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### Review

# The history of the cholinergic hypothesis

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#### ABSTRACT

The cholinergic hypothesis of cognitive impairment and Alzheimer's disease has been for decades a "polar star" for studies on dementia and neurodegenerative diseases. Aim of the present article is to briefly summarize its birth and its evolution throughout years and discoveries. Putting the cholinergic hypothesis in an historical perspective, allows to appreciate the enormous amount of experimental and clinical research that it has stimulated over years and the impressive extent of knowledge generated by this research. While some of the assumptions at the basis of its original formulation are disputable in the light of recent developments, the cholinergic hypothesis has, however, constituted an invaluable stimulus to better understand not only the anatomy and the biochemistry of the cholinergic systems of brain connections but also its developmental biology, its complex relationships with trophic factors, its role in cognitive functions. Thus, rather than being consigned to history, the cholinergic hypothesis will likely contribute to further understanding dementia and neurodegenerative diseases and will hopefully be integrated in novel therapies and treatments.

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### 1. Introduction

In 1936 Henry Dale and Otto Loewi shared the Nobel prize for their pioneering research on chemical neurotransmission and in particular for the discovery and the functional characterization of the first identified neurotransmitter, acetylcholine. The history of this neurotransmitter dated back to the crucial experiments performed by Dale, who identified acetylcholine as responsible of a strong vasodepressor effect [32] and by Loewi who demonstrated chemical neurotransmission in the frog vagus nerve-heart preparation [74]. This 15-year long story found a first conclusion with the demonstration that acetylcholine was actually present in mammalian organs [33]. Since then, the history of acetylcholine in neuroscience has been one of great advancement of our knowledge in many functions of the nervous system as well as in very harmful neuropathologies. The importance of cholin-

ergic neurotransmission is testified by the fact that it has led several investigators to elaborate "cholinergic hypothesis" for several brain functions and dysfunctions, from affective disorders, depression, schizophrenia and delirium to sleep regulation and brain traumatic injury [4,12,13,38,66,77,99]. There is no doubt, however, that the most famous in recent neuroscience history is the cholinergic hypothesis for impairment of cognitive function and dementia. Early studies based on pharmacologic interference with cholinergic function suggested a strong relationship between acetylcholine-mediated neurotransmission and cognitive function. A primary role for cholinergic function in cognition was initially supported by observations indicating that anticholinergic drugs had amnesic effect and reproduced memory deficits similar to those commonly observed in non-demented elderly subjects [40,75]. While these results were corroborated by primate studies, indicating the feasibility of pharmacologically reproducing in young and old animals some of the cognitive dysfunctions seen in elderly humans and Alzheimer's disease patients [7,11,111], the rationale of developing cholinergic pharmacotherapies for Alzheimer's disease was strongly questioned by some expert of the field [46]. The history

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of the most relevant steps, results and discrepancies arising by research efforts to elucidate relationships among cholinergic function, memory, cognition and dementia will constitute the bulk of the present article.

## 2. Emergence of the cholinergic hypothesis for Alzheimer's disease

The decades from mid sixties to mid eighties are with good reasons considered a sort of golden age for studies on neurotransmitters. During these years, an impressive convergence of studies based on biochemistry, electrophysiology, pharmacology and morphology, including immunohistochemistry, succeeded not only in discovering and characterizing the most important neurotransmitters and their receptors, but also in delineating the chemical neuroanatomy of the brain, i.e. the neurotransmitter systems underlying specific nervous connections and brain functions [64]. Evidence stemming from this worldwide research effort, strongly contributed to establish the idea that altered function of neurotransmitter systems was associated with neuropathologies and neural disturbances. Accordingly, neuropathologists started to look with increasing interest to neurochemical alterations present in autoptic samples obtained from post-mortem brain of patients deceased with diagnosis of various pathologies of the nervous system. The underlying therapeutic idea was that this knowledge could help to develop rational therapeutic disease approaches targeted at the correction of the neurochemical alterations found. When this approach was applied to examination of brain samples from Alzheimer's disease patients during seventies and early eighties, the cholinergic hypothesis did emerge and acquire momentum. Indeed, a specific cholinergic deficit, involving the cholinergic projection from a basal forebrain neuronal population, the nucleus basalis magnocellularis of Meynert, to the cortex and hippocampus was consistently found in autoptic material from Alzheimer's patients. The activity of the enzyme responsible for the synthesis of acetylcholine, choline acetyltransferase, a reliable marker of cholinergic neurons and synapses, was found to be remarkably decreased, sometimes in rather severe way, in pathological samples from the cortex and hippocampus of Alzheimer's patients [17,34,95]. Two other specific markers of the function of cholinergic synapses, depolarization-induced acetylcholine release and choline uptake in nerve terminals to replenish the acetylcholine synthetic machine, were also reduced in the same tissues [88,102]. The parallel observation of a substantially decreased number of the cholinergic projection neurons in the nucleus basalis of Meynert led to conclude that a major event in the pathogenesis of Alzheimer disease was represented by the degeneration of the cholinergic connection from the nucleus of Meynert to the cortex and hippocampus [125]. As memory impairment and dementia are primary symptoms of Alzheimer's disease, the then emerging role of acetylcholine transmission in cognitive functions supported this conclusion [40]. Further strength was provided by initial reports unraveling correlation between the damage of the basal forebrain cholinergic system and the burden of senile plaques in autoptic brains, both these markers being in addition apparently related to the dementia score evaluated from the patient during life [96,126].

