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Perspectives on the Intracellular Bacterium *Chlamydia pneumoniae* in Late-Onset Dementia

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Abstract

Purpose of Review Chronic diseases remain a daunting challenge for clinicians and researchers alike. While difficult to completely understand, most chronic diseases, including late-onset dementias, are thought to arise as an interplay between host genetic factors and environmental insults. One of the most diverse and ubiquitous environmental insults centers on infectious agents. Associations of infectious agents with late-onset dementia have taken on heightened importance, including our investigations of infection by the intracellular respiratory bacterium, *Chlamydia pneumoniae* (*Cpn*), in late-onset dementia of the Alzheimer's type.

Recent Findings Over the last two decades, the relationship of this infection to pathogenesis in late-onset dementia has become much clearer. This clarity has resulted from applying contemporary molecular genetic, biochemical, immunochemical, and cell culture techniques to analysis of human brains, animal models, and relevant in vitro cell culture systems. Data from these studies, taken in aggregate form, now can be applied to evaluation of proof of concept for causation of this infection with late-onset disease. In this evaluation, modifications to the original Koch postulates can be useful for elucidating causation.

Summary All such relevant studies are outlined and summarized in this review, and they demonstrate the utility of applying modified Koch postulates to the etiology of late-onset dementia of the Alzheimer's type. Regardless, it is clear that even with strong observational evidence, in combination with application of modifications of Koch's postulates, we will not be able to conclusively state that *Cpn* infection is causative for disease pathogenesis in late-onset dementia. Moreover, this conclusion obtains as well for the putative causation of this condition by other pathogens, including herpes simplex virus type 1, *Borrelia burgdorferi*, and *Porphyromonas gingivalis*.

Keywords *Chlamydia pneumoniae* · Infection · Pathogenesis · Late-onset dementia · Alzheimer's disease

Introduction

Historically, chronic disease genesis has been difficult to understand, and many diverse mechanisms have been proposed

to explain its occurrence. Most chronic diseases currently are thought to result from multifactorial interactions among host genetic factors and a variety of environmental insults [1]. This holds true for late-onset dementia of the Alzheimer's type (referred to as late-onset dementia below for simplicity), and quite possibly for other more generally delineated late-onset dementias. Unlike familial Alzheimer's disease (FAD), late-onset dementia is not a result of specific gene mutations; rather, it is an interplay between genetic risk factors, such as polymorphisms of the *ApoE* locus on chromosome 19, and environmental exposure to various influences, including infection [2–6]. Over the past 30 years, numerous reports have linked infectious agents with late-onset dementia [7••]. Various pathogens including viruses, bacteria, fungi, and parasites have been postulated to be associated with different populations of late-onset dementia sufferers. Importantly, critical questions have arisen regarding whether one or more of these

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agents is/are causative in disease genesis, or whether they are merely associated with disease as a result of brain changes obtaining during the process of disease genesis. These questions have led many researchers to evaluate the presence of proposed pathogens in brain tissues and fluids from late-onset dementia patients, most of which had been obtained at autopsy via a variety of technical approaches. The technical approaches to assessing pathogen presence and causation of damage have ranged from molecular genetic analyses to immunohistochemistry to biochemistry to culturing and various forms of ultrastructural analysis. Further, animal modeling studies have attempted to correlate infection with the pathology arising in late-onset dementia. We discuss these and other studies in detail in this review, and we emphasize those studies that focus on the possible role of infection with the obligate intracellular pathogen *Chlamydia pneumoniae* (*Cpn*) as an agent of disease genesis.

