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Cholinergic system during the progression of Alzheimer's disease: therapeutic implications

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Abstract

Alzheimer's disease (AD) is characterized by a progressive phenotypic downregulation of markers within cholinergic basal forebrain (CBF) neurons, frank CBF cell loss and reduced cortical choline acetyltransferase activity associated with cognitive decline. Delaying CBF neurodegeneration or minimizing its consequences is the mechanism of action for most currently available drug treatments for cognitive dysfunction in AD. Growing evidence suggests that imbalances in the expression of NGF, its precursor proNGF and the high (TrkA) and low (p75^{NTR}) affinity NGF receptors are crucial factors underlying CBF dysfunction in AD. Drugs that maintain a homeostatic balance between TrkA and p75^{NTR} may slow the onset of AD. A NGF gene therapy trial reduced cognitive decline and stimulated cholinergic fiber growth in humans with mild AD. Drugs treating the multiple pathologies and clinical symptoms in AD (e.g., M1 cholinergic and/or galaninergic drugs) should be considered for a more comprehensive treatment approach for cholinergic dysfunction.

Keywords

acetylcholine receptor; Alzheimer's disease; anticholinesterase; basal forebrain; choline acetyltransferase; cholinergic; galanin; gene therapy; mild cognitive impairment; NGF; nucleus basalis; p75^{NTR} receptor; proneurotrophin; sortilin; tau; TrkA receptor

Alzheimer's disease

Alzheimer's disease (AD) is a progressive and fatal neurodegenerative disorder manifested by cognitive and memory decline and progressive impairment of activities of daily living, as well as a variety of neuropsychiatric symptoms and behavioral dysfunctions. Prevalence studies

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suggest that approximately 18 million people worldwide have AD. In the USA alone there are more than 5 million people with AD. The percentage of people with AD increases by a factor of two with approximately every 5 years of increased age, meaning that 1% of those 60 years of age and approximately 30% of 85-year-olds have the disease. Without advances in rational drug therapy for the treatment of dementia, the number of symptomatic cases in the USA is predicted to reach 13 million by 2050. The cost of caring for patients with AD is extraordinary, with annual expenditures exceeding US\$100 billion [1,2]. These data highlight the immediacy of developing more effective therapeutic interventions for those in the early or prodromal stages of AD.

Prodromal AD

Although AD is a progressive neurodegenerative disorder characterized clinically by irreversible cognitive deterioration, evidence suggests that mild cognitive impairment (MCI) is one of the most common conditions affecting persons over the age of 65 years, and that the prevalence for MCI is more than double that of dementia. People with a clinical diagnosis of MCI comprise a heterogeneous cohort of which those with memory deficits only are classified as amnesic MCI (aMCI) and those with impairment in other cognitive domains lacking a clinical diagnosis of dementia are designated multi-domain MCI (mdMCI) [3,4]. Many individuals characterized with aMCI progress steadily to greater stages of dementia severity, and in many instances exhibit the neuropathologic, molecular and biochemical hallmarks of AD [5-9]. These clinical pathobiologic studies suggest that MCI, in general, represents a prodromal or preclinical stage of AD. Since it is believed that AD has an extensive preclinical phase, understanding the molecular pathogenesis leading to AD will require studying people during the early stages of the disease when brain pathology has been initiated prior to presentation of significant clinical symptoms [10]. Therefore, defining molecular and cellular mechanisms underlying the selective vulnerability of neurons associated with cognitive decline from people who died with an antemortem clinical diagnosis ranging from no cognitive impairment (NCI) to MCI to AD will be important for the development of novel therapeutics to arrest and/or slow the onset of AD. The current review will highlight research directed at understanding the neuropathobiology of well-characterized cholinergic deficits seen in patients with AD, with emphasis on molecular and cellular alterations early in the disease process, and how these findings translate to potential drug treatments for dementia.

Cholinergic basal forebrain system

Although there is a widespread decline in various neurotransmitter-containing cell bodies in end-stage AD, the most consistent losses throughout the progression of AD are seen in long projection neurons, including cholinergic neurons of the basal forebrain [11-13]. Cholinergic neurons within the nucleus basalis (NB) and the septal diagonal band complex provide the major source of cholinergic innervation to the cerebral cortex and hippocampus, respectively, and play a key role in memory and attentional function [11-13]. Cholinergic basal forebrain (CBF) cortical projection neurons contain the pathological AD hallmark, neurofibrillary tangles (NFTs), and undergo chemical phenotypic alterations during the progression of AD, making them an excellent natural model for studying mechanisms of cell death, survival and treatment approaches both *in vitro* and *in vivo*, including relevant animal models of neurodegeneration as well as human postmortem clinical pathological tissue studies [14]. The regions of the forebrain that contain cholinobasal and septohippocampal CBF neurons also display various non-cholinergic neurons, including GABAergic interneurons that innervate cholinergic perikarya [11]. In addition, neuropeptides often co-localize with CBF neurons. For example, the inhibitory neuropeptide galanin (GAL) is found in septohippocampal and NB neurons in rodents, but not in higher apes or humans where GAL fibers innervate CBF neurons. [15]. Therefore, it is likely that neurotransmitter interactions may provide novel

targets for the development of modulatory polypharmaceutical treatment approaches for cholinergic deficits seen in AD (see later).