These, and several other remarkable observations, constituted the basis of a seminal review article published in 1982 by Bartus et al. [11], that may be considered the first comprehensive and formal appraisal of the state of the art for the cholinergic hypothesis of age-related cognitive dysfunctions and dementia. While the amount of reviewed data was already impressive, a main problem considered by the authors was the almost complete lack of studies comparing control aged and Alzheimer's patient populations with a control young population, in order to better isolate the effect of the pathological dementia state from the age-related cog-

nitive impairment. The lack of this information and the fact that almost exclusively material from Alzheimer's patients deceased at advanced stages of the disease and aged controls had been examined at that time, is likely at the basis of some subsequent revision of the cholinergic hypothesis, as it will be discussed later on in this review. This possible source of confounding results clearly emerges from the first table of the article [11] which summarizes data on choline acetyltransferase activity in brain regions of aged rodents compared with young rodents, of aged humans compared with young humans and of Alzheimer's patients compared with age-matched elderly subjects. While only in a minority of studies significant age-related decrease of enzyme activity was detected in some brain areas, all the reported studies demonstrated significant impairment of the cholinergic markers in brain areas of Alzheimer's patients compared with elderly control subjects. A different situation did emerge from the analysis of data available at the time of publication, regarding density of muscarinic acetylcholine receptors. While data on aged rodents and humans converged to suggest an age-related decrease of muscarinic binding sites in several brain areas examined, no additive effect of Alzheimer's disease over aging was convincingly demonstrated [11]. The review of Bartus et al. [11] went on considering the possibility to reproduce cognitive impairment in young primates by pharmacologically interfering with the cholinergic function, with some encouraging results, or to restore cholinergic function [8] through administration of acetylcholine precursors, with almost entirely negative results (see, however, the modestly positive results reviewed in a more recent paper [3]). First encouraging evidence favorable to the use of acetylcholine hydrolysis inhibitors and acetylcholine receptor agonists, of which more will be said later on in this review, was also briefly discussed [11]. From the collection and discussion of data available at that time, the authors were convinced that: "Although it might be premature to draw any final conclusion from this circumstantial evidence, the data demonstrate that certain logical criteria, prerequisite for accepting the cholinergic hypothesis, have been satisfied and that continued empirical and therapeutic interest is therefore justified".