Implication of the Olfactory System in Brain Infection

Through detailed study of the brain from late-onset dementia patients, we obtained evidence for the association of specific infections with disease. Evaluation of particular regions of the brain for infection, as well as knowledge of structure-function relationships relevant to entry mechanisms to these regions, is vital to understanding the specificity of brain infection as it affects the onset of disease pathogenesis. The earliest damage in late-onset dementia is seen in the lateral entorhinal cortex, with synaptic changes followed by damage in the hippocampal formation; both of these function to receive olfactory information [8, 9]. Intriguingly, the “olfactory vector hypothesis” suggests that diseases such as Alzheimer’s disease (AD) and Parkinson’s disease (PD) may be caused or triggered by agents entering the brain through the nose. This hypothesis is not a new concept and follows from observations that smell loss and olfactory bulb pathology precede by a decade the symptomatic phase of AD and PD, along with evidence that numerous airborne xenobiotics can enter the brain through the nasal olfactory mucosa [10–13]. A large body of literature provides evidence that infectious agents, metals, herbicides, and prions utilize the olfactory network for access into the brain [14]. These findings do not unequivocally associate any of these agents with specific disease causation, however, and thus, there is a significant need to characterize more extensively infectious involvement of the olfactory network in late-onset dementia. We are not suggesting here that any single agent is responsible solely for non-genetic triggering or causation in late-onset dementia; however, a focus on underlying infections in the olfactory system is of real importance. The importance of infections and other environmental insults with relevance to olfaction is well-reviewed in a recent article on olfactory dysfunction in the pathophysiological continuum of dementia [15]. In this regard, our discovery of *Cpn* in the

late-onset brain is relevant, since this is a ubiquitous intracellular respiratory pathogen which has been shown to infect the olfactory regions of the brain [2]. *Cpn* has been identified in both human autopsy samples and mouse olfactory bulbs in experimental animal models [2, 16, 17, 18••]. In animals, the organism appears to spread in a centripetal fashion from the olfactory bulbs into the brain [16, 17, 18••, 19]. We do not understand completely the consequences of all of these data, but over the past 20 years, we have uncovered significant characteristics of this infection that may lead to a better global understanding of infectious insult in late-onset dementias in general [20••].

Alzheimer’s neuropathology has been identified in the olfactory mucosa [21], the olfactory bulbs [22, 23, 24••, 25], the anterior olfactory nucleus [23, 26], and deeper structures of the olfactory cortex [27–29]. Some debate exists as to whether pathology first appears in the peripheral olfactory structures or in deeper brain structures prior to olfactory involvement [30–32]. This is in part due to interpretation of the amount or density of pathology found in areas such as the amygdala and hippocampus, as compared to peripheral purely olfactory structures [9]. A xenobiotic using the olfactory pathway for entry to the brain may or may not initiate overt damage to olfactory peripheral cells prior to changes in the primary brain regions receiving input. Interestingly, in patients with mild cognitive impairment, β -amyloid aggregates have been shown in the olfactory mucosa [21], and sensory deficits including olfaction are detectable in the preclinical phase of late-onset dementia [33]. These observations suggest that peripheral olfactory structures may exhibit early insult prior to deeper brain changes. Taken together, these data may suggest that a relationship exists between the neuropathology in late-onset dementia and infection with *Cpn* in the olfactory network. Further characterization is critical to advancing our knowledge of how infection may trigger and/or exacerbate neuropathogenesis. These and other observations outlined below support the idea that infection by this pathogen is an early event in the initiation of neuropathogenesis, and not a consequence of prior damage providing access for infection of the central nervous system.

Evaluation of *C. pneumoniae* in the Human Brain

Our initial report described finding DNA of *Cpn* in 90% (17/19) of postmortem brain samples from patients with late-onset dementia, using specific PCR assays [2], but in only 5% (1/19) of postmortem, age-matched, non-demented, control brain samples. In 17/19 brains from dementia patients, positive samples were obtained from at least one area with neuropathology (e.g., temporal regions, entorhinal cortex, hippocampus, parietal cortex, pre-frontal cortex), and in four cases, from the cerebellum. In the latter brains, severe neuropathology existed throughout. In the two brains from dementia patients that were

PCR-negative, only mild pathology was observed, suggesting that a relationship exists between infection and significant pathological changes. Samples from PCR-positive brains also were immuno-positive for *Cpn* antigens in perivascular macrophages, microglia, and astroglia. Immuno-electron microscopy using anti-chlamydial antibodies revealed both the infectious form of *Cpn*, termed elementary bodies (EB), and the actively metabolizing form termed reticulate bodies (RB) in the PCR and antigen-positive brains [2, 34].

