J Mol Med (1998) 76:555–567 © Springer-Verlag 1998

REVIEW

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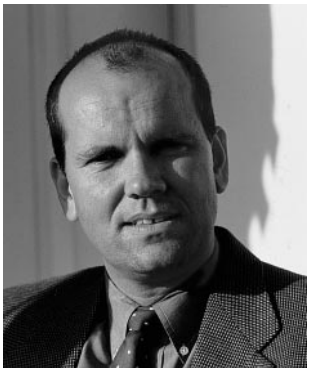
Cholinergic strategies for Alzheimer's disease

Received: 7 November 1996 / Accepted: 16 September 1997



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Abstract Alzheimer's disease is a devastating degenerative disorder of the central nervous system that results in gradual deterioration of cognitive function and severe alteration of personality. Degeneration of neurons in the nucleus basalis Meynert, the origin of the major cholinergic projections to the neocortex, occurs early in the course of the disease, and is correlated with the cognitive decline. This link between cholinergic dysfunction in the basal-cortical system and cognitive deficits has focused scientific efforts on developing tools to elucidate the neurobiological role of the cholinergic system in cognition and to develop therapeutic interventions in the disorder. An important step in understanding the mechanisms underlying cognitive dysfunction has been the development of in vivo rodent models that mimic some of the features of Alzheimer's disease. Acute excitotoxic or immunotoxic lesions of the nucleus basalis in rodents have revealed a role of the basal-cortical system in attention, learning and memory. More recent advances in developing mouse gene technology offer newer models to systematically examine the underlying neuropathological cascade leading to dysfunctions in mnemonic processing. Using in vivo rodent models, several cholinergic enhancement strategies have been tested and proven to be effective in alleviating lesion-induced cognitive deficits, including neuropharmacological approaches (acetylcholinesterase inhibitors), neurotrophic factor administration (nerve growth factor), and transplantation of cholinergic-enriched fetal grafts. Successful results have also been obtained using ex vivo gene transfer to deliver nerve growth factor or acetylcholine to compromised regions of the basal-cortical system. Gene therapy may be of particular interest for clinical applications, because this approach provides a method for topographically restricted and selective delivery of therapeutic genes and their products to afflicted areas of the brain. Advanced techniques in molecular biology (e.g., exogenous regulatable gene transfer) and newly developed tools of modern neuroscience (e.g., neural precursor cells) will be important contributions for deciphering the biological bases of neuronal degeneration and for refining therapeutic strategies for Alzheimer's disease.

Key words Acetylcholine · Gene therapy · Nerve growth factor · Nucleus basalis · Transplantation

Abbreviations *ACh* Acetylcholine · *AChe* Acetylcholinesterase · *AD* Alzheimer's disease · *AMPA* α-Amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid · *APP* Amyloid precursor protein · *CBF* Cholinergic basal forebrain · *ChAT* Choline acetyltransferase · *CNS* Central nervous system · *dChAT Drosophila* choline acetyltransferase · *IBO* Ibotenic acid · *ICV* Intraventricularly · *5′LTR* 5′ Long terminal repeat · *MLV* Murine leukemia virus · *MS* Medial septum · *NBM* Nucleus basalis magnocellularis · *NGF* Nerve growth factor · *QUIS* Quisqualic acid · *TET* Tetracycline

Alzheimer's disease

Diseases of aging have assumed major importance worldwide. Among these are neurodegenerative disorders such as Alzheimer's disease (AD) and Parkinson's disease [1]. From an epidemiological perspective these disorders share an increasing prevalence with aging, whereas clinically they are characterized by different clinical syndromes, unknown etiology, a progressive course of the disorder, and very limited potential for cure.

Of the infirmities of old age few are as dreaded as becoming demented and experiencing severe alterations of the personality. Based on several demographic studies in Europe, Asia, and the United States, the most prevalent of these aged-related dementias is AD, which primarily afflicts persons over age 65 [2]. Semi-log plots indicate that the prevalence of AD doubles for every 5-year epoch between age 65 and 85. Since the aging of the population will result in an increase in the elderly population in both proportional and absolute terms, AD become an even greater burden on our health care system in the future.

