Point of View

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Revisiting the Cholinergic Hypothesis in Alzheimer's Disease: Emerging Evidence from Translational and Clinical Research

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Abstract

Scientific evidence collected over the past 4 decades suggests that a loss of cholinergic innervation in the cerebral cortex of patients with Alzheimer's disease is an early pathogenic event correlated with cognitive impairment. This evidence led to the formulation of the "Cholinergic Hypothesis of AD" and the development of cholinesterase inhibitor therapies. Although approved only as symptomatic therapies, recent studies suggest that long-term use of these drugs may also have disease-modifying benefits. A Cholinergic System Workgroup reassessed the role of the cholinergic system on AD pathogenesis in light of recent data, including neuroimaging data charting the progression of neurodegeneration in the cholinergic system and suggesting that cholinergic therapy may slow brain atrophy. Other pathways that contribute to cholinergic synaptic loss and their effect on cognitive impairment in AD were also reviewed. These studies indicate that the cholinergic system as one of several interacting systems failures that contribute to AD pathogenesis.

Key words: Alzheimer's disease, cholinergic system, cholinesterase inhibitors, nucleus basalis of Meynert (NbM) degeneration, nerve growth factor, basal forebrain cholinergic system atrophy.

Highlights

- *The perspective review of the role of the cholinergic system on AD pathogenesis*
- *Discussion of cholinergic synaptic loss, neuronal atrophy, and cognitive impairment*
- *Cerebral cortex cholinergic innervation loss correlates with cognitive impairment*
- *Translational research on cholinergic drugs now suggest disease modifying benefits*
- *The cholinergic system is a systems failures model contributing to AD pathogenesis*

Introduction

The discovery, testing and validation of a broad spectrum of interventions to delay and eventually prevent neurodegeneration underlying chronic brain disorders such as dementia and Alzheimer's disease (AD) remain vital global strategic goals (1-3). This mega-challenge regarding current R&D paradigms for therapy development and the associated question of how to improve the productivity of drug development and accelerate the discovery of cures have been extensively deliberated at several meetings organized by the Alzheimer's Association's Research Roundtable, the Alzheimer's Association International Conference, Clinical Trials on Alzheimer's Disease, the Advances in Alzheimer Therapy conferences, and the EU-US Task Force (4-10). These deliberations have examined the challenges as well as potential solutions to the problems facing current therapy development efforts..

One critical factor that may account for the failure of current paradigms to yield effective disease-modifying treatments in recent years is the inadequacy of current concepts on the origins of neurodegeneration and the underlying pathogenic mechanisms (11). Now, there is growing recognition in the field for the necessity of a formal and comprehensive re-assessment of all major ideas or theories on pathogenesis and the need to foster new thinking on the neurobiology of neurodegeneration (2). The Cholinergic Hypothesis Workgroup [CWG] was convened to address this need. The current paper, which merely represents a 'work-in-progress', is an interim account based on a reassessment of the 'Cholinergic Hypothesis' in light of emerging evidence. Further revisions of this initial report are expected as this

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stocktaking exercise proceeds with specific feedback or general commentaries from the wider scientific community.

History of cholinergic involvement in AD

The concept of a significant cholinergic participation in the Alzheimer's pathology has its roots in neurochemistry, neuropharmacology and neuroanatomy. Some of these roots relate to the effects of scopolamine in memory mechanisms. Earlier rodent studies by Pazzagli and Pepeu proposed a correlation between the amnesic properties of scopolamine and brain acetylcholine content (12), while Bohdanecky and colleagues demonstrated, in primates, dose-related effects of scopolamine in impairing memory mechanisms in the delayed-to-matching paradigm (13). Since the mid-'60s, a number of related publications appeared in the literature including an opinion article by Deutsch in Science in 1971, signaling the cholinergic synapse as a possible "site of memory" (14). In the '60s and '70s there was a vague idea of the actual cholinergic pathways responsible for the above functions. Thus, most of the early anatomical information derived from histochemical techniques demonstrated in situ enzymatic activity of acetyl cholinesterase. Shute and Lewis early on proposed that the cortical cholinergic innervation was part of the "reticular ascending system" (15). The definitive conceptualization of the existence of basal forebrain neuronal cell body groups supplying cholinergic synaptic terminations to the hippocampus and cerebral cortex and its involvement in AD pathology had to wait for more advanced techniques, as discussed further below.

The attention on brain acetylcholine as crucial to the mechanisms of memory functions took on new momentum with the thorough and influential 1974 publication of Drachman and Leavitt, in which the authors studied the differential effects of scopolamine (central and peripheral effects), methscopolamine (peripheral effects), and physostigmine on cognitive performance. The performance of young subjects receiving scopolamine was comparable to that of untreated aged subjects on the same memory tests, suggesting possible cholinergic involvement in age-related cholinergic losses (16).

The seminal findings of Drachman and Leavitt were soon followed by the simultaneous discovery in 1976 by Davies and Maloney and by Bowen and collaborators demonstrating significant depletion in the cerebral cortex of the acetylcholine biosynthetic enzyme (choline acetyl transferase) in post-mortem brain samples identified as bearing AD pathology (17, 18). In the same year, Bartus and Johnson reinforced the concept of the cholinergic participation in short-term memory in studies applying scopolamine in primates (19). Bartus subsequently found that relatively high doses of physostigmine, by blocking acetylcholine degradation, would help memory outcomes in aged but not young primates, leading to the consequential proposition of a potential therapeutic avenue for age-related memory losses (20). These studies culminated with the much noted 1982 Science review by Bartus and colleagues on the cholinergic hypothesis of geriatric memory dysfunction (21).

At around the same time as research began to clarify the relationship between acetylcholine and memory, the nucleus basalis of Meynert (NbM) was identified as the source of cortical cholinergic innervation in the primate brain (22). In the same year, Whitehouse and collaborators provide neuropathological evidence of significant losses of "magnocellular neurons" of the NbM in patients with AD (23); a report soon followed by a review from Coyle and coworkers presenting arguments supporting the notion of AD as a disorder of the cortical cholinergic innervation (24).