Frozen brain samples revealed intact bacterial mRNA specific to *Cpn*, and recovery of viable bacteria was successful from homogenates of 2 brains positive by PCR and RT-PCR; however, it was negative from 2 control brains [2]. These findings suggest that *Cpn* as an intracellular bacterium infected the demented individual at some point during life and not simply by infection at the time of death, since the organism would not have had time to infect cells in the brain tissue. Analysis of the entire olfactory network from control, mild cognitive impairment, and dementia patients could address the correlation of infection to the onset of pathology. This approach would significantly assist in elucidating when infection occurs in relation to pathology and symptom diagnosis. Additional analyses in our original studies revealed that 11 PCR-positive samples had at least one allele for the *APOE* $\epsilon 4$ isoform (64%), consistent with that allele being a risk factor for late-onset dementia [6], as well as a modulator of the C-complement response to pathogens. Interestingly, a separate study in individuals with reactive arthritis showed *Cpn* DNA in their synovial tissues; 68% had at least one copy of the *APOE* $\epsilon 4$ allele [3]. These observations implicate a relationship between the *APOE* $\epsilon 4$ allelic genotype and infection by *Cpn* and that together both factors may confer increased risk for chronic disease genesis [2, 3].

Following our initial studies, other groups attempted identification of *Cpn* in brain tissue and other samples from patients with late-onset dementia. Those studies provided mixed results, with some reports giving positive identification [35, 36], and others failing to find DNA or antigens, for the most part, in their samples [37–40]; many different techniques were used in these studies, with no other study using identical methodology to our own. Specifically, positive and negative reports utilized PCR and immunohistochemical techniques that differed from one another with regard to protocols and samples examined. Some used formalin-fixed brain samples and others used frozen brain samples for PCR. For immunohistochemistry, different laboratories used different antibodies and dilutions thereof, antigen retrieval or not, and different preparations of sections with regard to fixation and thickness. In fact, one study used impression slides of frozen tissue in their immunohistochemical approach [40]. Knowing full-well of the capriciousness of the techniques used by different laboratories, these varied results are not surprising; our initial study used a variety of molecular, immunohistochemical,

ultrastructural, and culturing techniques for these very reasons. In a previous review of other literature in which *Cpn* was implicated as a factor in disease genesis, discrepancies in analytical methods among laboratories, and the variable data resulting from them, were pointed out [41].

Acknowledging these discrepancies, we continue to use, and have expanded upon, all techniques used in our initial studies to evaluate human brain tissues for infection. Other independent reports for *Cpn* in human cerebrospinal fluid (CSF) [42] and human brain tissues are noted [43, 44]. Interestingly, in one of these studies of atherosclerosis and *Cpn* involvement in which individuals died at a relatively younger age, little brain pathology was noted [44]. These findings suggest that *Cpn* may arise in the brain prior to observable pathological changes. In addition, our replicative studies published in 2006, employing new tissues obtained from brain banks not previously used by us, demonstrated *Cpn* in relevant brain samples from dementia patients [45]. In our replicative study, PCR analysis targeting two *Cpn* genes revealed PCR positivity in 20/25 late-onset brains, and from 3/27 control brains [45]. The organism was cultured from late-onset brains, and various chlamydial transcripts from those brains demonstrated viability and metabolic activity. Immunohistochemical analyses revealed that astrocytes, microglia, and ~20% of neurons were infected with *Cpn*. The finding of a large proportion of neurons positive for the organism in this study was unique [45]. As in our initial study though, infected cells were located in close proximity to both neuritic senile plaques and neurofibrillary tangle-containing neurons in the brain [2]. In a separate study from 2010 [46], intracellular and extracellular labeling for *Cpn* was found in the entorhinal cortex, the hippocampus, and the frontal cortex of dementia brains [46]. Serial sections from these areas exhibited both fibrillar amyloid (thioflavin S stained) pathology and *Chlamydia* immunoreactivity in apposition to one another. Two extracellular patterns of chlamydial immunoreactivity were observed: a punctate pattern and an amorphous foci pattern. These likely represent extrusion of whole organism (punctate) or secreted chlamydial products, e.g., lipopolysaccharide (LPS, amorphous foci) [47, 48]. These observations suggest that *Cpn* has a tropism for olfactory-connected structures such as the entorhinal cortex, amygdala, and hippocampus, brain regions demonstrating the earliest damage in late-onset dementia of the Alzheimer's type [8, 9]; infection at these sites may incite inflammation acting as a trigger for amyloid production and deposition. Other factors including other infectious agents may also incite inflammation and pathology in brains and regions in which *Cpn* is not involved as we readily acknowledge that *Cpn* would not be expected to be present in all cases of late-onset dementia.