Cholinergic dysfunction in AD

The loss of cholinergic markers within vulnerable neurons and their respective projection sites has been a major tenet in AD research for over 30 years. Specifically, progressive phenotypic downregulation of markers within CBF neurons as well as frank CBF cell loss has been observed consistently, along with an associated reduction of choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) activity within the cortex in AD [16]. Most researchers presumed that progressive disruption of cholinergic function underlies much of the short-term memory loss seen in AD. Delaying or preventing cholinergic neurodegeneration or minimizing its consequences is the mechanism of action for most currently available US FDA-approved drugs for the treatment of cognitive dysfunction observed in AD [17]. Notably, reports that physostigmine and oral anticholinesterases have beneficial effects for patients with AD suggest that the CBF system is somewhat preserved during the progression of dementia, despite well-documented loss of cholinergic biosynthetic machinery (including ChAT and AChE enzyme deficits) in patients with this disease. Interestingly, recent studies have shown that ChAT activity, which results in acetylcholine (ACh) synthesis, is preserved in the neocortex of people with MCI [18,19]. Therefore, cholinergic enzyme deficits are likely not the primary cause of memory loss observed in MCI, although these studies do not rule out other types of cholinergic dysfunction early in the disease course. In fact, our group found elevated ChAT activity in the hippocampus and frontal cortex of subjects with MCI [19,20]. These results suggest that cognitive deficits in MCI and early AD are not associated with a reduction in ChAT activity. Moreover, these data indicate that select components of the hippocampal and cortical cholinergic projection system are capable of compensatory and/or neuroplasticity responses during the early stages of AD. In MCI, increased hippocampal and frontal cortex ChAT tone may be important for promoting biochemical activity or compensating for neurodegenerative defects, which may delay the transition of these subjects to frank AD. Hippocampal ChAT activity was increased selectively in MCI cases with high Braak scores (Braak III/IV stage) indicative of advanced disease [19], suggesting that a compensatory upregulation of ChAT may be due, at least in part, to the disconnection of glutamatergic entorhinal cortex input to the hippocampus which occurs early in the disease process [21-23]. In this scenario, upregulation of hippocampal ChAT activity may be due to reactive synaptogenesis, the filling in of denervated glutamatergic synapses by cholinergic input arising from the septum [24]. This mechanism of cholinergic synaptic plasticity has been observed at the light and electron microscopic level in rodents with perforant path transections that demonstrate subsequent reactive synaptogenesis of cholinergic nerve terminals [25,26]. Reasons for the elevation of frontal cortex ChAT activity in MCI is less clear, but may be related to the observation that the anterior cholinergic subgroup of the NB, which projects to the frontal cortex, is least affected in AD and therefore most likely capable of cholinergic sprouting [27].

The enzyme that hydrolyzes ACh at the synapse, AChE, does not show a decline in cortical areas until at least moderately severe levels of dementia [28]. PET studies utilizing a ligand that labels AChE *in vivo* suggest that there is only a mild loss of AChE in MCI [29] and mild AD [29,30]. The manner in which this cholinergic enzyme impacts cognitive decline remains an area of great interest in AD. Studies utilizing AChE PET ligands in large sample sizes most likely will be undertaken in the future. A recent functional MRI study demonstrated that people with MCI treated with the FDA-approved anticholinesterase donepezil demonstrated increased frontal cortex activation relative to untreated controls, which was positively correlated with task performance [31].

Butyrylcholinesterase (BChE) is a serine hydrolase similar to AChE that is widely distributed throughout the CNS and also catalyzes the hydrolysis of ACh. BChE is localized to neurons and glia, and is associated with NFTs and senile plaques (SPs) in AD brain [32]. Interestingly, population-based genetic studies of AD have identified a point mutation that changes Ala539 to threonine in the K variant of BChE, which effectively reduces serum BChE concentrations, and may be associated with cognitive decline [33]. BChE activity also increases in AD brain whereas AChE activity remains unchanged or declines [34,35]. These data, and the finding of cholinergic plasticity in people with MCI, strongly support the continued use of cholinesterase inhibitor drugs as a treatment early in the onset of AD. Thus, AChE and BChE represent *bona fide* therapeutic targets for ameliorating cholinergic dysfunction associated with the cognitive and behavioral abnormalities in dementing illness. Since it has been suggested that some patients respond better to a particular cholinesterase inhibitor than others [36], functional MRI or related noninvasive imaging technologies could be a tool to match individuals to an optimal treatment regimen.

Cholinergic basal forebrain neuron defects in early AD

A second major tenet of AD is the loss of basal forebrain cholinergic neurons in end-stage patients [37-39]. During the past few years, numerous studies employing various phenotypic markers have shown that the alterations to the basocortical cholinergic system are more complex than originally proposed. For example, the vesicular ACh transporter (VACHT), which is co-expressed with ChAT in human CBF neurons and participates in loading ACh into synaptic vesicles in cholinergic terminals, is not severely altered in AD [40]. In this regard, pharmacological studies of VACHT in postmortem AD tissue or *in vivo* imaging studies using vesamicol and its analogs, suggest that VACHT levels remain steady or are minimally decreased coincident with a severe decline in ChAT activity in cortical areas [41]. The discordance between ChAT and VACHT in CBF neurons and the projection paths to the cortex is intriguing in light of the discovery that they are part of a single cholinergic gene locus with shared regulatory elements [42]. Moreover, there is evidence from experimental lesions in animals [43-45] and from postmortem human brain studies [46,47] suggesting that many cholinergic neurons shrink, are depleted of phenotypic markers, and/or persist in an atrophic state after injury or during the pathological process, rather than degenerate. Taken together, these observations suggest that CBF neurons may be viable, albeit dysregulated, early in AD, and amenable to pharmacotherapeutic interventions that may prevent or delay cognitive dysfunction associated with cholinergic deficits.

CBF cell survival in MCI & AD: sorting life from death

A central concept underlying the survival of CBF neurons is the observation that the neurotrophic substance NGF and its high (TrkA) and low (p75^{NTR}) affinity receptors play a crucial role in cellular function, and that dysregulation of NGF and its cognate receptors are crucial factors underlying CBF neuron dysfunction in AD [48]. NGF is synthesized as a precursor (proNGF) molecule that is proteolytically cleaved to a mature biologically active neurotrophin peptide [49]. Mature NGF binds to the TrkA receptor, which stimulates signal transduction pathways mediating the majority of the survival and growth effects of NGF [50], and to the p75^{NTR} receptor, which is a positive modulator of NGF/TrkA binding [50]. However, p75^{NTR} has multiple functions, including apoptotic or cell death actions [51-56], which are dependent upon its interaction with various receptor chaperones [57-59]. The physiological consequences of TrkA and p75^{NTR} signaling may depend upon their interactions with proNGF. In this regard, immunoblotting studies demonstrated that proNGF is the predominant form of NGF present in the cortex of aged cognitively intact humans [60]. ProNGF levels are increased in the cortex of subjects diagnosed with MCI or mild AD compared to those with NCI [61]. The biological consequences of proNGF are controversial, as is the