The severity of dementia in AD was found to have a positive correlation with the extent of the cholinergic loss (25, 26) and animals with cholinergic lesions and resultant learning impairments were characterized as models of AD (21). These developments culminated in the demonstration that inhibitors of acetylcholinesterase could lead to symptomatic improvement in patients with AD (27). It was subsequently found that neurofibrillary degeneration of the cholinergic NbM neurons, designated Ch4, was already present at the very early stages of AD, and that the extent of this neurofibrillary degeneration, even at a stage prior to cell death, is correlated with cognitive deficits (28, 29). Additional lines of research have also raised the possibility that this cholinergic lesion may influence AD pathogenesis through complex and poorly understood effects on amyloidogenesis, tau phosphorylation, and neuroplasticity (30). Although changes in other cortical neurotransmitters such a dopamine, norepinephrine, and histamine were also reported in AD, a review of the extensive literature led Geula and Mesulam to conclude that the cholinergic lesion is earlier, more widespread, and more consistent than the pathological alterations of other neurotransmitter systems (31, 16, 21).

The above body of ideas indicating that the decline in a system regarded as fundamental for memory mechanisms contributes to age-associated memory losses, along with evidence of atrophy of that system in AD, became by extension the simplified "cholinergic hypothesis of Alzheimer's disease" (32), and was understood as causative of the disease. However, that was not the message promulgated by proponents of significant involvement of the cholinergic system in AD, nor of Bartus et al., who in 1985, revised the historical status of "The Cholinergic Hypothesis", specifically clarifying that "it states nothing about etiological factors" but rather describes the role of cholinergic dysfunction in memory mechanisms (33).

To this day, cholinesterase inhibitors (ChEIs) remain the main approved pharmacological therapies

for cognitive deficits in AD. Currently, three ChEIs – donepezil, rivastigmine, and galantamine – are widely used as standard of care for the pharmacological treatment of clinical symptomatic stage AD. The efficacy of ChEIs has been demonstrated in multiple large-scale studies, although effect sizes are considered modest and ChEIs are traditionally conceptualized as "symptomatic" treatment. Critical questions remain to be answered, including whether it is useful to dichotomize pharmacological treatments in exclusive categories such as "symptomatic" versus "disease-modifying" and whether one can expect much higher effect sizes in co-primary outcomes of any treatment at the late and potentially largely irreversible stage of AD dementia. Moreover, as summarized in section 6, the use of ChEIs for more than two decades raises questions as to whether their effects are more significant and longer-lasting than has been assumed. Whether more effective or additional cholinergic interventions may have a more consequential effect on AD at symptomatic and pre-symptomatic stages is not yet clear.

Current status of the search for AD treatments

Success rates among AD drugs in clinical development are disappointing low (34, 35), much lower than for cancer and other complex diseases. In the past 25 years, the search for therapies aimed at slowing or halting AD progression has been dominated by the straightforward rationale of developing compounds targeting or disrupting amyloid beta (Aβ) formation (35). There is broad consensus that early onset autosomal dominant AD (ADAD) is caused by amyloidopathy. While the same may also be true for the more common late-onset, polygenic sporadic forms of the disease (LOAD), other factors may also contribute to or aggravate the disease. Indeed, the sporadic forms of AD have multiple variants, each with distinct clinical features and genetic associations (36). The pathophysiology of sporadic AD is therefore likely to be heterogeneous (i.e., in terms of disease onset, presentation and progression), and to reflect non-linear dynamic interactions among multiple factors. An integrated understanding of these genetic and biological interactions, processes, and feedback mechanisms is needed to develop more effective interventions based on the principles of biomarker-guided precision medicine tailored to individual patients at early disease stages (37, 38). The limitations of the amyloid cascade hypothesis and the failure of several Aβ-focused therapy development efforts, together with the recognition of the close relationship between tau pathology and cognitive status, have encouraged the development of other therapeutic approaches for AD, including tau-targeted therapies (39).

Cholinergic systems in aging, mild cognitive impairment and dementia

Anatomical studies conducted in the 1980s using choline acetyltransferase (ChAT) immunolabeling demonstrated that cholinergic innervation of the primate cerebral cortex begins in the basal forebrain (BF), specifically in Ch4 neurons within the NbM (40). Ch4 neurons project to all regions of the rhesus monkey cerebral cortex but receive cortical input only from limbic and paralimbic areas (41). Indeed, cortical cholinergic innervation arising from the NbM provides the largest component of the extra-thalamic ascending reticular activating system (ARAS); thus, its loss can result in impaired attention, memory, motivation, sleep, and plasticity (42).

Ch4 neurons are highly susceptible to degeneration in AD (28). In the advanced stages of AD, most Ch4 neurons contain a substantial number of neurofibrillary tangles; and neurofibrillary degeneration in the NbM appears to be responsible for destruction of outgoing axons. In the prodromal mild cognitive impairment (MCI) stage, there is also a marked increased density of neurofibrillary tangles in NbM neurons, and the amount is significantly correlated with performance on memory tests (28). Neurofibrillary tangles are even detected at low levels in cognitively healthy elderly (28). Altogether, these results indicate that neurofibrillary degeneration of cholinergic neurons in the NbM, which innervate the cortex, is a very early event in the age-MCI-AD continuum. However, neurofibrillary pathology of the NbM is not an isolated component of the healthy aging-MCI-AD dementia continuum, and there is no definite evidence that it drives other aspects of the pathophysiological cascade (30). The differential vulnerability of Ch4 sectors to AD pathology has been addressed by many investigators. Geula and Mesulam (31) reviewed 22 studies reporting Ch4 neuronal loss in AD and concluded that the posterior (Ch4p) and anterolateral (Ch4al) sectors tended to show the most pathology, and that these sectors were likely to project to areas known to display the greatest cholinergic denervation. These conclusions were offered with multiple caveats revolving around the heterogeneity of the histopathologic methods and of the patient populations.

While cholinergic neurotransmission is disrupted in individuals with AD dementia (43) and in the prodromal stage of AD (i.e., individuals with MCI) (44, 45), it is not clear whether such disruption also occurs in cognitively normal older adults who have increased levels of Aβ. Post-mortem studies of cognitively normal older adults have reported that decreased ChAT activity was significantly associated with increased concentration of Aβ (46, 47). Similarly, increased levels of Aβ were associated with accelerated loss of cholinergic fibers in the entorhinal cortex and inferior temporal gyrus (48) and recent studies have also shown that Aβ burden

correlates significantly with basal forebrain atrophy (49, 50). However, as normal cognitive function depends on adequate cholinergic neurotransmission, particularly the acquisition, storage and use of new knowledge (51, 52), the integrity of the cholinergic system in preclinical AD must be sufficiently intact to allow neuropsychological test performance to remain generally within normal limits (53).