Further development of the cholinergic hypothesis took place in the following years, some of them clearly supporting the idea while some resulting more equivocal. When detailed quantitative analysis were made regarding the cholinergic innervation to various brain areas, the loss of cholinergic fibers in Alzheimer's patients appeared particularly pronounced in areas concerned with memory and cognition, such as the hippocampus, the temporal and the frontal non-motor areas [39,53]. An essential enzyme for acetylcholine synthesis, the pyruvate dehydrogenase complex, which is highly active in the cholinergic neurons of the nucleus basalis of Meynert [79], was found to be reduced in activity in post-mortem brain tissue from Alzheimer's disease patients [55,110]. Another molecular step of cholinergic transmission, i.e. the acetylcholine vesicular transport essential to replenish synaptic vesicles, appeared to correlate with choline acetyltransferase levels in brain areas of Alzheimer's patients [44]. While further studies confirmed that muscarinic receptor subtypes were not significantly changed in Alzheimer's disease brain [91,122], at least one type of nicotinic receptors, the  $\alpha$ -4 subtype, was described as consistently reduced in patient's brain [18,106]. The renewed interest in neuronal survival factors, prompted by the 1986 Nobel prize awarded to Rita Levi Montalcini and Stanley Cohen for nerve growth factor discovery, stimulated novel research on possible involvement of nerve growth factor or other neurotrophins, and their receptors, in Alzheimer's disease. Nerve growth factor is a survival factor for cholinergic neurons of the nucleus basalis of Meynert, which express both high affinity and low affinity receptors for the neurotrophin. In particular the high affinity receptor TrkA was found to be decreased in these neurons in the brains from Alzheimer's disease patients [81,103]. However, no clear relation-

ship between production of nerve growth factor by brain regions which are targets of the cholinergic basal forebrain projection and Alzheimer's disease, was found [70,107]. To further complicate the issue, novel data in primates and humans confirmed that, similarly to the condition found in rodents [121], cortical cholinergic activity was actually decreased by aging [112,114] and that cholinergic deficits were also found in neurodegenerative diseases others than Alzheimer's disease [5,86,97]. In particular, it was reported that in the brain of patients with inherited olivo-ponto cerebellar atrophy, cortical choline acetyltransferase activity underwent a reduction similar to that of Alzheimer's disease patients, without resulting in patent dementia [71]. The same authors further reported that, at variance with Alzheimer's disease, hippocampal enzyme activity was unchanged in patients with olivo-ponto cerebellar atrophy, indirectly suggesting a prevalent involvement of the damage to the cholinergic septo-hippocampal projection in Alzheimer's disease.

These early developments of the cholinergic hypothesis had soon to confront with other ideas stemming from progress in the biochemistry of  $\beta$ -amyloid protein and of tau protein hyperphosphorylation, the two oldest stigmata associated with Alzheimer's disease since its early description from Alois Alzheimer [1,2] the senile plaques and the neurofibrillary tangles. These ideas gave rise to the amyloid cascade hypothesis for the pathogenesis of Alzheimer's disease [60,61,89]. In addition to the strong biochemical evidence, genetic studies of familial cases of Alzheimer's disease played an important role of support for this hypothesis, based on the demonstration that all main mutations found in these patients were related to genes involved in the production of the precursor of  $\beta$ -amyloid protein or in its processing to give final fragments [6]. Thus, the momentum acquired by the role of  $\beta$ -amyloid in the pathogenesis of Alzheimer's disease prompted research on the possible interactions between cholinergic mechanisms and the amyloid protein. Some of these studies provided evidence for regulation of the metabolism of  $\beta$ -amyloid by stimulation of muscarinic or nicotinic cholinergic receptors [30,90,119]. More often, however, the inverse relationship was evident due to the fact that in several cases cholinergic deficits appeared to be a secondary effect of  $\beta$ -amyloid toxicity [100]. Following these initial and largely contradictory results, further data have provided some more convincing support to the possibility of a bi-directional interaction between cholinergic function and processing of amyloid precursor protein, as reviewed in a recent article [93].

In addition to the pharmacologic studies considered above, evidence to support the cholinergic hypothesis was sought through the use of animal models in which the impairment of learning and memory resulted from the normal aging process or from lesions involving the cholinergic projection from the basal forebrain to the cortex and hippocampus. The controversial results regarding the correlation between aging and several aspects of memory formation have been extensively considered in a review from Bartus [10]. Some of the conclusions related to aging as well as to the pharmacologic manipulation of memory will be considered later on this article. Brain lesions targeted to the disruption of cholinergic function have been extensively used, based on the rationale suggested by the cholinergic hypothesis that in these animal models at least some of the cognitive deficits characterizing dementia and Alzheimer's disease could be reproduced and possibly counteracted by pharmacologic tools. Initial approach was based on the use of excitotoxins, such as ibotenic or quisqualic acid, stereotaxically injected in the nucleus basalis magnocellularis area of experimental animals, usually rats. While this experimental approach consistently results in large depletion of the basal forebrain cholinergic neuron population and substantial cortico-hippocampal cholinergic denervation [15,45,123], the relationship between memory impairment and depletion of cholinergic markers was far from convincing [42,43,123]. These contradictory results were, at least