function of its accumulation in the cortex during the prodromal stages of AD. Emerging literature suggests that recombinant proNGF binds TrkA and promotes neuronal survival and neurite outgrowth similar to mature NGF, but is approximately fivefold less active than the mature NGF peptide [62,63]. Although TrkA-mediated proNGF retrograde transport has not been demonstrated, proNGF accumulation in CBF cortical target sites may be due to reduced cortical TrkA levels and/or retrograde transport of TrkA to CBF perikarya [20]. Thus, poor utilization of proNGF in the face of reduced TrkA may result in CBF neurodegeneration. Significantly, while we have shown that reduced TrkA levels in the cortex were positively associated with lower cognitive performance as assessed by Mini-Mental State Exam (MMSE) test scores [64], increased cortical proNGF levels were negatively correlated with MMSE performance [61]. Thus, the concomitant reduction of TrkA and accumulation of proNGF in the cortex may be an early pathobiological marker for the onset of AD (Figure 1A). In fact, significantly increased cerebrospinal fluid (CSF) levels of NGF are detectable in AD [65], demonstrating the potential utility of NGF as a diagnostic biomarker. By contrast, several studies indicate that increases in cortical proNGF may result in proapoptotic signaling through binding to the p75^{NTR} receptor. In support of this, a different form of recombinant proNGF was shown to bind p75^{NTR} with high affinity and promote neuronal apoptosis [55]. Hence, increased proNGF in combination with reduced TrkA may result in enhanced binding between proNGF and p75^{NTR}, potentially shifting away from survival signaling to apoptotic proNGF signaling. It is also important to note that TrkA reduces and p75^{NTR} activates β -secretase strike (BACE) cleavage of the amyloid precursor protein (APP), which requires NGF binding and activation of the second messenger ceramide [66]. Aging may activate β -amyloid (A β) generation in the brain by 'switching' from TrkA to p75^{NTR}, suggesting that NGF receptor balance is a molecular link between normal aging of the brain and AD in relation to amyloid processing. Therefore, drugs that maintain a homeostatic balance between TrkA and p75^{NTR} may slow A β accumulation and SP deposition in the aged population, and delay and/or reduce the onset of MCI and, ultimately, AD.

In order to develop rational therapies that target the vulnerable cholinobasal projection system, it is incumbent upon the field of AD research to understand the full nature of proNGF signaling [67]. Recent findings indicate that the putative proapoptotic effect(s) of p75^{NTR}-mediated proNGF signaling is dependent on interactions between p75^{NTR} and the neurotensin receptor sortilin, a Vps10p domain trafficking protein that acts as a cell surface coreceptor with p75^{NTR} to mediate proNGF-induced cell death. This family of receptors is acquiring increasing importance owing to its potential involvement in AD [68]. A recent study provided genetic information for a role of the proneurotrophin receptor complex comprising sortilin and p75^{NTR}, in the mediation of neuronal viability *in vivo* [69]. Sortilin expression is required for p75^{NTR}-mediated apoptosis following proNGF treatment [57], suggesting that sortilin is a p75^{NTR} binding partner associated with the initiation of cell death [51,58]. In this regard, blocking this binding event precludes high affinity binding of proNGF to p75^{NTR} and subsequent cell death [50,57,70,71]. Thus, the outcome of p75^{NTR} signaling in response to proneurotrophin levels may depend on the identity and efficacy of the bound coreceptor. In summary, prosurvival or proapoptotic signaling in CBF neurons during the progression of AD may depend upon alterations in the stoichiometry of TrkA, p75^{NTR} and proNGF levels in cortical projection sites, the availability of various coreceptors, and the exact physiological role of proNGF within these different milieus (e.g., decreased neurotrophism or increased apoptotic signaling). A shift in the ratio of any of these factors during the development of AD may alter the functional outcome that proNGF binding would impart upon CBF neurons (Figure 1B). Therefore, defining these relationships *in vitro* and *in vivo* in relevant cell culture and animal models along with parallel studies in human postmortem brain tissues will be a major area of research in the development of neurotrophic mimetics for the treatment of dementia [72,73]. For instance, if proNGF binds with p75^{NTR} and induces apoptotic cell death [55,57], then it is crucial to design compounds that exert neuroprotection by blocking proNGF binding

to p75^{NTR}. Conversely, if proNGF binds with TrkA resulting in cell survival activity [62], then the development of drugs that enhance this binding event may provide neuroprotection in AD.

Increased proNGF levels in the MCI and AD brain suggest that alterations occur in the metabolic pathways regulating the maturation and degradation of NGF, which in turn may be pivotal for CBF neuron survival. Bruno and Cuellar reported that a protease cascade which converts proNGF to mature NGF (mNGF) and degrades mNGF in the extracellular space by the coordinated activity of plasminogen, tissue plasminogen activator (tPA), neuroserpin, matrix metalloproteinase (MMP)-9 and tissue inhibitor of MMP (TIMP)-1 may be defective in AD, resulting in NGF dysfunction [74]. We found an upregulation of MMP-9 protein levels and activity in both AD and MCI brains, which correlated inversely with cognitive status (Figure 1B) [75]. Since tissue alterations are often reflected in bodily fluids, determination of MMPs in blood, urine and CSF has been recommended as potential biomarkers to act as diagnostic measures to characterize the disease process that occurs in the brain [76-78]. Interestingly, plasma MMP-9 was increased in MCI and AD [77]. Our group is currently investigating whether MMP-9 CSF levels are a feasible biomarker for diagnosing MCI. These novel findings suggest additional possible biomarkers for the onset of AD as well as drug discovery.

Neurotrophin gene expression defects in CBF neurons in MCI & AD

Defining a genetic signature of vulnerable CBF neurons is rapidly becoming a major area of molecular clinical pathologic research for determining novel drug targets during the progression of AD. In this regard, our group has used single cell gene expression profiling coupled with custom-designed cDNA arrays and validation with real-time qPCR and *in situ* hybridization to evaluate the genetic signature of CBF neurons during the progression of AD. These studies have demonstrated a significant downregulation of *trkA*, *trkB* and *trkC* gene expression during the development of AD [7]. An intermediate reduction was observed in MCI, with the greatest decrement in mild AD compared to aged controls. Moreover, two separate expressed sequence tag (EST) cDNAs for each *trk* gene (e.g., ESTs targeted to the extracellular domain [ECD] and tyrosine kinase [TK] domains) were downregulated. By contrast, there was a lack of regulation of p75^{NTR} expression [7] in CBF neurons. A 'step down' dysregulation of *trk* expression may, in part, underlie CBF neuron demise associated with the clinical presentation of AD. Supporting this concept is the finding that *trk* downregulation is associated with measures of cognitive decline [7,14]. Hence, *trk* gene expression defects may provide a molecular marker for the transition from MCI to frank AD.