Receptor changes in AD reflect the degree of synaptic deafferentation and are secondary to neuronal, mainly synaptic, pathology. Therefore, muscarinic and nicotinic receptors are not equally affected at different stages of the disease (54). This difference may have therapeutic implications (55).

The cholinergic hypothesis implies that the efficiency of a reduction in the number or activity level of cholinergic synapses can be improved by either increasing the synaptic level of ACh or by allosteric modulation of postsynaptic cholinergic receptors with selective agonists. The second alternative can be best satisfied by stimulating or modulating either the M1 muscarinic receptor or some of the nicotinic cholinergic receptors subtypes. Both ways have been clinically exploited. M1 receptors play a role into memory and learning processes as demonstrated by effects in animal models and are impaired in AD at different stages of the disease (55, 56). The clinical experience with the first generation of M1 agonists has been largely disappointing, either because of clinically insignificant cognitive effects or side effects, often of cardiac nature (56). A promising extension of the concept of selective allosteric M1 receptor modulation is the recent development of muscarinic agonists targeting the sigma-1 receptor such as AF710B (57). In animal models, these compounds result in improved cognition, reduced synaptic loss, and reduced amyloid and tau pathologies (57). In this regard, it is encouraging that in a rat transgenic model of AD-like amyloid pathology, the AF710 B allosteric muscarinic and sigma 1 agonist, after a month of treatment interruption has shown "disease- modifying" sustained effects on diminishing the Aβ pathology and improving cognitive outcomes in a variety of tasks (58). However, these compounds have not yet been tested clinically.

In contrast to studies of muscarinic receptors, nicotinic receptor binding is reduced in autopsied AD brains (55, 59). Biopsy and PET imaging studies confirmed the severe loss of cortical nicotinic receptors in AD (60, 61), supporting the use of selective nicotinic agonists. Of particular interest have been alpha-7 nicotinic receptors, which are highly expressed in brain regions involved in cognitive processes and are particularly vulnerable to AD pathology (62). Encenicline, a partial selective agonist of the alpha-7 subunit, showed encouraging results in a phase 2 trial in mild to moderate AD patients but was put on hold in phase 3 trials because of rare but severe side effects (63). Thus, despite a solid pharmacological background and convincing implications in AD cognitive deficits, both muscarinic and nicotinic agonists or modulators have been so far disappointing in the treatment of AD.

During the last 10 years, neuroimaging studies have helped to chart the in vivo progression of neurodegeneration in the cholinergic system and to demonstrate its connection to anatomical and cognitive correlates of AD. For example, Teipel, Hampel, and colleagues developed innovative structural magnetic resonance imaging (MRI) analytic methods to demonstrate the presence of NbM atrophy in the AD brain as well as a decline in related cholinergic cortical projection areas. In a first study in 2005, they accomplished this stereotactic mapping of the basal forebrain cholinergic system (BFCS), which contains the NbM and Ch4, with the help of combined examination of histological sections and post-mortem MRI in a non-demented patient, and then applied this stereotactic map onto MRI scans of patients with probable AD (64). The same team replicated these results in an independent sample, additionally using diffusion tensor imaging (DTI), where they demonstrated an association with fiber tract disintegration within the ascending cholinergic pathways and confirmed that significant atrophy of the NbM occurs in patients with AD dementia and even in those with MCI (65). Together, these studies indicate that structural decline in the NbM may indeed be an early event during disease progression (66).

In addition, by studying MRI-based morphometry in a large cohort of healthy individuals across the adult lifespan, as well as patients with prodromal or mild AD dementia, Grothe and colleagues revealed that BFCS atrophy in AD occurs against a background of notable age-related degeneration and that exacerbated atrophy in AD progressed from the posterior NbM, at the prodromal stage, to include the entire basal forebrain in mild AD dementia (67). They also used serial scanning to show reduced baseline volumes and accelerated atrophy over time as the disease progresses, such that MCI-to-dementia converters show larger baseline decrease in BF volume compared with subjects that remained in the MCI range within the same time interval (45). A population-based study in healthy elderly subjects showed that changes in the BF may even precede symptom onset by 5 years (68).

Schmitz and Spreng asked the question whether longitudinal shrinkage of the basal forebrain region containing the NbM and entorhinal cortex grey matter volume were interdependent and sequential. Surprisingly, their data indicate that basal forebrain volume predicts longitudinal entorhinal degeneration. The Aβ positive cognitively normal subgroup, showed abnormal degeneration in basal forebrain, but not entorhinal cortex. Both abnormal basal forebrain and entorhinal cortex atrophy was exclusively observed among prodromal individuals, providing evidence that basal forebrain atrophy precedes and predicts

both entorhinal pathology and memory impairment, challenging the traditional view on brain regional AD pathological progression (66).

Kilimann and colleagues showed that NbM volume change was a robust neuroimaging marker even when probed with different scanner types in different medical research centers. In AD patients, significant volume reductions were observed in all sub-regions of the BFCS, with the posterior subregion of Ch4 (Ch4p) showing the best utility as a diagnostic classifier (69). BF atrophy was also associated with cognitive decline (44) as well as regional cortical degeneration (65), and glucose hypometabolism (50) that links to the cognitive deficits in a domain-specific manner; additionally, it correlated with Aβ burden in healthy controls, MCI subjects, and AD patients (49, 70). The mechanisms underlying these correlations are poorly understood and need to be investigated.

