmaceutical Sciences, Duquesne University, 407 Mellon Hall, Pittsburgh, PA 15282, USA

[2] C.S. Kubik Laboratory for Neuropathology, Massachusetts General Hospital, Boston, MA 02114, USA

[3] Civin Laboratory for Neuropathology, Banner Sun Health Research Institute, Sun City 10515, USA

[4] Brain and Mind Centre and Central Clinical School, University of Sydney, Sydney, NSW 2050, Australia

problem", in which investigators ignorant of the actual reasons for airbag use measure a robust correlation between airbag deployment and car crashes, and conclude that airbags are the cause of the crash, rather than being specifically manufactured as a defense mechanism [26].

In previous centuries, the field of microbial infections contended with similar problems in distinguishing correlation from causation. In the words of the physician and microbiologist Robert Koch,

> "…from the mere coincidental relation of tuberculous affections and bacilli, it may not be concluded that these two phenomena have a causal relationship, notwithstanding the not inconsiderable degree of likelihood for this assumption that is derivable from the fact that the bacilli occur by preference where tuberculous processes are incipient or progressing, and that they disappear when the disease comes to a standstill." [23]

Criteria that distinguish causal links from correlations

In his experiments that proved causality, Koch grew bacteria from patients' sera, for months in culture, which, when injected into animals, elicited a phenotypically analogous disease, whereas animals inoculated with serum lacking any bacterium failed to display similar symptoms. Sir Austin Bradford Hill subsequently described nine criteria for causal associations in epidemiological studies. The latter are copied and pasted below from Fredricks and Relman [13], to encourage their application to alpha-synuclein as a potential disease-causing entity:

1. Strength of association (relative risk)
2. Consistency of association
3. Specificity of association (outcome is unique to the exposure)
4. Temporality (exposure precedes outcome)
5. Biological gradient (evidence of dose–response)
6. Plausibility
7. Coherence (compatibility with present knowledge)
8. Experimentation (controlled manipulation of exposure should change outcomes)
9. Analogy (causal relationship conforms to previous relationships) [13]

According to criterion 8 and the very definition of a prion, scientists would need to show that alpha-synuclein protein itself, in the absence of other exogenous factors (e.g., nucleic acids, bacterial lipopolysaccharides, and proinflammatory cytokines) can initiate the histological and behavioral manifestations of Lewy body disorders, and that its removal from the host organism therefore abolishes the onset of disease. In the latter context (i.e., removal from host), two key experiments demonstrate a failure of transmission of alpha-synucleinopathy to *SNCA* knockout mice [22, 42], as well as after immunodepletion of alpha-synuclein from diseased human tissue that is then transplanted into mice [42]. These two observations lend credible support to the essential nature of alpha-synuclein protein for the emergence of disease—in the animal models. They do not, however, reveal whether other forms of Lewy-like pathology, consisting of ubiquitin, filaments, lipids, and organelles [46], can still materialize in the absence of alpha-synuclein protein.

Some reports suggest that the loss of neurons and the cardinal neurological deficits in Lewy body disorders are triggered by factors other than alpha-synucleinopathy per se [36, 37]. First, brain structures with Lewy inclusions do not necessarily display frank neuronal loss, and, conversely, Parkinson's patients may suffer cell loss in specific regions (e.g., presupplementary motor cortex) with sparse or no burden of Lewy bodies [8, 17, 20, 31, 35, 39, 43]. Second, neuronal loss may transpire before Lewy inclusions appear, such as in the substantia nigra during the early Braak stages I–II (flouting Hill's criteria 3–4) [32]. Third, A53E and G51D mutations in alpha-synuclein, which are associated with inherited forms of Parkinson's disease, delay—rather than accelerate—the fibrillization of alpha-synuclein [12, 14]. On the other hand, when minimal threshold levels of alpha-synucleinopathy are present, significant clinicopathological correlations are indeed observed, including deficits in movement and cognition [2] (fulfilling Hill's criteria 1–2). Additional research is required to determine if alpha-synuclein oligomers are a more reliable source of cellular injury than the relatively mature Lewy inclusions visualized by popular histopathological methods.