Defects in CBF neuron prosurvival signaling cascades

Since *Trk* signaling following NGF binding leads to the activation of specific downstream signal transduction pathways [79-81], it is of utmost importance to identify whether cognitive status is a dependent variable in terms of these signaling events. For example, NGF signal transduction activates the MAPK pathway, which participates in a wide array of biologic functions, including cell survival, differentiation and apoptosis [82-84]. MAPK is a serine/threonine protein kinase which becomes activated upon phosphorylation and affects a wide variety of transcription factors [85]. The classic MAPK cascade involves activation of the small GTPase Ras, and the kinases Raf and MEK [86,87]. Downstream consequences of MAPK activation include activation of the ribosomal S6 kinases (Rsk) and the MAPK-activated protein kinase 2 (MAPKAP2), which phosphorylates several transcription factors including Elk-1 and cAMP-regulated response element binding protein (CREB) [85]. The physiological significance of this elaborate NGF-induced network remains unclear, but the sustained activation of MAPK is linked to neurotrophin-mediated neurite outgrowth [88,89]. A second downstream pathway is the PI3K/Akt pathway that regulates neurotrophin-mediated survival

responses in PC12 cells [90,91]. Regulation of this pathway involves upstream elements including Ras/Gab1/IRS1 [92,93]. A third downstream mediator is the PLC γ pathway [94, 95]. PLC γ pathway dysfunction may play a role in Ca²⁺-mediated cellular degeneration in aging and disease by affecting oxidative stress systems [96].

With respect to p75^{NTR}-mediated signaling, the intracellular domain of this receptor lacks intrinsic catalytic activity and instead contains a death domain motif, which may signal via the association and dissociation of various death domain-containing adaptor proteins, potentially allowing p75^{NTR} to have multiple signaling capabilities impacting different cellular endpoints [51,58,97]. Recent evidence supports the notion that p75^{NTR} has an intrinsic signaling capacity including:

- Sphingolipid metabolism [98-100]
- Activation of the JNK pathway [52,54,56,101-103]
- Activation of the NF- κ B pathway [104-109]
- Activation of the Akt pathway [110]
- Activation of the MAPK pathway [97,111]

Despite the phenotypic discordance between ChAT and NGF receptor expression in select CBF neurons early in AD, virtually nothing is known about how disease progression affects downstream signaling pathways normally activated within NGF-dependent CBF neurons in preclinical AD. This will be an active area of research in the near future. Studies are needed to determine whether classes of genes involved in cell survival signaling, regulation of transcription and membrane trafficking transcripts are differentially altered depending upon the chemical phenotype of CBF neurons during the onset of AD. The results of molecular analysis discussed herein will play a pivotal role in the development of future pharmacological treatments for CBF cell survival in the field of AD. Based on animal models and clinical studies, the delivery of NGF [17,112,113] or brain-derived neurotrophic factor (BDNF) [114] has been proposed as treatments to prevent or delay the onset of AD. There have been significant issues with NGF delivery, including the difficulty of using a polypeptide as a CNS drug, *in vivo* instability, poor bioavailability and proliferative effects during intracranial delivery [17,84]. Because neurotrophins do not pass the BBB, and CNS transplantation with genetically engineered cells that secrete neurotrophins is technically challenging and face the same limitations as infused neurotrophins, small molecule partial agonist activators of trk receptors are being designed and tested for the treatment of AD [115]. A high-throughput screening assay of small-molecule agonists for the TrkA receptor has identified gambogic amide, an alkaloid used in traditional Chinese medicine [116]. Gambogic amide binds selectively to TrkA (but not TrkB or TrkC), phosphorylates TrkA tyrosine residues, and activates the Akt and MAPK TrkA-mediated NGF signaling pathways. Gambogic amide has been demonstrated to ameliorate excitotoxic damage and promote neurite outgrowth in PC12 cells [116], making this a potential lead compound for chemical modification and clinical trial assessment. In summary, pharmacotherapeutic interventions need to be developed that fall into the category of disease-modifying therapies, which slow or arrest disease progression by interrupting the early pathophysiologic process(es) underlying CBF degeneration and subsequent cognitive dysfunction.

Cholinergic receptor expression deficits in MCI & AD

Two classes of receptors involved in cholinergic transmission have been suggested as drug targets for AD: nicotinic ion channels, which mediate fast postsynaptic transmission and muscarinic G protein-coupled ACh receptors. The ionotropic nicotinic ACh receptor (nAChR) is a pentameric membrane protein composed of four polypeptide subunits designated nAChRs

α , β , δ and γ , which are expressed at varying levels throughout the CNS [117]. The muscarinic ACh receptor (mAChR) family includes five members, M₁, M₂, M₃, M₄ and M₅, which are also topographically distributed within the CNS [118]. Single cell expression via microarray analysis was used to determine whether expression levels for nAChR and mAChR receptors, as well as ChAT, were differentially regulated within individual CBF neurons harvested from NCI, MCI and AD cases. ChAT mRNA expression levels did not differ across clinical conditions (Table 1). However, there was a significant upregulation of $\alpha 7$ nAChR subunit expression in AD compared with NCI and MCI. No differences were found for other nAChR subunits across clinical groups (Table 1) [119]. This increase in $\alpha 7$ nAChR expression levels within CBF neurons was inversely associated with cognitive performance. Increased $\alpha 7$ nAChR expression in CBF neurons may signal a compensatory response to maintain basocortical cholinergic activity during the onset of AD. Upregulation of the $\alpha 7$ nAChR within individual CBF neurons is also consistent with reports of increased $\alpha 7$ nAChR mRNA and protein expression levels in hippocampal neurons, astrocytes and peripheral blood leukocytes in AD [120-122]. The observed increase in $\alpha 7$ nAChR in early AD may regulate basocortical cholinergic tone through pre- and/or postsynaptic mechanisms within cholinergic NB neurons prior to their frank degeneration in the later stages of AD. Despite a putative beneficial role for increased CBF neuron $\alpha 7$ nAChR expression in AD (that may also be relevant to smoking behavior), evidence suggests that increased $\alpha 7$ nAChR expression contributes to cellular degeneration. Notably, $\alpha 7$ nAChR binds and/or interacts with APP and A β peptides [123-125]. Increased NB neuronal $\alpha 7$ nAChR expression may arise as a compensatory response that is offset by aberrant A β - $\alpha 7$ nAChR interactions, leading to cholinergic dysfunction.