A recent overview describing late-stage AD drug trials conducted in the period 1984 - 2013, indicates that there has been a recent surge in interest in therapies targeting a range of receptors to modulate cholinergic or other neurotransmitter systems (35). These recent studies indicate that cholinergic therapies may have heretofore unsuspected disease-modifying properties and that, administering them early in the disease process and relying on compounds that are both more selective and more potent, might represent one of the many interventions that will be needed to delay the progression of cognitive impairment in asymptomatic individuals at risk for AD. The most recent evidence has been presented by the Hippocampus Study, a double-blind randomized controlled trial of donepezil with primary MR imaging outcomes. Dubois, Hampel, Cavedo, and colleagues showed a significant treatment effect of donepezil in reducing rate of atrophy in the hippocampus, cortex, and basal forebrain volume, areas that are strongly involved in the pathophysiology of AD and cholinergic innervation (71). In particular, individuals in the treatment group with suspected prodromal AD showed a 45% reduction in the rate of hippocampal atrophy compared to the placebo group after one year of treatment (72). In a follow-up study by the same group, donepezil-treated participants also showed reductions in the annualized rate of change in regional cortical thickness compared to participants receiving placebo. The main cortical areas revealing reduced cortical thinning in the treatment group were the rostral anterior cingulate, the orbitofrontal, the right inferior frontal cortices and the right insula (71). Recently, Cavedo and Hampel and colleagues presented evidence reporting reduced basal forebrain system atrophy after one year of donepezil treatment in the same study population. Notably, the exploratory analysis on the basal forebrain cholinergic system nuclei revealed a specific effect of donepezil in reducing rate of atrophy in the NbM, representing the region with the most elevated concentration of cholinergic neurons projecting to the cortex, and in the medial septum/diagonal band nuclei connected to hippocampus and entorhinal cortex (73). These remarkable effects cannot be explained by currently known mechanisms of cholinergic biology and deserve further investigation. Although trials exploring the effectiveness of cholinesterase inhibitors in MCI have yielded variable results, and a recent practice guideline update could find no Level A evidence that cholinesterase inhibitors offer symptomatic improvement at the MCI stage, it is encouraging that some multicenter, double-blind, placebo-controlled trials of cholinesterase inhibitors in MCI suggest that conversion to clinically diagnosed AD may be delayed in Apolipoprotein Eε4 carriers on cholinesterase inhibitors as compared to placebo. In a rat transgenic model with AD-like amyloid pathology, an allosteric muscarinic and sigma 1 agonist reportedly showed "disease-modifying" effects, diminishing the Aβ pathology and improving cognitive outcomes (41).

Obviously, no therapeutic strategy can overlook other aspects of AD pathophysiology. For instance, neurofibrillary degeneration and neuritic amyloid plaques are present in areas of the brain beyond the NbM, including the entorhinal cortex, amygdala, and hippocampus, as well as other areas of the cortex. Treating the loss of cholinergic innervation might therefore have only a partial effect on overall pathogenesis and symptomatology (30). Inflammatory pathways also appear to play an important role in disease timing and severity. For example, pharmaco-epidemiology data suggest that long-term use of non-steroidal anti-inflammatory drugs (NSAIDs) may protect against AD (74, 75); and at preclinical stages, NSAIDs may diminish significantly AD prevalence (42), while they are ineffective after its clinical presentation (43).

In individuals with intact NbM and intact cortical cholinergic axons, ChEIs are expected to have cholinomimetic effects on cortical neurons by delaying the hydrolysis of presynaptically-released ACh. However, since few if any cholinergic axons remain intact in advanced AD, ChEIs are unlikely to have the same type of cholinomimetic effect. In fact, the bulk of cortical cholinesterases in advanced AD are found in plaques and tangles (13). Since the cholinergic innervation of the striatum and thalamus are largely intact in AD, ChEIs in these patients could potentially exert their symptomatic effects on cognition by altering cholinergic activity along these subcortical pathways (13).

The CNS cholinergic phenotype: implications of a novel nerve growth factor (NGF) metabolic pathway in Alzheimer's pathology

Nerve growth factor (NGF) retrogradely transported from cortex has been known for many years to be necessary for the maintenance of BFCS.

During development, NGF is essential for survival, differentiation, and establishment of synaptic density, while in the mature central nervous system (CNS) it is critical for preserving the neuronal phenotype of cholinergic NbM cells and synaptic density (76). This knowledge, as well as the experimental evidence that lesion-induced cholinergic atrophy and death could be prevented by the application of exogenous NGF, stimulated interest in a possible neurotrophic therapy in AD (77). Unfortunately, the earliest interventions with the direct intracerebral application of exogenous NGF in AD patients had a number of undesirable effects. Based on a single case observation of positive cognitive outcomes on verbal memory (78, 79), the Karolinska team embarked in a larger study with exogenous intracranial NGF application in AD. At the time, the outcome of the early clinical attempts to treat Alzheimer's disease with intracerebroventricular exogenous NGF was discussed by Cuello and Thoenen (80), highlighting the significant adverse effects such as hyperalgesia, anorexia and herpes zoster. A full report of the three cases studied with such approach was published in 1998 (81), indicating that no significant improvement was noticed in the MMSE scores of the treated patients.

More recent attempts for neurotrophin-oriented therapies involved the implantation of NGF-secreting cells or the actual local transfection of cells to release neurotrophins in the selected brain areas. Thus, a series of AD patients have been treated with encapsulated NGF-transfected cells to secure neurotrophin secretion at the basal forebrain (NbM and diagonal band regions) (82). Another recent alternative approach has been applying adeno-associated virus vectors expressing NGF (AAV-NGF) in the NbM. Such studies were initiated in 2001, demonstrating in a 2005 report a slower cognitive deterioration in some AD individuals thus treated (83). A more recent report on post-mortem material of AD sufferers who received AAV-NGF transfections revealed trophic responses in cholinergic neurons of the NbM (84).

The above approaches, while encouraging, are rather invasive and rely entirely on the application of exogenous NGF, as a pharmacological agent, at non-physiological sites of release. The discovery of a novel NGF metabolic pathway offers the theoretical possibility of modifying the rate of production of endogenous NGF at its physiological sites of release, i.e. the cerebral cortex for the cholinergic neurons of the nucleus basalis and the hippocampus for septal/diagonal band neurons.

The discovery of this novel metabolic pathway resolved the apparent paradox in AD of marked cholinergic atrophy of the NbM despite an abundance of the NGF precursor and no compromise of NGF synthesis. This pathway revealed that pro-NGF is released in an activity-dependent form, converted to mature NGF in the extracellular space, and eventually degraded by a complex CNS metabolic pathway (85). In brains with AD pathology the conversion of the released pro-NGF is compromised, resulting in the pathological brain build-up of pro-NGF and very limited production of the biologically active mature NGF. This deregulation can be provoked by oligomeric amyloid peptides and likely aggravated by Aβ –induced neuroinflammation (86, 87). The possibility that other inducing factors, such as metabolic stress, may precede the aggregation of Aβ peptides has not yet been explored.