James Surmeier argued that varying degrees of vulnerability of neuronal subpopulations better explain the non-contiguous emergence of Lewy inclusions across space and time within the host, rather than prion-like transmission [47]. To date, it also remains unclear to what degree alpha-synucleinopathy contributes to—rather than correlates with—neuronal demise and an idiopathic clinical syndrome, particularly if few inclusions are present. Although behavioral deficits in patients may be triggered by surpassing a threshold of neuronal loss rather than Lewy bodies and neurites, it also seems likely that alpha-synuclein aggregates perturb axonal function, which might trigger cell death, if sufficiently chronic or intense [51].

Is the debate merely semantic?

The most significant human evidence favoring the prion-like properties of alpha-synuclein derives from transplant studies, which demonstrated that embryonic tissue engrafted

into the striata of Parkinson's patients acquired Lewy inclusions over the course of a decade or more, with the inclusions increasing over time, in a roughly "dose-dependent" manner, from 3 to 5% of grafted cells to ~ 30% [24, 25, 27, 28] (Hill's criteria 4, 5, and 8). However, whether these observations reflect the prion-like transmissibility of alpha-synuclein, or the cellular toxicity of other factors present in the diseased host brain, including inflammatory mediators, reactive oxygen and nitrogen species, and bioenergetic disequilibria, continues to be debated. No overt cell death was apparent in the transplanted human tissue, again supporting the uncoupling of Lewy inclusions from cell loss (see Hill's criteria 1–3).

After a spate of tissue transplant studies, it became important to examine whether relatively pure preparations of alpha-synuclein could induce de novo alpha-synucleinopathy. In a series of landmark studies from the Lee lab, Volpicelli et al. and Luk et al. collected support for the idea that recombinant, preformed fibrillar alpha-synuclein protein can induce disease in wildtype cells and animals [30, 52]. Compelling evidence favoring transneuronal transport of alpha-synuclein into the brain from the periphery, including the gut, has also been collected [22, 50]. Indeed, alpha-synuclein assemblies appear to cross into the brain from the oral, intravenous, intraperitoneal, intraglossal, and intramuscular routes [7, 29, 38, 45]. Some of the latter data have been collected in transgenic animals, in which Beekes et al. argued the inoculum might simply increase or accelerate the preordained precipitation of forcibly overexpressed alpha-synuclein in vulnerable brain regions, rather than representing an "infectious trigger" [3]. These transgenic animals are, after all, genetically engineered to display amyloidogenic proteins, and, despite this experimental design, do not faithfully recapitulate the human alpha-synucleinopathies anyway.

The appropriateness of the term prion versus prion-like is not an inconsequential debate, but one with significant implications for our understanding of the etiology of Lewy body disorders, and how patients and their biopsied tissues are treated in the home, clinic, hospital, surgery room, and research laboratory. In the words of Adriano Aguzzi, "prionoids sport predatory behavior akin to that of prions," but they "do not spread within communities or cause epidemics" [1]. In contrast, prions such as serum amyloid A display true elements of infectivity in geese and cheetahs, including uptake, replication in permissive hosts, and excretion [1]. It is worth noting that excretion of infectious PrP^{Sc} from the host may represent the mechanism of spread for scrapie and chronic wasting disease in the wild [16, 48]. However, patients are not infected by casual contact with nasally secreted or aerosolized prions, although nasal brushings collected from the olfactory epithelia of Creutzfeldt–Jakob disease patients have prion-seeding activity in real-time quaking-induced conversion tests [34]. Rather, patients seem to acquire the transmissible disease by consuming prion-infected brain matter or being subjected to invasive medical procedures with infected biological materials or surgical instruments.

Are there inherited, sporadic, and acquired forms of Lewy body disorders?