Cpn has been demonstrated in both human and animal olfactory bulbs [2, 16, 17]; in mice, the organism appeared to spread centripetally from the olfactory bulbs into the

piriform cortex and to secondary olfactory centers such as the thalamus [16, 17, 19]. Uptake of organisms by the olfactory neuroepithelia also may lead to amyloid peptide generation at this peripheral site and may affect the overall integrity of this region. Others have shown experimentally using intranasal delivery of either polyinosinic:polycytidylic acid (PolyI:C) [49] or LPS [50•] that immune cell infiltration, inflammation, and levels of degeneration are prominent at the entry levels of the olfactory network. In the aged human brain, in which regeneration capacity of the olfactory neuroepithelia has been shown to be compromised [51], infectious and other insults may lead to a more prominent damage response than that seen in younger individuals. Importantly, both olfactory and lung routes for infection of the central nervous system are supported by DNA sequencing studies in which the organism isolated from late-onset brain samples was shown to be more closely related to respiratory than to atherosclerotic strains [52]. Molecular genetic and cell biological characteristics for two isolates of *Cpn* from late-onset brains were found to be genetically diverse (i.e., not clonal), as with most respiratory isolates [53]. Analyses for single nucleotide polymorphisms (SNPs) indicated several differences from standard respiratory isolates, but no genetic attributes suggested a specific neurotropism [52]. Intriguingly, infection of the olfactory network effectively would allow infection to bypass the blood brain barrier, suggesting that olfactory insult could be the primary pathway by which pathology is initiated in late-onset dementia.

Animal Models of *C. pneumoniae* Infection in the Brain

Previous models of late-onset dementia of the Alzheimer's type have utilized transgenic mice which overexpress mutants of presenilins, β -amyloid protein precursor, and *tau* genes [54]. Overexpression of amyloid results in development of amyloid plaques in the brain, paralleling the pathology observed in FAD. However, these systems do not address the initiating events of late-onset disease, in which mutations in these same genes are not present. As proof of principle for involvement of infection in late-onset dementia, we developed a non-transgenic animal model to address whether infection with a relevant brain isolate of *Cpn* in naïve BALB/c mice would promote damage in the animal brain similar to that identified in human late-onset dementia [2]. Since non-transgenic mice do not normally develop relevant neuropathology, they are a suitable host for analyzing whether infection leads to pathological change(s) in their brains. We used BALB/c mice, which are susceptible to, and can maintain, a persistent respiratory infection with *Cpn* [55]. We found evidence supporting our contention that infection with the organism can initiate processes resulting in the development of relevant pathology in the brain [16]. In the current iterations of

our animal model, we are assessing behavioral changes including learning and memory (Skinner operant chamber) of mice infected with *Cpn*. Our intent is to determine whether infection induces functional changes which parallel the pathological changes occurring during the progression to dementia. These data will address the contention that *Cpn* can induce both pathology and behavioral changes similar to those observed in late-onset dementia of the Alzheimer's type.

Precedents for infection in the exacerbation of dementia-related neuropathology have been reported for other pathogens in other animal models [56, 57]. Once infection has been controlled, levels of soluble amyloid apparently decrease, resulting in fewer deposits at 3–4 months [58]; nevertheless, targeting strategies at and around the time of infection have not been tested and could finally resolve whether *Cpn* is the triggering factor for the neuropathology. In mice infected with a *Cpn* brain isolate in our studies, β -amyloid deposits were identified as early as 2 months post-infection, with the greatest number of deposits identified at 3 months [16] causing a progressive Alzheimer-like pathology. Further, models utilizing direct injection of microbial products causing a sterile infection have shown induction of amyloid production and deposition [59, 60]. However, not all studies have reached the same conclusion, as a prior study by others failed to identify substantial pathology in the brain following infection with a different laboratory strain of *Cpn* [61]. The authors noted that discrepancies could have resulted from use of the laboratory isolate, which may have had different virulence properties than the human brain isolate.