The degeneration of basal forebrain cholinergic neurons observed in AD is also seen in patients with Down syndrome (DS) (88), who typically develop neuropathology nearly identical to that reported in AD by their 30s; and, by age 70, they will most likely have dementia (89). In both AD and DS, dysregulated metabolism or retrograde transport of NGF to the NbM may provide a mechanism for cholinergic synaptic loss, neuronal atrophy, and eventually cognitive impairment. NGF metabolic deficits, including increased pro-NGF concentration and reduced tissue plasminogen activator (tPA) activity appear at early stages of Aβ accumulation in DS, before the onset of dementia (87). Moreover, longitudinal studies of asymptomatic DS and AD patients have shown that a rise in plasma pro-NGF from baseline to year one predicts a greater cognitive decline at year two (90). These data suggest that the NGF metabolic pathway is likely to be substantially compromised during the "silent stages" of AD, which may offer opportunities to search for novel biomarkers (i.e. a raise of pro-NGF in body fluids anticipating worsening of AD pathology at preclinical stages). The therapeutic possibilities of manipulating this CNS metabolic pathway responsible for the endogenous production of mature NGF has not been explored. However, there is already data demonstrating that both conversion and degradation of NGF are amenable to pharmacological manipulation in naïve rats with an impact on the cortical cholinergic synaptic phenotype (91).

Identifying preclinical AD through pharmacologic action on the cholinergic system

Following recent clinical trials of anti-amyloid therapies that failed to demonstrate significant therapeutic benefits, positron emission tomography (PET) imaging with amyloid ligands revealed that up to a quarter of the participants in those trials did not have fibrillary amyloid pathology targeted by the drug's mechanism of action, despite a clinical diagnosis of mild-to-moderate AD (92, 93). This has highlighted the need for better patient selection based on biomarker positivity. The problem, however, is that PET imaging is expensive, hardly generalizable, invasive and labor-intensive. One question worth addressing, based on the hypothesis that there is subtle disruption to cholinergic system integrity during the preclinical stage of the disease, is whether a

challenge test can be created and effectively utilized that would allow for more reliable subject selection for clinical trials (and eventually as a screening test for preclinical AD).

Snyder and colleagues have suggested that it may be possible to transiently disrupt cholinergic neurotransmission in a stress test designed to unmask very early, otherwise undetectable cognitive impairment in a modestly compromised system such as preclinical AD (53). They have developed what they call a "cognitive stress test" analogous to the cardiac stress test that is used to unmask preclinical cardiac physiologic markers of early cardiovascular disease.

The procedure relies on the subcutaneous administration of a low dose of scopolamine hydrobromide, a muscarinic cholinergic antagonist that has short-term negative effects on cognition, as the temporary stressor (94). For a cognitive assay that is sensitive to subtle perturbation of the cholinergic system, they use the Groton Maze Learning Test (GMLT), which has been shown to detect subtle alterations in working memory and problem solving in the visual-spatial domain (95). The test is available in 20 well-matched alternate forms and can be completed in five minutes. Approximately 45 validation and factor analytic studies, as well as approximately 60 phases 1 and 2 clinical trials support the validity of the test to assess two cognitive domains: error monitoring and learning efficiency. The GMLT has also been shown to detect specific working memory impairments following low-dose scopolamine challenge in healthy elderly adults (96).

Using amyloid PET imaging to identify healthy elderly adults with AD risk factors but low cortical Aβ burden, Snyder and colleagues showed that a very low dose of scopolamine leads to cognitive impairment at 3 hours that can be detected by the GMLT, with full recovery within 5 hours (97). The idea of the stress test is that individuals in the early stages of AD will recover less quickly. At present, they are conducting a 27-month double-blinded, controlled longitudinal study in adult caregivers of 1st degree relatives of individuals with AD who have subjective memory impairment but do not (yet) qualify for a diagnosis of mild cognitive impairment (MCI). In this sample, they have shown that subjects' ability to recover rapidly from the pharmacologic challenge is correlated with neocortical amyloid burden (39). Snyder and colleagues suggest that patients who present with relative decrements in cholinergic tone, identified by this stress test, may represent a clinically specific subpopulation who would respond best to secondary prevention treatment with a potent cholinergic agonist and/or with novel ChEI (39).

Best possible utilization of cholinesterase inhibitors in AD therapy

The use of ChEIs as a treatment for AD derives from the hypothesis that deterioration in cholinergic neuron function causes the cognitive and behavioral impairments of AD. Twenty years of experience with these drugs show that they can be of benefit for patients in all stages of AD, including those with progressively worsening disease. However, while long-term follow-up assessment of AD patients treated with ChEIs have suggested a delay in cognitive deterioration, long -term effects of such therapy on disease progression have not been systematically explored, and flaws in study designs have made it difficult to interpret existing studies difficult.

Higher doses of ChEIs show increasing clinical benefits; however, increasing dose has been limited by greater adverse effects. Long-term efficacy studies suggest that cognitive benefits may persist for up to five years (98-101). Longer-term open-labeled studies suggest that there is a subgroup of patients with stabilized cognition and activities of daily living, presumably in response to ChEIs, although this could reflect a selection bias. Moreover, several studies have shown that patients with moderate or moderately-severe AD exhibited greater response to ChEI therapy (102, 103), thus suggesting that cholinergic deficits progress during the latter course of disease (104). As discussed above, however, it is not entirely clear that ChEIs in advanced disease can have the type of cholinomimetic effect on the cerebral cortex that is seen in the normal brain.

ChEIs may also have beneficial effects on sleep, which is commonly disrupted in individuals with AD. Indeed, ChEIs have been shown to produce positive effects in cognitive function and sleep in elderly and non-demented individuals with sleep disorders (105). However, most of these benefits were observed during the day while at night there was some exacerbation of sleep disorders. In AD patients, adverse sleep-related events are rare and short lasting and generally attenuated with prolonged use of ChEI (106). Continuous monitoring of AChE and butylcholinesterase inhibition in CSF and plasma, following administration of rivastigmine showed no differences throughout the day and directly correlated with cognitive benefits in AD patients (107).