in part, attributed to the modest specificity of the treatments (non-cholinergic neurons were affected together with the cholinergic ones) and erratic involvement of cholinergic populations projecting to different brain areas. Subsequent studies, therefore, adopted a more specific immunotoxic strategy, based on injection of an immunotoxin composed of the ribosome-inactivating toxin, saporin, coupled to an antibody against the low affinity p75 nerve growth factor receptor, which is expressed by cholinergic basal forebrain neurons [127]. While this more selective technique was able to restrict lesions more precisely to cholinergic neurons, evidence for direct relationship with memory impairment was still disputable [10,50,124].

Notwithstanding the many conceptual problems examined above, cholinomimetics appeared as potentially useful drugs for symptomatic treatment of Alzheimer's disease and, in particular, of the cognitive decline associated to it. On this basis, several drugs targeted to enhancing cholinergic transmission were introduced in therapy of Alzheimer's disease patients [41,52]. In particular, to compensate for decreased synthesis and synaptic availability of acetylcholine, interest was focused on inhibitors of cholinesterases, the enzymes hydrolyzing acetylcholine released in the synaptic cleft. Four cholinesterase inhibitors, tacrine, donepezil, rivastigmine and galantamine, have received approval by US Food and Drug Administration (FDA) and have been widely used for years in many countries, in particular for patients diagnosed with mild-intermediate forms of Alzheimer's disease based on Mini-Mental State Examination [87]. The modest results of these therapies have been repeatedly discussed [10,49] and the results of clinical trials conducted for 6 months or longer with standard dosages, have been recently reviewed [14,108]. In most studies, in particular those not involving advanced stages of the disease, the decline of cognitive functions resulted moderately slowed down and behaviors related to daily life were somewhat improved. Adverse side effects were acceptable and treatments with donepezil carried on for more than 2 years did not show significant increase of mortality risk [76]. While responder analysis from some large clinical trials suggested that the response to cholinergic therapy was greater in about 15% of patients, it was not possible to find predictive elements for early identification of responder populations in which to start early therapy [49]. Furthermore and unfortunately, data from clinical trials based on long-term administration of donepezil, rivastigmine and galantamine to individuals diagnosed with Mild Cognitive Impairment, a state considered to be prodromal to dementia, were negative about the possibility to reduce the risk or delay the onset of Alzheimer's disease [98]. In conclusion, while cholinesterase inhibitors are therapeutically valuable in modestly ameliorating conditions of patients with mild to moderate dementia, they cannot counteract or significantly delay its insurgence. Alternative ways to target the cholinergic system for memory improvement in dementia through nicotinic or muscarinic agonist are still in early stages of clinical testing.

### 3. Recent developments of the cholinergic hypothesis

The conceptual consistency and the therapeutic potential of the cholinergic hypothesis have continued to be intensively investigated during the recent years. Although this effort did not seem able to provide so far innovative therapeutic approaches, it has been of great value in expanding our knowledge of many molecular and pathologic aspects of dementia and Alzheimer's disease.

One of the most interesting novel contributions is represented by the extension of studies on cholinergic deficits to early and prodromal stages of the disease. This was largely made possible by the so-called religious orders study with the participation of over thousand aged nuns, brothers and priest all over United States convents, monasteries and churches, under funding from the National