In our animal model, *Cpn* was identified in the olfactory epithelia and the olfactory bulbs by both light and electron microscopy following intranasal infection [16]. Analysis of pathology in the brain revealed $A\beta_{1-42}$ deposits that resembled Alzheimer amyloid plaques. Intranasal infection results in cumulative pathology in the brains of BALB/c mice, and subsequent inoculations result in an additive effect in the degree of pathology. Activation of astrocytes and co-localization of reactive astrocytes with amyloid deposits suggested that a cellular inflammatory response was being initiated. This response may be focused on *Cpn* alone, or it may be directed against amyloid deposits or to soluble amyloid. These observations suggest that $A\beta$ generation is a response to the infectious insult and lend support to the hypothesis that $A\beta$ can act as a "biofloculant" [62]. Other studies have suggested that $A\beta$ -amyloid may have antibacterial properties, providing support for its occurrence as a result of infection in the brain [63, 64]. Induction of amyloid deposits in the brains of non-transgenic BALB/c mice further supports our contention that infection with *Cpn* in the brain is causative for the initial Alzheimer-like neuropathology that evolves. This is further corroborated by our earlier study using Moxifloxacin in *Cpn*-infected mice, where minimal amyloid deposition was observed in infected animals that received antibiotics within

7–21 days post-infection. In comparison, animals not receiving antibiotics, or those with delayed treatment at 56–70 days post-infection, amyloid plaque numbers were 8–9-fold higher [65].

Our observations indicate that isolates of *Cpn* differ in the ability to establish persistent infection and to promote progressive neuropathology. Thus, we have focused on using both a brain isolate (i.e., obtained from late-onset dementia brains) and a laboratory isolate (AR-39) originally obtained from a respiratory infection. Further, a critical issue in development of late-onset dementia is the age of the individual, with greater age demonstrating greater risk for disease. In this regard, the age at which *Cpn* infection occurs may also influence likelihood of brain infection. With this in mind, an earlier study from our group indicated that *Cpn* infection of older as compared to younger animals resulted in more prominent establishment of a brain infection that was statistically significant following intranasal inoculation [17]. Also, we inoculated a small group of BALB/c mice with the AR-39 isolate either twice or three times at 30-day intervals, then sacrificed at day 90. Animals inoculated twice displayed an average of 68 amyloid deposits, and those inoculated 3 times averaged 177 deposits (unpublished observations). Mice receiving only a single intranasal inoculation showed an average of 17–18 deposits at 3 months post-infection, suggesting that multiple inoculations exacerbate damage in the brain. Although we do not know how dosing or multiple exposures to infectious agents through olfaction may affect the human population, our findings may have implications for how the risk of neuropathogenesis may arise following multiple exposures to infecting agents.

In the late-onset brain, inflammation is thought to result from A β deposition, which has been advanced as the primary mechanism in late-onset dementia pathogenesis [66]. Clinical trials investigating the effects of non-steroidal anti-inflammatory drugs (NSAIDs) also implicate inflammation as a factor, since some have shown that these drugs can delay onset of disease [67]; however, they appear to be ineffective as a therapeutic once the disease becomes manifest. Interestingly, some untreated immunosuppressed individuals appear to have an increased risk of AD, but with treatment with anti-TNF α agents, the risk for AD is lowered [68]. At this time, the factors leading to increased risk in the immunosuppressed population are unknown. The resident cells in the brain responsible for inflammation are typically microglia and astroglia. Both are activated in the late-onset brain and often are identified in and around amyloid plaques [69]. Microglia and astroglia respond to insult by producing proinflammatory cytokines and reactive oxygen species (ROS). Our identification of *Cpn* in the central nervous system (CNS) in microglia, astroglia, perivascular macrophages, and neurons suggested that infection-initiated inflammation could be involved in the early neuropathology of late-onset disease [2, 45]. Contributions also could come

from *Cpn*-infected monocytes and endothelial cells that we observed in late-onset brains [2, 70]. As proof of concept for our observations, others have shown that proinflammatory molecules are significantly higher in culture supernatant fluids of *Cpn*-infected murine microglial cells compared with controls [71]. Further, infected murine astrocytes showed higher levels of MCP-1 and IL-6 compared to controls. Neurons exposed to conditioned supernatant from infected murine microglial cells showed increased cell death compared with mock-infected supernatants. These data may reflect what is occurring following infection in situ.