Although ChEIs are still largely believed to provide "symptomatic" benefits only, evidence discussed above suggests that they may affect disease progression. For example, a very small preliminary study in mild AD demonstrated preserved cognitive function after 12 months of treatment, which was associated with levels of ChE inhibition in plasma (108). Open-label extensions of short duration trials and long-term observational controlled studies have provided further evidence suggesting sustained benefits for patients who take ChEIs for many years (109). These and other studies have prompted the search for novel «cholinergic-

oriented» treatments with higher clinical efficacy than ChEIs. Thus, it would be of interest to assess the possible clinical efficacy of new and under development allosteric modulators of muscarinic and nicotinic receptors. Ideally, drugs needed to substantially slow or block NbM neuronal atrophy or loss during the asymptomatic preclinical phase would represent a logical strategy to delay progression of cholinergic denervation and its effects on cognition.

As described above, a placebo-controlled French multi-center donepezil MRI Trial reported a considerable effect of one year of donepezil treatment on reduced rate of hippocampus and basal forebrain atrophy, as well as on regional cortical thickness, suggesting a potential slowing of brain structural decline in treated versus placebo control patients with suspected prodromal AD (71). The mechanism underlying this unique finding needs further elucidation. The pharmacological intervention for correcting the AD pathology by targeting the deregulated NGF metabolic pathway is also being investigated.

Such pharmacological treatments may also be of benefit for other neurodegenerative diseases involving the cholinergic system, such as dementia with Lewy bodies (DLB), Parkinson's disease dementia, mixed AD and vascular dementia, and traumatic brain injury. Antagonists of 5-HT6, combined with ChEIs, higher doses of ChEIs combined with peripheral blockers of undesirable side effects also appear to have therapeutic potential. Nicotinic or muscarinic agonists are also likely to be promising if side effects can be managed. There is also evidence that a loss of calcium binding proteins such as calbindin may precede neurofibrillary degeneration and loss of neurons in the NbM (13). Calcium channel blockers may therefore also have a role in preventive cholinergic therapies.

Why therapy development efforts have failed – the need for novel concept and models of Alzheimer's disease

One of the major barriers to effective therapy development for complex neurodegenerative disorders is the lack of appropriate models or modeling systems. The entire enterprise of developing therapies, ranging from drug discovery to testing for the efficacy of treatments, is in urgent need for substantially more accurate models of disease than those currently available.

Prospective experimental prototypes necessary for the study of complex neurodegenerative disorders such as dementia should not only account for heterogeneous biological phenotypes but also must explain the full spectrum of clinical features across the complete spectrum of the syndrome. For example, a future 'ideal model(s)' (110) should address, and if possible, account for:

- Why the pathobiology preferentially affects specific locations, or neural systems in the brain
- How different pathologies shape pre-clinical and clinical phases of disease
- How individual environmental and genetic factors contribute to the development of clinical symptoms
- The relationships between risk factors and underlying pathophysiological mechanisms
- The preponderance of mixed pathologies
- The role of existing medical comorbidities

A vital limitation of current models of AD, and associated paradigms for therapy development, is their unquestioned acceptance of the idea that a unitary etiological pathway or a single causative factor underlies the neurobiology of the disease, including all its heterogeneous forms of expression. The key problem is that although the theory of a single pathogenic mechanism might adequately explain some forms of the disease (e.g., ADAD), it may not be necessary or sufficient to account for the underlying complex biology of all other forms of the disorder (e.g., LOAD) (2). Increasing evidence indicates that chronic neurodegenerative disorders and dementia, particularly late onset sporadic form of AD, are polygenic disorders that entail complex pathogenic paths with multiple components and mechanisms that are yet to be sorted out. Thus, there is the need for critical re-evaluation of all old assumptions and the adoption of radically new thinking about the origins of complex neurological disorders.

The recent sequential failure of treatment trials for LOAD, which uniformly have relied on interventions derived from extrapolations of a unitary pathogenic pathway model, have reinforced growing doubts about the validity of current theories on therapy development and the prevailing assumptions that one mode of action fits all forms of AD. This commonly held implicit supposition has contributed to unrealistic expectations for efficacy in the current drug development environment and may also explain the selection of inappropriate treatment targets that fail to consider the complex interactions among multiple pathogenic pathways or the intricate cascade of molecular events leading to late-stage dementia of AD.

A more productive future R&D enterprise for discovery and testing of therapies for complex neurological disorders may require radically different ways of conceptualizing these disorders. For example, AD might be characterized not as a disease but as a clinical-pathological syndrome that reflects varying patterns of failure in various related neural systems that underlie behaviors such as memory, language, attention, affect, motor function, etc. The critical feature of such a systems failure model is that this approach to thinking about the disorder does not rely on a unitary etiologic factor or a linear pathogenic process but rather requires dissecting the key components of the system and understanding the complex interactions among the

constituents of the systems.

The field is still at an early stage in the comprehensive exploration of the complex spectrum of neurobiological mechanisms that underlie chronic neurodegeneration or the intricate pathogenesis of Alzheimer syndrome. To move forward in this direction, we need to adopt new concepts and principles from other fields that also grapple with understanding the behavior or functioning of complex non-linear systems; for example, studies or research based on general systems theory, complexity science, and computational biology. Novel conceptual models or theories of the Alzheimer syndrome, which take into account the complex polygenic nature of underlying biology, may well change current paradigms and lead to successes in treatment and/or prevention. However, such a radical change in thinking or a tectonic shift in current research philosophies will not be easy.

In particular, discovering and testing new therapeutic targets focusing on disease progression (and eventual prevention) will require addressing the full spectrum of pathophysiologic mechanisms, as well as the biomarkers associated with these neuropathologic process across all involved systems. This will require identification of all critical components of these systems, along with time-space relationships of key elements involved in the clinical manifestations of the disease.

The fragmented R&D enterprise – including drug discovery, clinical testing, biomarker development, molecular genetics/genomics, neurochemistry, and neuroimaging – needs to be integrated to enable problem solving based on systems approach. Better alliances and collaborative models will need to be coordinated between industry and academia. Current paradigms of therapy development need to be expanded to include the concept of a complex, multifactorial brain failure involving a wide array of interlinked systems and brain regions. The continuation of traditional search for magic bullets or "one-size-fits-all" drugs will not likely succeed in treating sporadic AD, which largely includes clinically heterogeneous cohorts with multiple underlying genetic and biological variants.

The road ahead will likely mirror other advanced fields, such as oncology, using genomic screening and biomarker-guided targeted therapies tailored to an individual's biological makeup. This will include understanding how the pathophysiology of the cholinergic system differs among individual patients, potentially explaining the spectrum of responders and non-responders according to neural network paradigms (37, 38).