Conversely, M1 subunit gene expression in single CBF neurons is preserved during the progression of AD (Table 1) [119,126]. The M1 receptor is a most interesting drug target as it links several of the major hallmarks of this disorder, including cholinergic deficiency, cognitive dysfunctions, A β and tau pathologies. Therefore, it has been argued that restoring cholinergic tone via activation of M1 mAChR may alter the onset and/or the progression of AD [127]. Despite this intriguing concept, clinical use of muscarinic agonists in AD has been limited owing to the adverse effects seen at high doses [128]. Recently, a novel group of M1 partial agonists was developed (AF102B, AF150(S) and AF267B-i) [129]. In a series of studies using the 3x transgenic-AD mice, which recapitulate the major pathologies of AD [130], chronic AF267B treatment rescued cognitive impairment and decreased A β 42 and tau pathologies in the cortex and hippocampus. These changes were associated with M1 mAChR-mediated activation of the TNF α -converting enzyme ADAM17/TACE, decreased BACE1 steady state levels and inhibition of GSK3 β [130]. At this point, clinical trials are recommended to determine whether the M1 mAChR low molecular weight partial agonist AF267B will become a viable treatment candidate and the first monotherapy to treat multiple pathologic and biochemical deficits during the progression of AD.

Synaptic & trafficking expression deficits in CBF neurons in AD

It is well known that synapse loss is the strongest correlate of cognitive decline in AD [16, 131-133]. Unfortunately, to date there are very few drugs that target synaptic function as a treatment approach for AD. Single cell gene array studies have shown that synaptic transcripts are selectively downregulated in CBF neurons in AD, with significant reductions in synaptophysin and synaptotagmin but not synaptobrevin or SNAP29 mRNA [134,135]. Intriguingly, synaptotagmin function is related to vesicle-presynaptic membrane fusion and neurotransmitter release, suggesting that perturbations in presynaptic vesicle trafficking comprise a common event in vulnerable neuronal populations in AD. In contrast to synaptic transcripts, mRNAs encoding APP and Notch were unchanged between control and AD subjects, whereas acid hydrolase cathepsin D mRNA was upregulated in AD [134-136]. In addition, subunits of protein phosphatase PP1 (Unigene-NCBI annotation PPP1CA and

PPP1CC) mRNAs were downregulated in CBF neurons in AD [135]. This observation is interesting in light of the observation that PP1 can phosphorylate tau on several serine/threonine residues and experimental downregulation of PP1 activity leads to increased tau hyperphosphorylation [137,138], which may affect NFT formation in CBF neurons.

Tau gene expression in CBF neurons in AD

Cortical and CBF neurons display NFT formation in the MCI brain [9,139,140], suggesting a concomitant alteration in tau gene expression during the early stage of AD. The adult human brain contains six tau isoforms ranging from 48 to 67 kDa, which are expressed through alternative splicing of a single tau gene on chromosome 17 [141,142]. Three of these tau isoforms contain three tandem repeats in the carboxy-terminus end of the molecule (3Rtau), while three isoforms display four tandem repeats (4Rtau) in this region. Expression levels of the six tau transcripts within CBF neurons do not differ significantly during the progression of AD [6]. However, a calculation of the ratio of 3Rtau/4Rtau revealed a significant shift in the 3Rtau/4Rtau ratio, with a decrement in 3Rtau in relation to 4Rtau levels for each tau transcript analyzed within CBF perikarya obtained from MCI and AD cases (Table 1) [6]. A similar shift did not occur during normal aging. These data suggest a subtle, yet pervasive shift in the gene dosage of 3Rtau and 4Rtau within vulnerable CBF neurons in MCI and AD [6]. Shifts in the ratio of tau transcripts may be a fundamental mechanism whereby normal tau function is dysregulated, not only in CBF neurons, but may be a more widespread process contributing to the selective vulnerability of neurons to NFT formation (Figure 1B) [143-145].

Galanin plasticity within the CBF in AD

Pharmacotherapeutic research has concentrated on developing monocholinergic compounds for the treatment of the cholinergic deficits seen in AD. This narrow vision may have prevented the creation of novel polypharmaceutical approaches for the alleviation of these deficits in AD. For example, the neuropeptide GAL, which functions via the interaction with three G protein-coupled receptors termed GALR1, GALR2 and GALR3, has multiple biological actions, including effects on cognition and neuroplasticity [15,146,147]. In the late [148-150] but not early [151] stage of AD, fibers within the basal forebrain containing the neuropeptide GAL thicken and hyperinnervate surviving CBF neurons. Although animal and cell-culture studies have shown that GAL plays a crucial role in the regulation of CBF neuron activity [152] and rescues cholinergic cells from amyloid toxicity [153], the molecular consequences of this unique plasticity response upon CBF neurons in AD remain unclear. Gene expression studies of cholinergic transcripts have shown that GAL hyperinnervated, but not nonhyperinnervated, CBF neurons display an upregulation of ChAT expression in AD compared to controls [126]. These observations suggest that GAL overexpression regulates cholinergic tone of CBF neurons in AD. Intriguingly, GAL overexpression may also regulate genes associated with NFT formation in CBF neurons. For example, clinical pathological studies have shown a relationship between GAL hyperinnervation and different NFT-related tau epitopes in CBF neurons during the course of AD [154]. CBF neurons displaying the tau C3 epitope, a marker for early stage NFT formation, were often hyperinnervated by GAL-containing fibers, whereas CBF neurons displaying the tau epitope MN423, an end-stage NFT marker, were not associated with GAL. Single cell gene expression studies have demonstrated that the levels of mRNAs encoding select subclasses of PP1 subunits (e.g., PP1 α and PP1 γ) are stable in GAL hyperinnervated but downregulated in non-hyperinnervated CBF neurons in AD [149]. Hence, GAL may prevent the potential involvement of PP1 and PP2A subunit activity implicated in tau hyperphosphorylation [155]. Taken together, these observations suggest that GAL remodeling may increase cholinergic function and at the same time delay NFT pathology in CBF neurons in AD. This suggests that GAL is in the unique position of treating multiple AD-

related pathological events simultaneously, most likely via interaction with cognate GAL receptors with varying downstream effector pathways.