AD is clinically characterized by the development of a progressive dementia, with memory loss, disturbances in language, visiospatial relations, and behavior [3, 4]. Neuropathological examination of AD brains reveals neuronal and synaptic loss, amyloid-containing plaques, and neurofibrillary tangles. These characteristic neuroanatomical changes are most prominent in the association areas: the parieto-temporal neocortex, the hippocampus, the entorhinal cortex, and the amygdala. Several specific neurotransmitter systems are regularly and substantially altered in AD brains. One of the most prominent systems affected in the course of AD are the cholinergic neurons of the nucleus basalis magno cellularis (NBM) [5–7]. The NBM is part of the cholinergic basal forebrain (CBF) system, which extends from the medial septum (MS) through the diagonal band of Broca to the NBM [8, 9]. The NBM innervates the cortex and the amygdala while the MS and diagonal band of Broca innervate primarily the hippocampus. Degeneration of the CBF system not only plays an important role in AD but is also observed in other disorders such as Parkinson's disease, dementia pugilistica, Korsakoff's disease, progressive supranuclear palsy, and olivopontocerebellar atrophy [10].

Cholinergic neurons in the NBM are reduced very early in the course of AD, and the dramatic loss of these cholinergic neurons is more severe than the neuronal loss related to other neurotransmitter systems (e.g., serotonergic cells of the dorsal raphe and noradrenergic neurons of the locus ceruleus) [5–7]. Choline acetyltransferase (ChAT), the biosynthetic enzyme for acetylcholine (ACh), is reduced 60–90% in the cerebral cortices and the hippocampus of AD brains, and the degree of its loss is correlated with the severity of the observed cognitive impairments [11–14]. These specific neuroanatomical and neurochemical changes led to the foundation of the "cholinergic hypothesis of geriatric memory dysfunction" almost two decades ago [6]. Cholinergic deficiencies do not account for all of the deficits observed in AD, however, and undoubtedly other neurotransmitter systems and complex interactions between systems play a vital role in maintaining cognitive processing. Loss of neurons and synapses appears to be the basis for cognitive dysfunction, but we are still far from fully understanding the pathological cascade that leads to neuronal loss and deterioration of learning and memory. Currently there does not exist a universally accepted theory of age- and AD-related cognitive deficits, and major controversies regarding the underlying pathogenesis remain. However, the marked decrease in cholinergic neurotransmission and its strong association with cognitive decline remain a compelling focus of investigation.

Neocortical cholinergic hypofunctioning: potential model for AD-related cognitive deficits

This review focuses on the cholinergic neurons of the NBM, one of the systems implicated in AD-related cognitive deficits. First, we describe some of the rodent models of cholinergic hypofunctioning in the NBM-neocortex system that have been useful for clarifying the functional role of this system in cognitive processes (Table 1). The development of such models has also been essential for evaluating the potential of cholinergic enhancement strategies for reversing cognitive deficits associated with NBM damage. It should be noted, however, that although these animal models try to mimic the major clinical features of AD, none of these models provides an exact replica of AD.

Excitotoxic lesions of the nucleus basalis magnocellularis

A variety of methods have been used to lesion the NBM, including radiofrequency [15], ethylcholine aziridium [16], and a variety of excitotoxins [17]. Excitotoxins, potent agonists for glutamate receptor sites, provided a considerable methodological improvement over mechanical lesions because only perikarya, and not fibers of passage, are destroyed [18]. Three axon-sparing neurotoxic amino

Table 1 Rodent models of cholinergic hypofunctioning in the basal-cortical system; the table is not comprehensive, and characteristics have been chosen to illustrate the text. (+++ Presence of behavioral deficit, # behavioral deficits due to peripheral neuropathy, ± controversial findings, Intrapar = intraparenchymal)