Institute of Aging. Participants underwent periodical medical and psychological evaluation and agreed to donate brain after death, thus allowing extensive evaluation of the transition from normal function of aging brain to Mild Cognitive Impairment and further progression to Alzheimer's disease. While, as described above, cholinergic deficits are consistently detected in the brains of patient Alzheimer's disease cases, when reliable diagnosis of early stages of the disease became available it was realized that no significant decrease of choline acetyltransferase activity could be reproducibly measured in brain samples of these patients [35,118]. In the framework of the religious orders study, this was confirmed in samples from the hippocampus and various cortical areas of patients diagnosed with mild Alzheimer's disease [37]. Furthermore, and quite surprisingly, samples from the hippocampus and frontal cortex of patients deceased with Mild Cognitive Impairment diagnosis, actually showed an increased activity of choline acetyltransferase [37]. When the study was extended to the evaluation of the basal forebrain neuronal population giving rise to the cholinergic projection to the cortex and hippocampus, conflicting results did emerge. In samples from Mild Cognitive Impairment and early Alzheimer's disease patients, cholinergic neurons of the basal forebrain, identified through immunoreactivity for choline acetyltransferase or the vesicular acetylcholine transporter were not reduced in comparison to control individuals [56]. However, when these neurons were marked in the same patients through the expression of the high affinity or the low affinity nerve growth factor receptors, *trkA* and *p75*, a significant decrease was apparent [23,83,84]. These neurons usually show co-labeling with these markers [80] and the observed dissociation suggested that downregulation of nerve growth factor receptors is not necessarily representative of cholinergic dysfunction at prodromal or early stages of Alzheimer's disease [85]. Alternative explanation for this discrepancy is that to find choline acetyltransferase activity not impaired or even increased at an early stage of Alzheimer's disease does not necessarily imply the occurrence of a normal cholinergic function, as other pre- or post-synaptic mechanisms of cholinergic transmission may be compromised [104,117]. Nerve growth factor is an essential survival factor for cholinergic neurons of the basal forebrain, being synthesized in the hippocampus and cortex and retrogradely transported to the cell bodies [113]. Multiple evidence suggests that the deficit found in Alzheimer's disease is not due to reduced synthesis of the peptide from the target areas, but rather to impairment of its retrograde transport to basal forebrain neurons [29,31,82]. Thus a further explanation for the observed discrepancy among different markers for the nucleus basalis cholinergic neurons, may be that altered receptor response to nerve growth factor marks an initial stage of neuronal damage when cholinergic machinery is still fully active in the same neurons. Several hypotheses have been advanced to explain the increased choline acetyltransferase activity in the hippocampus and areas of the frontal cortex in patients diagnosed with Mild Cognitive Impairment. An attractive idea is that this finding may result from compensatory response to the loss of hippocampal input from the entorhinal cortex, which is known to be already present in Mild Cognitive Impairment patients [37,68]. According to this idea, the impairment of short term memory characterizing the Mild Cognitive Impairment may be attributed to reduction of the entorhinal-hippocampal connection rather than to alteration of the septo-hippocampal cholinergic system [57,73]. While not yet demonstrated, this hypothesis is consistent with the well documented ability of the hippocampal cholinergic innervation to sprout in response to the depletion of other connections, such as the one from the entorhinal cortex to the dentate gyrus [115].

Genetically engineered mouse models for Alzheimer's disease have been introduced in research in recent years based on the knowledge of mutations found in familial, early-onset Alzheimer's disease cases. Single, double and triple transgenic mice were

created targeting genes for the amyloid precursor protein and presenilins 1 and 2. These mice develop in adult age brain alterations resembling those found in Alzheimer's disease, such as deposition of  $\beta$ -amyloid plaques, altered synaptic function and impaired learning and memory [16,22,54,92,120,129]. Whether and to what extent this is accompanied by actual neuronal loss is more controversial [19,69,105,116]. Whether neurodegeneration occurs among cholinergic neurons of the basal forebrain and involves their projections to the cortex and hippocampus is even more doubtful [63,69,123,51,55]. Thus, rather surprisingly, transgenic mice models for Alzheimer's disease obtained through direct targeting of well established genetic mutations found in human patients, do not convincingly support the cholinergic hypothesis. In these mice, development of  $\beta$ -amyloid plaques appears to not profoundly affect cholinergic function and to not result in clear degeneration of the basal forebrain-cortico/hippocampal projection. A different mouse model, based on creation of a nerve growth factor-deficient phenotype, results in better correlation with cholinergic deficit. These mice undergo with age  $\beta$ -amyloid deposits, development of neurofibrillary tangles, cortical neuronal loss and parallel degeneration of the basal forebrain cholinergic neurons [20,101]. Furthermore, cholinomimetics ameliorate in these mice the synaptic plasticity which is found to be deteriorated in cortical slices [94].