Culture Studies of *C. pneumoniae* Infection

Monocytes are known to be involved with the expression of cytokines, apoptosis, and β -amyloid clearance in AD [72–75, 76•], and our observations indicate that transcription of monocyte genes encoding inflammatory products changes significantly at 48 h post-*Cpn* infection. Infected cells maintain proinflammatory cytokine secretion over 5 days, including IL-1 β , IL-6, and IL-8 [76•]. High levels of IL-1 β are correlated with neuroinflammation in the late-onset brain [77–80]. This cytokine activates nitric oxide synthase, which has been implicated in hippocampal neuronal cell death [81, 82]. Other studies have implicated IL-1 β in promotion of the neuronal synthesis of the β -amyloid precursor protein [80]. Such observations provide a rationale for triggering events in which the production of A β would be a consequence in late-onset disease.

Interestingly, four genes were upregulated after 48 h infection in our in vitro studies, each of which encodes a product involved with host defense against bacterial infection [76•]. One, *DEFB4*, encodes a defensin protein with anti-microbial activity linking innate and adaptive immune responses [83]. Another encodes inflammasomes, *IPAF* and *AIM2*, which are associated with toll-like receptors and which mediate the response to both extracellular and intracellular pathogens [84]. *NLRC4* can be activated by type III secretion systems characteristic of *Cpn* and other gram negative bacteria [85]; this system acts to transfer effector proteins from the bacteria into the cytosol of the host cell, resulting in generation of reactive oxygen species (ROS). ROS are thought to result from the assemblage of another inflammasome complex, *NLRP3* [86, 87], which also is activated by chlamydial infections [88, 89]. Upregulation of the *AIM2* inflammasome transcript may have been the result of detecting double-stranded DNA from the organism in the cytosol [90, 91]. The fourth transcript encodes MCP1/CCL2, a key chemokine for recruiting monocytes and macrophages; this was increased 1000-fold following infection of monocytes with *Cpn* [76•, 92]. This gene product is an important contributor to the neuroinflammatory process observed in late-onset dementia and is increased in both CSF and plasma from individuals with MCI and dementia [93,

94]. CCL2 may allow increased monocyte migration into brain tissues; it also may affect production and clearance of A β from the brain [93–95].

Cell biological studies have demonstrated standard inclusion and chlamydial morphology for both isolates in human epithelial cells (HEp-2), astrocytes (U-87 MG), and microglial cells (CHME-5), as in our previous studies [96]; this is also the case for standard *Cpn* inclusion morphology in the human microglial cell line HMC3 in our current studies. *Chlamydia*-induced disease is largely a result of immunopathogenesis. Chlamydial infection promotes secretion of proinflammatory cytokines [97]; strong inflammatory responses are initiated by chlamydial LPS, heat shock proteins, and outer membrane proteins. LPS alone may account for many aspects of late-onset dementia pathology, as studies have shown that *Escherichia coli* LPS, when injected at low dose into the brains of rats, results in inflammation characterized by increased cytokine production and microglial activation [98]; induction of the β -amyloid precursor protein also was observed in the rat temporal lobe, suggesting that products of infection alone could stimulate cellular changes resulting in neurodegeneration.