Model	Mechanisms of cell death/dysfunction	Behavioral deficit	Disadvantage
Excitotoxin (IBO, QUIS, AMPA)	Ca^{2+} influx upon glutamate receptor binding	+++	Loss of GABA-ergic neurons; Structural damage to striatal/pallidal neurons
Immunotoxin (192IgG-saporin)	Ribosome inactivation by retrograde transport of p75NGFr complex	ICV: +++ Intrapar: ±	Purkinje cell loss; Structural damage
Aging	Unknown: possibly glutamate/oxidative neurotoxicity	+++	Lack of AD-related neuropathology
Transgenic			
APP	Age-dependent amyloid toxicity; synaptic loss	+++	Lack of tangles
NGF/trkA/p75NGFr	Trophic factor dependent cell populations	+++#	Preserved CBF system

acids – ibotenic acid (IBO), quisqualic acid (QUIS), and α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid (AMPA) – have been most commonly used to produce the NBM lesions [19]. In rats IBO lesions of the NBM, the rodent homologue of the nucleus basalis of Meynert in humans, have been reported to induce numerous cognitive changes, including deficits in passive avoidance retention [20–22], active avoidance [22], T-maze performance [23], and spatial memory in the radial arm maze and Morris water maze [24–27].

Such work supported a role of the basal-cortical system in learning and memory. However, this conclusion has been questioned in subsequent studies with other excitotoxins. Specifically, experiments using QUIS and AMPA to damage the NBM revealed greater loss of cholinergic markers with these toxins than with IBO but less, or in some cases no, cognitive impairment [28–31]. A lack of correlation between cholinergic toxicity and behavioral performance is particularly evident in the Morris water maze, a task that is widely used to measure learning and memory. In this task animals are trained to locate a submerged invisible platform in a circular pool by means of environmental cues for spatial orientation. The rate of acquisition and the ability to remember the platform location after training have proven to be a reliable and consistent measure of spatial memory. The lack of behavioral impairment in this spatial memory task prompted studies to explore the involvement of the NBM-cortical system in other cognitive processes, such as attention. A role of this system in attention has been supported by findings that the degree of cortical cholinotoxicity is correlated with the extent of impairment in a visual discrimination task that measures reaction time and selective attention under several experimental conditions [32–34].

In general, several factors have contributed to conflicting interpretations of the specific function of cholinergic neurons after excitotoxic lesions of the NBM [35]. First, excitotoxic lesions damage both cholinergic and noncholinergic systems. In particular, these lesions have been found to decrease monoamine levels in brain regions and to destroy GABAergic neurons at the injection sites [36, 37]. Secondly, the deficits could result from damage to noncortical NBM efferents, such as the amygdala [38]. Thirdly, histological evidence has indicated that striatal and pallidal neurons, the amygdala, and the thalamic reticular nucleus are also often damaged by excitotoxic NBM lesions [28–30]. These nonspecific and nonselective effects of excitotoxins on other neurobiochemical and neuroanatomical systems that are also involved in cognitive processes make a definitive interpretation of the functional role of the cholinergic basal-cortical system very difficult and controversial.

Immunotoxic lesions of the nucleus basalis magnocellularis

Cholinergic neurons in the basal forebrain differ from other cholinergic populations in the central nervous system (CNS) by possessing a high density of the low-affinity nerve growth factor (NGF) receptor (p75NGFr) on their surface [39–41]. The most recent development in the search for a specific cholinolytic agent has exploited this property with the immunotoxin 192IgG-saporin [42]. Immunotoxins are conjugates of a monoclonal antibody targeting a specific antigen and a ribosome-inactivating protein. In this case 192IgG is a monoclonal antibody to the rat p75NGFr. This compound is endocytosed upon binding to the p75NGFr receptor and is retrogradely transported from axon terminals to the neuronal cell body [43]. When radio-iodinated and injected intraventricularly (ICV), 192IgG accumulates preferentially in cholinergic neurons throughout the entire CBF system [44]. Saporin is a 30-kDa ribosome-inactivating protein derived from the plant *Saponaria officinalis* [45]. Coupling of saporin and 192IgG affords saporin preferential access to the intracellular compartment of p75NGFr-bearing neurons of the CBF system, where it halts protein synthesis by inactivating the 60S ribosomal subunit thus destroying cells [46].