Conclusion and future efforts

The present provisional report reassessing the 'Cholinergic Hypothesis' is intended as initial phase of what would be an ongoing and an expanded effort to re-formulate this 'hypothesis'. We expect to enlarge the CWG by inviting wider participations and contributions of differing perspective, via formal commentaries, in the preparation of a final version of this hypothesis and its putative linkages to other ideas or theories on dementia. Here we discuss some of the major unanswered questions and challenges facing this enduring effort to revitalize the 'hypothesis'. For example, how do we understand the cholinergic hypothesis and explain to clinicians the novel features learned from emerging evidence?

Re-formulation of the 'Cholinergic Hypothesis'

Thus far, the primary focus of the CSW was to re-evaluate existing information pertaining to the role of the cholinergic system in memory dysfunction. The remaining essential task is to re-define the so-called 'Cholinergic Hypothesis' as a formal premise to explain neurodegeneration in the pathogenesis of the Alzheimer's dementia syndrome.

The first formal formulation of the ''Cholinergic Hypothesis' in the 1982 Science article by Bartus et al was not a truly formal 'hypothesis' but rather offered a review of the associations between cholinergic system and geriatric memory dysfunction (21). Thus, one of the unfinished critical tasks is to characterize objectively the potential pivotal role of the early structural (i.e., anatomical) and functional (i.e., neurochemical) changes in the cholinergic system as an important component of AD pathogenesis.

The remaining challenge for the CSW is to integrate the emerging understanding of the cholinergic system into a formal hypothesis that specifies some major testable assertions or predictions about the role or place of the cholinergic system in neurodegeneration and dementia (112). Then we will need to identify the crucial experiments that will be required to validate the major postulated or the central claims of the revised hypothesis and test its key predictions (113, 114).

One important paradox to be resolved is whether to emphasize the putative role of the cholinergic system as a key component of dementia pathogenesis via amyloid-tau pathways or to stress the strong relation of the cholinergic system to the hallmark behavioral or clinical features of the disorder e.g., memory or cognition, affect, agitation etc.

If the focus of the revised hypothesis is to explain the etiologic role of the cholinergic system via the 'amyloid-tau' story, then this proposition will be obliged to elucidate how (mechanistically) cholinergic system dysfunction leads to synapse loss or cell death. However, if the amended hypothesis focuses on explaining cognition, then the questions to be addressed are how the cholinergic hypothesis explains memory change associated with Parkinson's, ALS or any other neurodegenerative disorder; whether different cholinergic neural nets are involved in these different disorders; and whether the cholinergic hypothesis

accounts for other related symptoms with these disorders, such as motor and sensorimotor dysfunction and changes in affect. The answers to these questions could provide new therapeutic targets or different strategies for tweaking the cholinergic system as interventions for these and other related burdensome conditions.

What are the linkages between 'Cholinergic Hypothesis' and other theories on neurodegeneration and dementia?

Where does the notion of cholinergic deficits fit among the universe of all the different theories about the pathobiology of the disease, including amyloid, tau, calcium, inflammation, vascular changes, and metabolic dysfunction? Although the CWG discussed some possible linkages between cholinergic deficits and other neurobiological markers of dementia, associations do not indicate causal relationships. A future challenge for a reformulated cholinergic hypothesis is to postulate specific mechanistic relationships. For example, how does the cholinergic hypotheses account for synaptic loss and/or in what way is this effect (if any) similar to or different from synaptic loss resulting from amyloid- or tau-induced pathobiology?

Given that cholinergic loss in AD is confined to cortical structures (e.g. cerebral cortex, basal forebrain, and hippocampus), and that cholinergic innervation of subcortical structures remains spared, any mechanistic explanation must recognize that the cholinergic lesion in AD reflects vulnerability of the NbM and cholinergic medial septum neurons rather than a general failure of cholinergic neurotransmission. While there is growing knowledge about the anatomy and structural changes in the cholinergic system, this is only part of the story; there are yet additional unresolved questions regarding functional and mechanistic aspects of these alterations. Thus, the key research themes emerging from the CWG deliberations include a need to focus on these questions:

- A critical issue for any hypothesis is the specificity of the damage, i.e., why certain cells in some specific structures are affected while others are not. For example, cholinergic neurons in the hypothalamus and some in the thalamic nuclei ones are not affected in AD, whereas cells in NbM begin to gradually deteriorate. The question is, what starts that process?
- What are the upstream events that trigger the cholinergic changes? Is there something about specific cholinergic cell types that renders them more vulnerable, or are these specificities due to some unique features of the signals that initiate the degenerative processes?
- A related challenge is how to reduce the loss of forebrain cholinergic neurons or how to enhance the cholinergic activation of the remaining NbM neurons, for example, by stimulating still viable cortical and hippocampal muscarinic and nicotinic receptors.

Delaying the progression of disease represents an important goal: The question of whether cholinergic therapies can play a central role remains a crucial subject for future investigation, which warrants further validation in well-powered, longitudinal follow-up studies. At present, the weight of evidence suggests the potential for use of ChEIs in patients with MCI or dementia due to AD pathology, although Level A evidence to recommend this practice in MCI is not yet available. Possibly this lack of evidence may reflect the heterogeneity of MCI (115). In the future, studies restricted to MCI patients with positive AD biomarkers may yield more encouraging results. Evidence that some ChEIs have shown an apparent protective effect on cholinergic neurons (72) and that they may delay or reduce amyloid deposition (111) is already most encouraging.

Potential utility of early alterations in cholinergic functions as a biomarker

A huge unanswered question is whether markers of cholinergic function change, including clinical-behavioral changes, can accurately predict with great precision what is going to happen clinically down the road. If so, this would argue forcefully for intervention as early as possible.

Thus, a future reformulation of the 'Cholinergic Hypothesis' should incorporate a mechanistic explanation for the emerging evidence from in-vivo brain imaging studies that the cholinergic system undergoes particularly early degeneration even at the preclinical stage of AD. The finding of these relationships opens the door for further research on the impact of cholinergic therapy at asymptomatic stages.