Kuru was transmitted in the natural world due to ritualized mortuary cannibalism in the Fore region of New Guinea. Prions may also be transmitted across the species barrier, such as when humans consume PrP^{Sc}-infected tissues. It is easy to imagine that calorically dense and prion-rich brain tissues would have been consumed at every opportunity by prehistoric hominid hunters. Even modern societies have consumed goat and sheep brains as a part of their traditional diet, including cervelle de veau, part of the French cuisine. The bovine spongiform encephalopathy outbreak was traced to infected cattle meat, and the Centers for Disease Control states that ~ 230 patients with variant Creutzfeldt–Jakob disease have been reported worldwide since 1996. In addition, > 450 cases of Creutzfeldt–Jakob disease were traced to clinical treatment with cadaver-derived human growth hormone or gonadotrophin, dura mater or cornea transplantations, implantation of surgical or EEG recording tools, and blood or plasma transfusions [3]. Prion infections are, therefore, a rarity, compared to the incidence of Parkinson's disease, which affects more than 1200 individuals per 100,000 in industrialized nations [40].

It is not known why alpha-synucleinopathies do not naturally emerge in other vertebrates, whereas prion diseases clearly do. Given the human-specific nature of Lewy body disorders and the paucity of dietarily acquired prion diseases, the statistical probability that alpha-synuclein aggregates within consumable animal products give rise to Lewy body disorders seems low (see Hill's criterion 6), even if meat consumption is correlated with a higher risk of Parkinson's disease [21]. Prospective studies on this topic are limited. On the other hand, amyloid proteins produced by gut bacteria can seed alpha-synucleinopathy in the nervous systems of vertebrate and invertebrate models [9].

If future experiments demonstrate that exposure to brain or other tissues transmits Lewy body disease, even if iatrogenic, we could state with greater confidence that there are three forms of Lewy body disorders—inherited, sporadic, and acquired. One 2013 study tested this hypothesis, but failed to gather support for the idea that Parkinson's disease could be acquired from cadaver-derived human growth hormone [18]. It is unfortunate that the authors of this study did not include the terms "multiple system atrophy" in their search for iatrogenic transmission, given the work of the

Prusiner lab on this condition [41]. In their report, Irwin et al. cited unpublished findings from the National Institutes of Health, in which there was no evidence of Parkinson's pathology in primates inoculated with diseased human tissue [18]. More recently, however, exposure to cadaver-derived human growth hormone has been associated with the development of Alzheimer's disease [19, 44].

Conclusions

Few would insist that similar molecular processes are not at play with alpha-synuclein and PrP^{Sc}. Nevertheless, given the lack of unequivocal evidence that exposure to alpha-synuclein aggregates is the cause of a communicable form of disease in the natural environment, many authors employ the term "prion-like" for alpha-synuclein. We have learned that preclinical models of disease suffer from grave limitations, and that the most parsimonious narratives about human conditions (i.e., Occam's razor) can be fundamentally wrong. It is exceedingly difficult to prove causality when incubation periods are long, when there are confounding effects of host factors, such as inflammation, and when reliably translatable preclinical models are largely unavailable. Further complicating matters, humans likely display robust immune responses against foreign protein aggregates, given the phylogenetically ancient nature of amyloid, and our bodies may clear protein aggregates unexpectedly well. If this were true, prionoids might struggle to gain a foothold in the human body, unless, perhaps, the concentration surpasses a threshold or aging processes diminish protein clearance.

Guest et al. wrote that infection is not simply a matter of biochemistry, but a "multidimensional question of biological, epidemiological, and sociological origin" [15]. Koch's avoidance of casual use of the word infectious therefore seems instructive today. Obviously, we must avoid the application of Koch's postulates with a "mathematical zeal not warranted in the biological world", as maintained by Fredricks and Relman [13]. For example, disregarding the prion-like properties of alpha-synuclein might lead to accidental human disease and decelerate progress in the field of rational drug design, such as anti-alpha-synuclein vaccination therapies. On the other hand, Fredricks and Relman also concluded, "the power of Koch's postulates comes not from their rigid application, but from the spirit of scientific rigor that they foster. The proof of disease causation rests on the concordance of scientific evidence, and Koch's postulates serve as guidelines for collecting this evidence."

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