ICV infusions of 192IgG-saporin have induced a selective and substantial loss of neurons in the CBF [47, 48], a marked and prolonged depletion of ChAT activity in the neocortical and hippocampal projection fields of the CBF [49], and dose-dependent impairments in the Morris water

maze [50]. Behavioral deficits in a delayed match-to sample task (T-maze) [51], passive avoidance retention [52], habituation in startle response, and hyperactivity in an open field task [50] have also been reported. Although 192IgG-saporin shows high specificity for the CBF [47–49, 53], there are two disadvantages to the ICV route of administration. First, Purkinje cells express p75NGFr and are destroyed after ICV administration at doses required to obtain significant behavioral impairments [50]. It is therefore unclear to what extent the destruction of cerebellar structures contributes to observed impairments in the behavioral tests. Numerous behavioral studies (classical conditioning of the eyelid response and motor learning of the vestibulo-ocular reflex) suggest an involvement of the cerebellar cortex in learning and memory for cerebellum-dependent motor functions and in cognitive processing in general [54, 55]. In fact, the cerebellum with its deep nuclei projecting via the mesencephalic tegmentum and thalamus to the neocortex has been shown to be involved both in associative and nonassociative learning processes [56]. Secondly, all parts of the CBF system are affected by ICV injections, and the specific contribution of the MS-hippocampal and NBM-cortical systems cannot be separated. Thus more recent studies have focused on intraparenchymal injections of 192IgG-saporin into discrete regions of the CBF [57–61]. Other than minimal gliosis at the injection site, damage to noncholinergic neurons (neurotensin, galanin, somatostatin, NADPH-diaphorase, calbindin, parvalbumin, and neuropeptide Y) or loss of Purkinje cells does not occur [61].

Intraparenchymal immunotoxic lesions of the NBM have produced inconsistent behavioral consequences in the Morris water maze: some investigators have reported impairments [57, 59] whereas others have not [60]. Such findings may reflect the use of different testing paradigms in the Morris water maze and/or differences in the extent of the NBM lesions (e.g., different stereotaxic coordinates, toxin concentrations, and injection volumes). The amount of immunotoxic damage clearly impacts cognitive performance, since an almost 90% reduction in cortical and hippocampal ChAT activity is required after ICV injections to produce substantial behavioral effects [50]. The CBF may thus have a considerable reserve, and a high degree of damage may be necessary before cognitive dysfunction arises. Alternatively, compensatory mechanisms may prevent behavioral impairment for major but incomplete loss of cholinergic fibers. Although work with 192IgG-saporin is still in early stages, the use of this toxin to achieve cholinergic specific and topographically restricted lesions provides a powerful tool to assess the role of the cholinergic system in cognitive processing and in particular may help clarify the involvement of the NBM-cortical system in attention.

Cholinergic hypofunctioning in the neocortex during aging

The age-related cognitive impairments of AD are characterized by an insidious and slow onset, progressive and time-dependent deterioration, and permanence. Since none of the existing neurotoxins fully mimic these features, aging animals may provide better in vivo models for understanding the role of different anatomical, neurophysiological, and neurochemical systems in cognitive dysfunction [62]. Extensive work has documented that subpopulations of aged animals show an age-dependent decline in performance in various learning and memory tasks [63–66]. Cognitive dysfunction in these animals has been linked to atrophic/degenerative changes in the CBF. The number and size of cholinergic neurons in the NBM show the highest correlation with age and Morris water maze performance [66]. For example, old rats with the fewest and smallest cholinergic neurons in the NBM perform most poorly in the Morris water maze. In addition, a substantial decrease in ACh synthesis of up to 70% has been observed in aged rats [67].

Although these findings support the role of cholinergic NBM neurons in this task for learning and memory, the magnitude of cell loss and/or shrinkage has not appeared substantial enough by itself to explain the severe impairments observed during senescence. Indeed, available data strongly suggest that cognitive impairments in aged animals reflect a multisystem deficit involving monoaminergic neurotransmission as well [66]. General performance deficits in aged animals may also be related to changes in other factors such as vision, motor performance, sensory processing or more complex behaviors such as motivation. Although different brain areas in aged rodents show neuronal and synaptic loss, thus far amyloid-containing plaques or neurofibrillary tangles, the classical neuropathological hallmarks of AD, have not been observed in senescent rats. Deficits observed in aged animals may therefore reflect a physiological age-related decline in learning and memory rather than a rapid progression of cognitive deficits related to the neurodegenerative process that takes place in AD.