If GAL inhibits ACh release, as some have suggested based upon findings from animal studies [146], it is possible that GALR subtype-specific antagonists may enhance cholinergic transmission by decreasing the inhibitory influence of GAL on the firing rate of CBF neurons. If, however, GAL promotes the survival or cholinergic tone of CBF neurons, then a GALR agonist may prove efficacious. Gene expression profiling studies revealed that human CBF neurons express mRNAs encoding all three GALRs [151]. However, the predominant GAL-mediated signal elicited in innervated and hyperinnervated cholinergic neurons is unclear. The peptidergic compounds, AR-M1896 and AR-M961, which are selective agonists for GALR2 and GALR1/GALR2, respectively [156], have been used in rat models to identify GALR2 subtype specific activities including neurogenesis in cholinergic sensory neuron explants [157]. The ongoing search for selective GALR ligands in drug discovery programs will hopefully provide new research tools to understand GALR subtype-specific pharmacology with respect to cognitive processes mediated by CBF neurons in AD. Since AD appears to arise from multiple etiologies, a rational treatment strategy might include high-affinity GALR ligands used in combination with anticholinesterases and perhaps other compounds, such as memantine (an NMDA receptor antagonist) and modulators of A β aggregation or clearance. In this vein, intraventricular infusion of NGF increased hippocampal GAL mRNA expression in rats [158], suggesting that the use of NGF for the treatment of AD [113] may indirectly increase brain GAL providing a dual therapeutic benefit for the treatment of CBF dysfunction in AD. We suggest that the polypharmacological use of such compounds may ameliorate cholinergic hypofunction in AD and perhaps benefit other aspects of this heterogeneous disorder.

Current cholinergic drug targets

The ultimate goal of AD research including cholinergic therapies is to prevent further impairment as well as restore the decline in memory and cognitive function that occurs over the course of the disease. During the last several years, the vast majority of clinical trials aimed at treating the cholinergic deficit in AD have concentrated on testing the efficacy of cholinesterase inhibitor drugs (e.g., donepezil, galantamine, rivastigmine or huperzine A), which are derivatives of tacrine, the prototypical cholinesterase inhibitor (Table 2). Recent studies report that anticholinesterase drugs reduce circulating A β deposition in several dementia types, including AD [159]. Evidence from clinical trials [160], noninvasive functional imaging [161] and basic science research suggest that cholinesterase inhibitors might alter APP processing and therefore provide some degree of neuroprotection [162,163]. In this regard, AChE has been localized to SPs in the vicinity of cholinergic synapses, and experimental evidence suggests that AChE promotes A β fibrillization [164], suggesting that a further benefit from AChE inhibitor therapy may be to prevent continued A β deposition in cholinergic projection sites. Other mechanisms by which cholinesterase inhibitor drugs may affect AD pathology include the promotion of nAChR or mAChR actions on nonamyloidogenic APP processing, the interruption of cholinesterase-amyloid interactions associated with SP formation or by anti-inflammatory actions. In general, long-term clinical assessments indicate that the main effect of anticholinesterase drugs is symptomatic treatment with limited disease-modifying actions [165]. Since the cholinergic deficit is not an early defect in the progression of AD [18-20], the use of these drugs in the prodromal stages of AD should be continued. However, the limited effect of cholinesterase inhibitors for the treatment of cognitive decline in AD coupled with unwanted side effects such as diarrhea, nausea, insomnia, fatigue and loss of appetite indicates the need to move beyond this conventional drug treatment with somewhat circumscribed efficacy.

Neuroprotective strategies for the treatment of CBF degeneration

A large body of scientific evidence suggests that NGF can prevent cholinergic neuron atrophy and correct the behavioral deficits caused by experimental injury or associated with normal aging in animals [166-173]. Since recent findings indicate a defect in NGF receptor expression in CBF neurons early in the onset of AD [7,48,64,174-176], treatments aimed at facilitating NGF actions may prove highly beneficial in counteracting the cholinergic dysfunction found in AD and attenuating the rate of degeneration of CBF neurons (Table 2).

NGF therapy has been tested in clinical trials of diabetic peripheral neuropathy and HIV-associated neuropathy, as well as patients with AD [177]. Each of these clinical trials met with disappointment owing to lack of efficacy, toxicity or both. Recently, biotechnology and pharmaceutical companies have attempted to extend the success of neurotrophin treatment found in animal models of AD to the clinic. A careful examination of past clinical trials reveals several common weaknesses [178] including that neurotrophins were administered systemically, with little understanding of their function outside of the CNS and the bioavailability of actual neurotrophin reaching the target neurons. In addition, indirect effects and unwanted side effects appeared with systemic administration including unregulated neurotransmitter release, hyperinnervation, sprouting of neurons, sympathetic stimulation, induction of antibodies, cachexia and hyperalgesia [178-181]. Although reducing the dose seemed to overcome some of these problems, intraventricular or intrathecal administration directly into the CNS did not improve the outcome [178,182]. Moreover, intracerebroventricular administration of NGF was shown to initiate sprouting of sensory and sympathetic fibers into the CNS [183]. These unsuccessful clinical trials after a period of high expectations led to a degree of pessimism in the field [182]. However, after further testing in rat and primate animal models [170,184-192], and taking into account several of the lessons learned from past failures in clinical trials (e.g., poor drug delivery and unwanted systemic side effects), a new Phase I clinical trial was undertaken to examine the utility of *ex vivo* NGF gene therapy for AD [113].

The *ex vivo* Phase I trial attempted to move beyond currently available treatments for AD [113]. The goal of this NGF trial was to protect CBF neurons from degeneration, as well as augment the function of remaining cholinergic neurons by delivery of human NGF. After obtaining informed consent, skin biopsies were attained to generate primary cultures of autologous fibroblasts that were transfected to produce human NGF [184]. If fibroblasts were found to be acceptable based on NGF production rates, then grafts were stereotactically placed into multiple locations within the region of the CBF neurons. Following a period of 22 months, no long-term post-surgical adverse effects were found and the rate of cognitive decline appeared to be reduced [113]. There was also evidence of cholinergic fiber sprouting into the region of the graft [113]. It should be kept in mind that this was not a double-blind study. Further clinical investigation of this gene therapy approach is clearly warranted.