Persistence of *C. pneumoniae* Infection and Host Responses

Many studies have shown that under certain conditions and/or within specific host cell types, *Cpn* alters its biologic state to generate persistent, long-term infections [99–101]. Chlamydiae undergoing such infections are morphologically aberrant and display an unusual transcriptional profile [99–101]. Importantly, the mechanisms of pathogenesis differ between active and persistent chlamydial infection, and it is in *the persistent state that these organisms are thought to elicit chronic disease* [102, 103]. *Cpn* has been associated with several chronic pulmonary diseases [104] and an array of non-respiratory diseases, including atherosclerosis, inflammatory arthritis, multiple sclerosis, and others [105–108]. Studies relevant to late-onset dementia have included analyses of *Cpn* infection in vitro in both neuronal [109] and astrocyte cell lines [110••]. In SK-N-MC neuronal cultures at 3 to 10 days post-infection, cells were resistant to apoptosis when induced by staurosporine, suggesting that *Cpn* may induce chronic infection by interfering with apoptosis, a feature found in the late-onset dementia brain, in which apoptosis can be initiated but does not necessarily go to completion [111]. With regard to astrocytes, *Cpn* infection in culture promoted transcriptional upregulation of genes involved in neuroinflammation, lipid homeostasis, microtubule function, and amyloid precursor protein processing. Protein levels for the secretases BACE1 and PSEN1 were twofold higher than in controls. BACE1 enzymatic activity was also shown to be increased, suggesting that infected cells promoted a pro-amyloidogenic pathway [110••]. Together with evidence in the late-onset dementia brain of infection in astroglia, microglia, and neurons, a rationale for involvement of *Cpn* infection in

neuroinflammation and amyloid protein processing and generation of β -amyloid is compelling.

Proof of Principle of *C. pneumoniae* as a Causative Agent of Late-Onset Dementia

Traditionally, proofs of infection causing disease have invoked Koch's Postulates, which were a set of rules for which infectious agents were associated with disease causation. Interestingly, Koch himself realized that not all organisms which caused a particular disease fell within the confines of his particular postulates [for review see 112]. In this regard, viruses and obligate intracellular bacteria, which are not free-living (a condition of one of the four postulates), cannot be cultured without parasitizing another host cell or organism. Thus, proof of concept studies for these types of infectious agents cannot fit easily into Koch's postulates, but rather require a modification or redefinition by which proof of causation should now be accepted. We contend that the postulates derived by Koch should now be addressed in the following manner, given our and others' data.

1. The microorganism must be found in abundance in all organisms suffering from the disease, but should not be found in healthy organisms.
 - a. We first found *Cpn* in 17/19 late-onset dementia brains and only 1/19 control brains [2]. In a replicative study, we found *Cpn* in 20/25 dementia brains and only 3/27 control brains [45].
2. The microorganism must be isolated from a diseased organism and grown in pure culture.
 - a. *Cpn* is an obligate intracellular pathogen that requires a host cell for culturing, which cannot be grown in pure culture, however
 - b. *Cpn* was isolated from a human brain, cultured in human THP1 monocytes, and subsequently identified by immunohistochemistry, PCR, and electron microscopy [2].
3. The cultured microorganism should cause disease when introduced into a healthy organism.
 - a. The human brain isolate of *Cpn* that was cultured in THP1 monocytes was purified and inoculated into normal BALB/c mice and resulted in A β -amyloid accumulation in the mouse brain [16].
4. The microorganism must be re-isolated from the inoculated, diseased experimental host, and identified as being identical to the original.
 - a. The human brain isolate that created "AD-like" pathology was purified from the infected mouse brain. It

was clearly identified by immunohistochemistry and PCR from the mouse brain isolate, and

- b. Human brain isolates of *Cpn* were sequenced and resemble human respiratory strains of *Cpn* [52, 53].

Conclusion

The observations summarized here demonstrating an association of *Cpn* infection and late-onset dementia of the Alzheimer's type suggest that this infection could be causative for disease pathogenesis if we accept the outlined modifications of Koch's postulates addressed herein. Without demonstration that treatment of late-onset dementia patients for infection can correct the problem, we cannot unequivocally claim that disease is caused by *Cpn* infection or any other infectious agent. Only through initiating clinical trial approaches in which anti-infectives and possibly anti-inflammatory regimens are tested in the late-onset population will we have more definitive answers as to whether infection is a significant cause underlying and eliciting late-onset dementia of the Alzheimer's type.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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