Transgenic models of Alzheimer's disease

Significant progress has been made in the development of transgenic models of AD, and this technology may provide an excellent opportunity to study the underlying neurobiological mechanisms of AD-related cognitive deficits. Since the CBF system consists of NGF-responsive cholinergic neurons, initial efforts have been directed toward the development of mice lacking NGF producing cells (i.e., NGF knock-outs) [68]. As expected, the phenotype of these mice is characterized by a severe loss of sensory and sympathetic neurons, two cellular populations that are known to be highly dependent on NGF [69]. Surprisingly, however, CBF neurons persist throughout the short life span of the transgenic mice (4 weeks) and no obvious cholinergic hypofunctioning is evident [69]. Similarly, mice lacking the high-affinity receptor for NGF *trkA* appear to have a normal complement of neurons within the CBF although a substantial decrease in acetylcholinesterase (AChe) immunoreactive fibers that project from the

CBF to the hippocampus and cortex is evident [70]. Finally, adult p75NGFr-deficient mice (low-affinity knockout) appear to have even more ChAT-positive neurons in the MS than their controls, indicating that p75NGFr may mediate apoptosis of CBF neurons during development [71]. Gene-targeted deletions for NGF, *trkA*, and p75NGFr have led to a better understanding of their role during embryogenic and perinatal period and resulted in substantial neurological deficits due to the sensory and sympathetic neuronal loss. The CBF system itself, however, is much less affected during development than originally anticipated. Future conditional transgenic strategies may allow the selected deletion of a single gene in a spatial and temporal-specific manner in adult and aged mice and offer thus the potential to model the precise function of a certain gene both at any given site in the CNS, and at any given stage during life.

Enormous efforts have focused also on generating mice that overexpress amyloid precursor protein (APP), a transmembrane protein that is considered one of the primary pathogenic candidates of AD. Recently two studies were successful in showing AD-like neuropathology in transgenic mice [72, 73]. Mice overexpressing a human mutant APP form ("Swedish" mutation of APP: V717F, valine at residue 717 substituted by phenylalanine) under the control of a platelet-derived growth factor-β promotor have been found to display extensive, age-dependent AD-type pathology, including extracellular β-amyloid deposits, neuritic plaques, and neuronal and synaptic loss with astrocytosis and microgliosis [72]. Behavioral characterizations of these mice has not yet been reported. In the second study a double mutation of a human APP form lacking the Kunitz-protease inhibitor (early-onset AD in a large Swedish family; mutation of APP: K670N, lysine at residue 670 substituted by aspargine, M671L, methionine at residue 671 substituted by leucine) was inserted into a hamster prion protein cosmid vector [73]. Mice overexpressing the mutant APP showed age-dependent behavioral impairments in the Morris water maze and Y-maze that was associated with robust pathological AD-like abnormalities. These transgenic APP mice thus provide important contributions for exploring the biological bases of cognitive deficits in AD.

Cholinergic enhancement strategies for the neocortex

"Classical" neuropharmacological approach

The hypotheses that AD-related cognitive deficits are due to the destruction of cholinergic neurons in the NBM, and that postsynaptic receptors remain intact have fostered a therapeutic focus on augmenting cholinergic neurotransmission to improve or reverse cognitive deficits [74]. Thus far, however, neuropharmacological replacement strategies for ACh have met with limited success in AD patients. Three approaches for enhancing the cholinergic system have been pursued: (a) administration of precursors, (b) use of enzymes to block ACh degradation, and (c) administration of direct cholinergic receptor agonists. Of these three, precursor therapy has clearly proven to be ineffective and has been abandoned. Agonists appear promising, and studies with selective direct-acting agonists for M_1 and M_3 muscarinic receptors are currently underway [74].