Expert commentary

The main targets of cholinergic therapy continue to be the use of drugs with anticholinesterase activity despite their inability to overcome the cognitive deficits associated with the cholinergic hypofunction seen in AD (Table 2). During the past few years, it has become evident that dysfunction of the cholinergic projection system is mainly a later stage event in the development of AD [18,19]. In fact, there is phenotypic dysregulation, but not a frank loss of CBF neurons early in the progression of AD [40,175,176,193]. Therefore cholinesterase inhibitor therapy may be useful early rather than late in the course of the disease. Compounds need to be developed not only to enhance cholinergic activity, but preferably to slow or prevent the degenerative processes underlying the eventual extensive loss of CBF neurons in AD.

Perhaps the use of a M1 mAChR agonist free of the drawbacks of the current generation of M1 drugs which treat the multiple pathologies and clinical symptoms in AD will become a standard treatment for a comprehensive approach defined as “one compound against AD hallmarks” (Table 2) [128]. Recently, the novel non-selective antihistamine dimebon (2,3,4,5-tetrahydro-2,8-dimethyl-5-[2-[-6methyl 3-pyridnyl]ethyl]-1H-pyrido[4,3-b] indole) was shown to inhibit BChE and AChE, block the NMDA receptor signaling pathway, inhibit mitochondrial permeability and provide neuroprotective effects in models of AD [194]. Dimebon is an orally available small molecule which is well-tolerated by patients and appeared to have positive effects on cognition for patients with AD in Russian clinical trials [194]. This multifaceted drug will require extensive investigation at the mechanistic and clinical levels in the future. Although technically not a polytherapy, dimebon is a compound that interacts with multiple transmitter systems and may pave the way for additional studies that selectively target multiple systems (e.g., cholinergic, glutamatergic and galaninergic) using specific agonist/antagonist compounds in a combined dosing therapy (Table 2).

Growing evidence supports the neuroprotective effects of NGF as a treatment to rescue CBF neurons in AD. Future directions in gene therapy clinical trials including NGF and TrkA mimetics will likely become a standard for the treatment of cholinergic cell death in AD. These studies should incorporate several important considerations. First, the therapy should enable ongoing regulation of gene expression allowing for increasing or decreasing expression levels as the course of the disease advances [195]. Second, the duration of gene expression must be considered since sustained, long-term expression is crucial for continued success of therapy. Third, the site specificity of expression is crucial for the success of the therapy. In addition, CBF phenotype specific expression can be achieved through the determination of unique cell surface epitopes against which viruses and/or delivery modules may be targeted. Fourth, the immunogenic potential of the virus should be a major consideration as seen in the first amyloid vaccine trials [196,197]. Fifth, expression of specific receptor or pathway agonists/antagonists to facilitate a given cellular end point, including enhanced survival, decreased apoptosis, neurite outgrowth or neurotransmitter production and secretion should be investigated. In this regard, the combinatorial targeting of multiple pathways, for example, BACE1 inhibition to block the amyloid cascade and downstream inflammation, ACh synthesis to improve cholinergic tone in addition to those mediated by NGF and/or TrkA mimetics, may allow a synergistic approach to facilitate the maintenance of CBF function in AD [115]. Finally, a greater understanding of how neurotrophins function at the receptor level within vulnerable cell types and their downstream signaling pathways will allow for the development of new ligands with novel activity profiles, which will then allow for better therapies. Combining basic knowledge of neurotrophin receptor biology with the continued advances in drug-delivery methods, such as gene therapy and combinatorial chemistry, will enhance the ultimate and pressing goal of amelioration of neurologic disease processes for millions of people worldwide.

Five-year view

Since there are no universally accepted animal models of AD currently available that exhibit the complex interactions underlying the cholinergic deficit seen in AD, the gold standard will continue to be clinical neurobiological investigations of the cholinergic system comparing findings derived from brain tissue harvested from people who died with a clinical diagnosis of NCI, MCI or AD. During the next few years it is envisioned that multimodal, combinational therapies will become more prevalent to include neuroprotective strategies, cholinesterase inhibitors, novel drugs to be used in conjunction with esterase inhibitors, and the development of unique gene therapy approaches. It is likely that these and other treatments will be directed at people with MCI and hopefully extended to primary prevention in high-risk populations [1]. Relatively recent advances in molecular and cellular biology have introduced gene therapy as a potential means of delivering substances to the CNS. Gene therapy offers the prospect of

delivering neurotrophic factors and/or their cognate receptors directly into the brain parenchyma, in a well-targeted, regionally restricted, long-term and safe manner. A new series of NGF gene therapy clinical trials will be undertaken and some are currently in progress. From a more conventional drug-design pharmacological approach, the continued development of a TrkA mimetic and/or an M1 mAChR agonist (or partial agonist) free of the side effects of the current M1 drugs which treat multiple pathologies and clinical symptoms in AD may become a standard treatment for a more comprehensive approach (Table 2). The diversity of knowledge gained from recent molecular and cellular investigations based upon the study of humans during the early phases of dementia will lead to breakthrough therapeutic strategies that will become standard-of-care disease-modifying treatments for AD.

Key issues

- Acetylcholine is a major neurotransmitter in the brain and cholinergic deficits occur during the progression of Alzheimer's disease (AD), which lead to widespread cognitive dysfunction and decline.
- Cholinergic basal forebrain neurons, which provide the major cholinergic innervation to the cortex and hippocampus, may be viable, albeit dysregulated, but do not display a frank loss in the prodromal stage of AD, making them a prime target for early-stage pharmacotherapeutic development for the amelioration of AD symptomology.
- Cortical choline acetyltransferase reduction is a late-stage event in AD, suggesting that cholinergic drug treatment should be initiated early in the course of the disease.
- Hippocampal choline acetyltransferase activity is upregulated in prodromal AD, suggesting a compensatory and/or neuroplasticity response to the disease process, which may stabilize cognition under the appropriate conditions where cholinergic tone is normalized.
- Cholinergic basal forebrain neurons display a phenotypic downregulation of NGF receptor protein expression in early AD.
- Cholinesterase inhibitors remain the most common form of drug therapy prescribed for symptomatic relief of cognitive dysfunction in AD, and this concept is being challenged as the most effective treatment modality for AD based upon basic science, translational and clinical studies during the progression of AD.
- Novel drug therapies for the treatment of the cholinergic deficit in AD need to be developed and tested, including galanin receptor agonists, TrkA mimetics and M1 muscarinic acetylcholine receptor partial agonists.
- A Phase I NGF gene therapy trial shows promise for the treatment of cholinergic cell degeneration and cognitive recovery in patients with mild AD.