A big challenge for the field, as well as for the prospective hypothesis, is to identify the earliest measurable changes in the cholinergic system – or perhaps a combination of several early markers -- that could accurately predict the onset of symptoms or cognitive decline in asymptomatic people. Presently, several biomarkers have been identified with high degree of confidence for significant associations with various aspects of the pathobiology of the Alzheimer dementia syndrome. However, virtually all these observed correlations are derived from cross sectional data; their putative causal relationships have yet to be demonstrated. None of these biomarkers has been validated by prospective longitudinal studies to determine whether, or to what extent, the patterns of biomarker changes will enable accurate prediction on an individual, rather than population, level.

Emerging new knowledge about the cholinergic system, e.g., retinal imaging is beginning to provide the tools for measuring biomarkers derived from the

cholinergic hypothesis. The future validation of such biomarkers as predictive tests will require studies with: a) huge numbers of subject, b) very large numbers of measurements, c) follow-up longitudinal recording of several biomarkers from different domains, and d) new computational algorithms to detect meaningful patterns of changes in several biomarkers rather than relying on a single marker in a particular domain. Thus, we will need to link the cholinergic hypothesis with advances in computational biology to develop the algorithms for predicting risk.

A revised 'Cholinergic Hypothesis' from the vantage point of systems biology

A crucial question for the development of future or second-generation interventions based on the 'Cholinergic Hypothesis' is how to account for the fact that cholinergic therapies have demonstrated relatively minimal clinical effects given that acetylcholine affects multiple mechanisms including neuroprotection, inflammation, and neuromodulation. How can we enhance the robustness of the effect and make cholinergic therapy more effective? Perhaps combining treatments is the answer, for example, by using a 5-HT6 blocker in addition to cholinesterase inhibitors. Determining whether combination therapy adds clinical value will require a revised view of the cholinergic hypothesis that incorporates a systems approach to therapy development.

The adoption of a systems point of view will require the prospective revision of the 'Cholinergic Hypothesis' to explain the complex interrelationships between multiple variables. Thus, the reformulation of the hypothesis needs to focus on identifying the essential components of the system as well as their complex relationships, including the dynamic interactions among these key components that underlie the system's functionality. For example, testable assertions are needed regarding the functionality of the cholinergic system with respect to the production or processing of amyloid or the tau, including (perhaps more importantly) its role in maintaining synaptic integrity.

A 'Systems Theory' perspective in redefining the cholinergic hypothesis will require the field to consider novel ideas, such as the notion that multiple seemingly disparate pathways can lead to a common endpoint such as synaptic loss and changes in neural connectivity. For example, cholinergic mechanisms for synthesis, re-uptake, and receptor-turnover could interact with calcium toxicity and inflammatory activation of one or more components, leading to dendrite pruning and synapse loss.

The adoption of notions from 'systems theory' to reframing the cholinergic hypothesis could provide the mechanistic rationale for combining cholinergic treatments with other interventions as a means to tweak the cholinergic neuron through different pathways to get better results. For example, one might envision combining a cholinergic agonist with neurotrophins or some of the metabolic enhancers.

In summary, substantial work is required to position the cholinergic system within the context of other pathophysiological mechanisms. Progress in this direction could pave the way for novel and more effective interventions across the continuum of the disease but will require filling a number of research gaps, including:

- Identifying, developing, and validating novel imaging and CSF biomarkers to quantify the "intactness" of cholinergic system.
- Developing tools to assess cholinergic activity in vivo.
- Understanding the "neurotransmitter-independent" effects of cholinesterases.
- Developing novel cholinomimetic agonists with tolerable side effects.
- Understanding the factors that make the BFCS selectively vulnerable to degeneration.
- Understanding of the mechanisms of how ChEIs impact progression of regional brain atrophy.
- Seeking corrective therapies for mechanisms leading to degeneration of BFCS.

Table 1. Abbreviations used in this manuscript

5-HT6	5-hydroxytryptamine receptor 6
AAV-NGF	Adeno-associated virus vectors expressing nerve growth factor
AChE	Acetylcholinesterase
Aβ	Amyloid beta
AD	Alzheimer's disease
ADAD	Autosomal dominant Alzheimer's disease
ARAS	Ascending reticular activating system
BF	Basal forebrain
BFCS	Basal forebrain cholinergic system
CCB	Calcium-channel blockers
ChAT	Choline acetyltransferase
ChEIs	Cholinesterase inhibitors
CNS	Central nervous system
CWG	Cholinergic System Working Group
DAT	Dementia of the Alzheimer's type
DLB	Dementia with Lewy Bodies
DS	Down syndrome
GMLT	Groton Maze Learning Test
LOAD	Late-onset Alzheimer's disease
MCI	Mild cognitive impairment
MRI	Magnetic resonance imaging
NbM	Nucleus basalis of Meynert
NGF	Nerve growth factor
NSAIDs	Nonsteroidal anti-inflammatory drugs
PET	Positron emission tomography
RES	Reticuloendothelial system
SD-OCT	Spectral-domain optical coherence tomography
tPA	Tissue plasminogen activator

Disclosure: The present paper was originally submitted for publication in Alzheimer's & Dementia [A&D]; because it fit the new initiative the journal had launched to propose novel 'hypotheses' of AD pathogenesis and also to reevaluate existing theories related to tau, amyloid, cholinergic systems, an so on. The first version of this paper was rejected after peer review but with sufficient constructive criticism to warrant a resubmission. The revision underwent a second peer review, which generated a positive decision. This led to a pre-publication posting on the A&D website (October 10, 2017, DOI: https://doi.org/10.1016/j.jalz.2017.08.016). At that time, colleagues outside of the coauthor group, pointed out two potential conflicts of interest. First, four of the authors were on the A&D editorial board, including the Chief and Executive Editors. Secondly, the meeting that brought the coauthors together for a brainstorming session had been funded by Axovant, which at that time was developing a cholinergic agent which has since been shown ineffective for treating AD. Although the Editors and other coauthors believed that the A&D peer review had been conducted impartially and free of interference (appropriate recusals and appointment of an independent Editor), and even though Axovant neither compensated the authors for the writing of the paper nor had any input into its contents, the authors felt that it would be in the best interest of all to withdraw the paper from A&D and resubmit it for an additional independent assessment to Journal of Prevention of Alzheimer's Disease that may also offer the additional benefit of open peer commentary from the community as a whole. Although unusual and potentially confusing, we feel that this course of action prevents the appearance of conflict related to the contents of the paper.

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