Most effort has focused on the use of AChe inhibitors to augment cholinergic function. In experimental studies systemic administration of such compounds to animals with excitotoxic lesions of the NBM has partially reversed deficits in water maze performance [75, 76], spatial alternation in a T-maze [77], radial arm maze [78], and passive avoidance [79, 80]. Reasons for incomplete recovery in these tasks are unclear but may reflect an inability of the drugs to compensate for nonspecific excitotoxic damage to noncholinergic cells or problems inherent to the drug and/or delivery method. For example, difficult pharmacokinetics of these compounds, undesired peripheral cholinomimetic effects, and nonselective stimulation of central cholinergic systems outside of the CBF may contribute to the limited effectiveness of this approach.

Human clinical trials have been carried out using a variety of cholinesterase inhibitors. Three large-scale multicenter trials of tetrahydroaminoacridine (tacrine) demonstrated that use of this cholinesterase inhibitor results in modest cognitive improvement in patients with AD [81–83]. However, the use of tacrine was associated with an elevation in liver enzymes in almost 50% of patients exposed to this drug. Three pivotal trials led to the approval of tacrine for the treatment of AD in the United States in 1993. A subsequent study suggested that the use of tacrine is associated with a reduced likelihood of nursing home placement [84]. However, this study did not have an appropriate control group, and the results remain suggestive and in need of confirmation. In late 1996 a second cholinesterase inhibitor, donazepil, was approved in the United States for the treatment of AD. This cholinesterase inhibitor produces similar improvement in cognitive functioning to tacrine [85]. Unlike tacrine, however, it rarely produces hepatotoxicity and has a much reduced incidence of gastrointestinal distress. Additionally, it can be administered once daily, an ideal dosing pattern for patients with cognitive impairment. Several other cholinesterase inhibitors are reported to result in cognitive improvement, including controlled-release physostigmine [86] and exelon [87]. At present more than a dozen cholinesterase inhibitors are currently in clinical trials. Additional therapeutic benefits for AD may occur by pharmacological manipulation of multiple neurotransmitter systems, particularly serotonergic, glutamatergic, or noradrenergic function. However, this approach has not yet been widely explored.

Nerve growth factor

Neurotrophic factors promote the maintenance and survival of numerous peripheral and CNS cells during adulthood. One of the most therapeutically promising of these

molecules is NGF, originally discovered four decades ago [88]. Substantial knowledge has been accumulated about NGF-responsive neuronal populations in the periphery and CNS [89, 90]. In the basal forebrain the receptor tyrosine kinase *trkA*, the high-affinity receptor for NGF, is abundantly expressed on the surface of cholinergic neurons [91, 92]. These neurons are highly responsive to NGF, as revealed in several animal models of cholinergic hypofunctioning in which NGF administration prevented degeneration of CBF neurons [90]. In rats with IBO lesions of the NBM, NGF reversed behavioral impairments in the water maze [93, 94], increased ChAT activity [95] and ACh synthesis in the cortex [96], and produced a hypertrophy of remaining neurons in the NBM [95].

Neurons in the NBM of humans also possess receptors for NGF [97], reflecting not only the capability of the adult basal forebrain to respond to NGF but also the potential to prevent neuronal degeneration of the NBM in AD. NGF does not cross the blood-brain barrier and must be delivered directly into the CNS [98]. Continuous ICV infusions of NGF are capable of preventing degeneration of NBM neurons, but possible undesirable effects of continuous ICV administration of NGF must be addressed when considering its clinical use in humans. For example, pumps and ICV delivery systems are adequately tolerated in humans, but pose risks of infection and perioperative complications. Further, continuous ICV administration of NGF has been associated with Schwann cell hyperplasia, aberrant sprouting of sensory and sympathetic neurites, and weight loss [99–103]. One potential explanation for these adverse effects is the stimulation of NGF-receptive populations exposed to cerebrospinal fluid. Better spatial and temporal delivery of NGF to the brain, through intermittent and/or intraparenchymal infusions, may prove more efficacious in minimizing undesirable effects by decreasing the exposure of nontargeted brain structures to NGF. In support of this notion Swedish patients who received intraparenchymal administration of NGF into the putamen have not suffered the weight loss and back pain that has been reported by AD patients treated with ICV infusions of NGF [104, 105]. Although a promising therapy, the method of NGF delivery to the CNS needs to be adequately addressed in animal models before initiating full-scale clinical trials in humans.