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- of considerable interest

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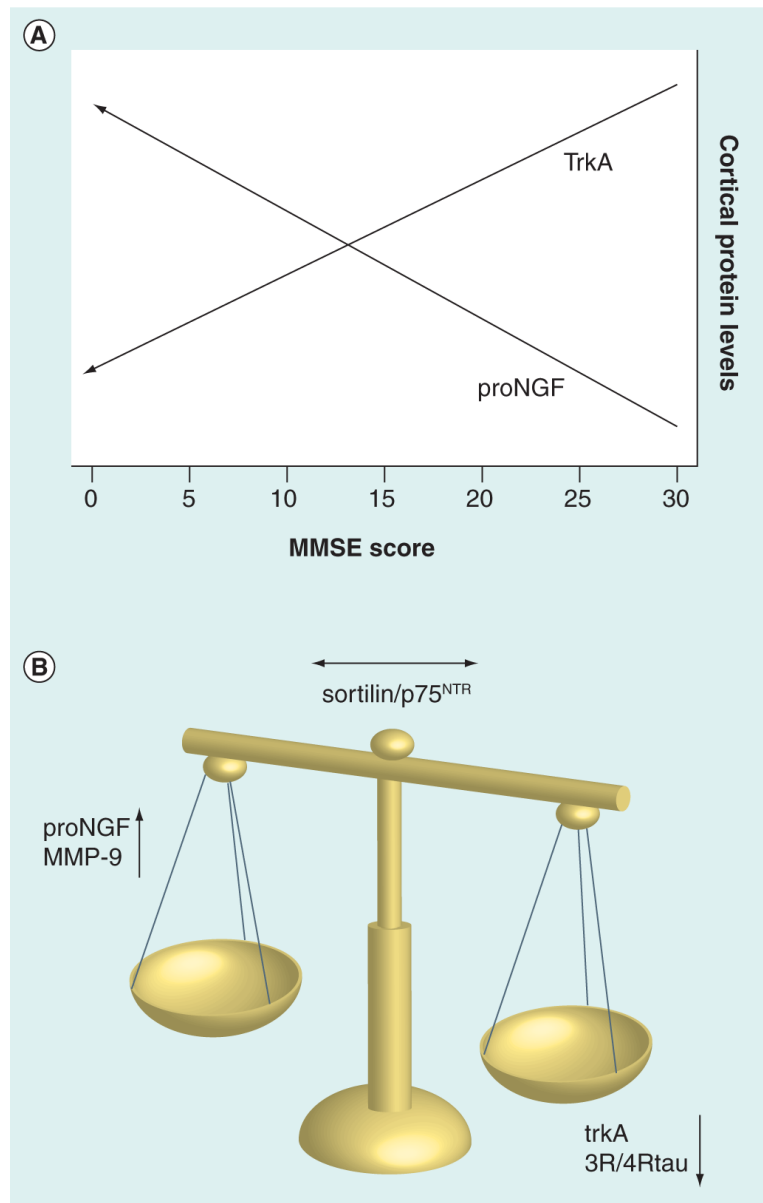


Figure 1. Schematic diagrams show the shifting stoichiometric relationships of several gene products underlying cholinergic basal forebrain (CBF) survival during the progression of Alzheimer's disease (AD)

(A) The relationship between cortical levels of TrkA and proNGF and MMSE scores reveal that poorer performance on the MMSE is associated with decreased TrkA and increased proNGF protein levels in the cortex. **(B)** Shifts in the balance of NGF metabolism, NGF receptor and coreceptor expression, and tau isoform expression may contribute to the degeneration of CBF neurons in AD. MMSE: Mini Mental State Exam.

Table 1

Alterations in cholinergic mRNA expression levels within single cholinergic nucleus basalis neurons during the progression of Alzheimer's disease.

mRNA expression level measured in single cholinergic NB neurons	Change across NCI, MCI and AD diagnostic groups
Choline acetyltransferase	No change
Low-affinity neurotrophin receptor p75 ^{NTR}	No change
High-affinity NGF receptor trkA	NCI > MCI, AD
High-affinity BDNF receptor trkB	NCI > MCI, AD
High-affinity neurotrophin 3, 4/5 receptor trkC	NCI > MCI, AD
Muscarinic acetylcholine receptor subtypes M1-M5	No change
Nicotinic acetylcholine receptor subunits α 1–6	No change
Nicotinic acetylcholine receptor subunit α 7	AD > NCI, MCI
Nicotinic acetylcholine receptor subunits β 1–4	No change
Tau 3R isoforms/4R isoforms	NCI > MCI, AD

Differences in mRNA levels among the diagnostic groups were measured by one-way ANOVA with Newman-Keuls post hoc testing ($p < 0.001$). 3R: 3-repeat; 4R: 4-repeat; AD: Alzheimer's disease; BDNF: Brain-derived neurotrophic factor; MCI: Mild cognitive impairment; NB: Nucleus basalis; NCI: No cognitive impairment.

Table 2

Cholinotropic therapies for the treatment of Alzheimer's disease.

Compound	Mechanism of action
Anticholinesterases [*]	Inhibit breakdown of synaptic ACh; may prevent A β fibrillization
Dimebon [‡]	Inhibits A β toxicity; anticholinesterase properties
NGF gene therapy [‡]	Targeted delivery of NGF to CBF perikarya
M1 agonists [§]	Stimulation of excitatory cholinergic signaling; neuroprotective signaling; nonamyloidogenic APP processing
TrkA agonists	Prosurvival signaling within CBF perikarya
MMP-9 inhibitor	Prevent NGF degradation
GALR ligands	Stimulate prosurvival GALR2 or antagonize inhibitory GALR1
NFT inhibitors	Prevent tau fibrillization in CBF neurons

A β : β -amyloid peptide; ACh: Acetylcholine; APP: Amyloid precursor protein; CBF: Cholinergic basal forebrain; GALR: Galanin receptor; M1: M1 muscarinic acetylcholine receptor; MMP: Matrix metalloproteinase; NFT: Neurofibrillary tangle; TrkA: High affinity nerve growth factor receptor.

^{*} Phase I.

[‡] Phase II.

[§] FDA-approved.