#### 4. Lessons from Down's syndrome and Down's syndrome models

Down's syndrome, the most frequent genetic type of mental retardation [62], is caused by trisomy of chromosome 21 and shows interesting relationships with Alzheimer's disease. Many affected subjects, indeed, display around the fourth decade of life a clear Alzheimer's disease-like neuropathology with appearance of  $\beta$ -amyloid plaques, neuronal fibrillary tangles, degeneration of basal forebrain cholinergic neurons and consequent decrease of cholinergic activity in their target areas [21,48,58,78,128]. While in some brain regions, such as the hippocampus and cerebellum, reduced neuron number is due to a defect in neurogenesis [26,27] this is not the case for basal forebrain cholinergic neurons as they are normal in Down's children [72]. The fact that the cholinergic deficits appear in affected individuals in rather precise temporal relationship with plaques and tangles, strengthens the similarities between Down's syndrome and Alzheimer's disease. As the homologous of human chromosome 21 is the murine chromosome 16, genetic mice models with total or segmental triplication of this chromosome have been created [36,47,65]. Segmental trisomic mice with triplication in chromosome 16 of many genes present in human chromosome 21, Ts65Dn mice, have been extensively used for research. These mice survive up to around 2 years of age and start to display learning and memory deficits relatively early (by 6–8 months of age). They also undergo cholinergic deficit with age but this happens considerably later. While some reports suggested shrinkage and reduction in number of cholinergic neurons of the basal forebrain by 6 months of age in Ts65Dn mice [59,67] other studies led to conclude that decreased number of basal forebrain cholinergic neurons and reduction of choline acetyltransferase activity in the hippocampus and cortex, only became significant after 12 months of age [25,28,109]. Surprisingly, by 10–12 months of age, choline acetyltransferase activity was actually found to be increased in the hippocampus and some cortical areas [25,109]. Furthermore, at 24 months, an age close to their maximal life span, Ts65Dn mice did not show any tendency to undergo increased age-related cholinergic deficit, but actually displayed a slowing down of this process in comparison with age-matched wild type mice [24]. Thus, while it is clear that Down's syndrome reproduce some characteristic features of Alzheimer's disease in relatively young individuals, animal mod-

els of the syndrome, similarly to most animal models of Alzheimer's disease, fail to convincingly support the cholinergic hypothesis. Indeed, while some cholinergic deficit may be found in these animals, impairment of cholinergic function due to degeneration of basal forebrain cholinergic neurons does not seem to play a pivotal role, appearing relatively late in life and being present in mild form.

## 5. Final (not conclusive) remarks

Every history in science is a never-ending story as it does not allow to draw any final conclusion or any happy (or not-so-happy) end. Scientists know this very well and are ready to accept this truth without discouraging themselves. Luckily, in addition to science, also philosophy comforts bench investigators, by teaching them with the authority of Karl Popper that any conclusion in science is such only if it can be, in principle, falsified. The history of the cholinergic hypothesis is a remarkable example of how a scientific hypothesis which can be considered at least questionable with respect to its original formulation and its deepest meaning, has produced over more than 30 years an impressive amount of science and a substantial improvement of our knowledge of one of the most devastating human diseases. It seems clear that in its original formulation, i.e. of a primary and driving event in the pathogenesis of Alzheimer's disease, the cholinergic hypothesis is not convincingly tenable in the light of the recent developments of research and clinical data, as outlined in the previous paragraphs. It has, however, constituted over years an invaluable stimulus to better understand not only the anatomy and the biochemistry of the cholinergic systems of brain connections but also its developmental biology, its complex relationships with trophic factors, its role in cognitive functions. These developments were clearly forecasted more than 20 years ago by Bartus [9] when he argued that: "... the cholinergic hypothesis necessarily suffers from a certain degree of inherent oversimplification and possible myopia. Yet, advantages gained by the research activities it helps stimulate and direct must not be underestimated. It seems clear that the opportunity it provides for increasing our understanding of age-related memory problems and for developing an effective mean of reducing the severity of the cognitive symptoms of aged brain and Alzheimer's disease, provide adequate testimony for the continued value of the hypothesis as an important heuristic tool". There is, furthermore, no doubt that cholinergic impairment is an important event in the progression of Alzheimer's disease and a valuable target for palliative therapies to be integrated in a polypharmacy approach to enhance efficacy, as predicted by Bartus in his recent reappraisal of the cholinergic hypothesis [10], while waiting for efficient tools to prevent Alzheimer's disease pathogenesis. When these tools will be available, which unfortunately is not likely to occur soon, a reappraisal of the cholinergic hypothesis will tell us whether it should be consigned to history or, as I personally hope and believe, fruitfully integrated in novel therapies and treatments.

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