Cellular replacement therapy

Intracerebral transplantation has proven to be an effective technique for restoring or preserving function after brain damage in numerous experimental models of CNS disorders [106–110]. This work suggests the potential clinical usefulness of grafts for neurodegenerative disorders of the human CNS. Cellular replacement strategies have in fact shown promise in initial trials with parkinsonian patients [111]. In animal models of AD grafts of fetal neural tissue (cholinergic-rich grafts) or genetically engineered cells have been used to repair cholinergic damage and/or restore cognitive function. The transition from experimental models to the treatment of AD patients must be approached cautiously, but results obtained with grafts may help to develop a therapeutic concept for ameliorating the devastating cognitive impairments associated with this disorder.

Fetal cholinergic grafts into the neocortex

Transplantation of fetal neural tissue takes place in two basic steps: first the removal of neural tissue from one site of the embryonic brain and then the grafting of this tissue to one or more sites of the host brain. Numerous studies have demonstrated that fetal cholinergic-enriched grafts in the neocortex survive well, anatomically integrate into the host parenchyma, and modify the function of the basal-cortical system after damage [105, 106]. In addition, animals with NBM lesions have shown significant behavioral improvements after grafting. For example, grafts of embryonic basal forebrain tissue to the neocortex of rats with excitotoxic NBM lesions improve Morris water maze performance [112], passive avoidance retention [113], T-maze performance [114], and visual attention [115]. Similar results in other models (e.g., septo-hippocampal lesions, aged rats), support the general utility of cholinergic-rich transplants for treating cognitive dysfunction [106, 107].

Several mechanisms have been suggested by which grafted embryonic basal forebrain tissue might influence basal-neocortical function, including the establishment of functional graft-host connections, graft augmentation of ACh in the neocortex, graft release of growth factors, and nonspecific effects of grafted cells in the damaged host brain [106]. Fetal grafts contain a variety of noncholinergic neuronal populations (peptidergic: neuropeptide Y, enkephalin, and somatostatin) and glial cells that may contribute to the functional recovery following transplantation [116]. Thus it has been difficult to conclusively link a particular factor(s) to graft-induced improvements in behavior. Recently there has been progress in identifying some of these mechanisms by using genetically modified cells as graft material. This newer approach for understanding and repairing cholinergic damage also has sparked interest in gene therapy as a potential clinical application for AD.

Ex vivo gene transfer into the basal-cortical system

The underlying hypothesis of gene therapy as a treatment strategy for neurodegenerative disorders such as AD is that the transfer of genes to the brain will either protect against disease-related cell death, repair neuronal cell damage, or replace missing neurotransmitter function [108–110, 117]. The goal of this strategy is to delay disease-related functional decline or reverse some of the deficits associated with the disease-related decline. In comparison with all previously described therapeutic approaches, the major advantage of gene therapy in the CNS is the ability to deliver a single or several well-defined molecules in a highly specific spatial and temporal fashion.

Conceptually, gene transfer can be divided into the ex vivo and in vivo approaches. For the ex vivo technique, cultured cells are genetically modified to express a foreign gene ("transgene") of interest and then grafted into a predetermined site in the brain. In contrast, the in vivo approach is a direct gene transfer system in which viral vectors are injected into the brain to deliver transgenes into endogenous host cells. While significant interest has been generated concerning both gene transfer strategies as a therapeutic approach for neurodegenerative disorders, the majority of experimental studies have focused on the use of the ex vivo technique to explore and repair the damaged basal-cortical system. One reason for this emphasis is the ability to generate large numbers of genetically modified cells that can be fully characterized in vitro prior to grafting.

The identification of genes relevant to the neurodegenerative process, the development of efficient vector systems for gene transfer, and the selection of appropriate donor cells for genetic modification have been the necessary steps for successful implementation of the ex vivo technique. To date attention has primarily focused on the use of retroviral-based vectors as gene transfer vehicles [118]. These vectors can be easily modified to accommodate a relatively large transgene (DNA insert: 4–8 kb). Retroviral vectors are made by using retroviral packaging cell lines in the absence of replication-competent helper virus [119]. Improvements in the design of packaging cell lines and retroviral vectors have ensured that only replication-defective virus is generated. The retrovirus has the capacity to infect a variety of proliferative cell types and once inside the cell integrates into random sites within the genome [118]. To date a variety of cell lines and primary cells of neural and nonneural origin have been successfully transduced with retroviral vectors and used for gene transfer to the CNS [110].

Nerve growth factor-producing cells implanted in the nucleus basalis magnocellularis

Ex vivo gene transfer techniques have targeted two intervention points in the compromised cholinergic basal-cortical system: (a) to prevent the degeneration of neurons in the NBM (Fig. 1) and/or (b) to enhance cholinergic neurotransmission in the neocortical projection field (Fig. 2). Based on the unique capacity of NGF to rescue damaged cholinergic cells in the adult basal forebrain, initial work focused on the genetic modification of cells to produce NGF. Transduced cells offer powerful means for achieving site-specific and regionally restricted delivery of NGF to the brain, thus potentially avoiding adverse side effects associated with cannula infusions (see above).

Fibroblast cell lines were first used as target cells for NGF gene transfer since they were readily available and easily grown and manipulated in culture. Cells were transfected using the Moloney murine leukemia virus (MLV) containing the mouse NGF cDNA (777-bp *Hga*I-*Pst*I fragment) under the control of the viral 5′ long terminal repeat (5′LTR) [120]. In vitro assessments confirm that the modified cells produce and release NGF (50 pg NGF/h per 10^5 cells). Using the lesioned septo-hippocampal pathway as an in vivo bioassay for the transduced cells, sufficient NGF was produced in vivo (approximately 0.2 ng NGF/h per rat, or 4.8 ng/day) to substantially prevent the degeneration of cholinergic neurons after damage. However, the cell lines either survived poorly within the brain or formed tumors. This work thus proved the validity of this approach but indicated that cell lines are not appropriate cellular vehicles for CNS gene transfer.

Subsequently focus shifted to primary skin fibroblasts. This cell type is of particular clinical relevance since autologous cells can be obtained from small skin biopsies of patients and used for genetic manipulations to avoid postgrafting immunological problems. Primary rat fibroblasts show stable growth in vitro, good survival in a nonmitotic state for up to 2 years after grafting, and excellent secretory properties, a feature that makes these cells an ideal cellular "minipump" for delivering NGF to the cholinergic basal forebrain system [108–110, 117]. For in vivo applications, primary fibroblasts derived from Fischer 344 rats were infected with MLV-derived vectors containing human β-NGF cDNA (1100-bp *Sma*l-*Apa*I fragment of human cDNA) under the control of the viral 5′LTR promotor [121]. A neomycin resistance gene (dominant selectable marker: transposon Tn5 neomycin-resistant gene) driven from an internal Rous sarcoma virus promotor facilitated selection of infected cells with the neomycin analog G418. A two-site enzyme-linked immunosorbent assay for NGF indicated that transduced cells secrete 154–172 pg/h per 10^5 cells into the culture medium. Bioactivity of the cells in vivo was confirmed by showing that NGF grafts into the posterior/medial septum protect approximately 75% of the cholinergic population following septo-hippocampal axotomy. This protection was obtained with doses that were a magnitude lower than those necessary with ICV infusions, showing the functional efficacy that can be achieved with precise targeting of NGF.

In two model systems of basal-cortical degeneration NGF-producing fibroblasts into the NBM have shown functional effectiveness (Fig. 1). In one study, rats with bilateral IBO lesions of the NBM were implanted with either NGF cells or control fibroblasts transduced to express β-galactosidase (β-gal) into the lesioned nuclei [122]. NGF grafts produced approximately 130 ng per day per rat, a dose nearly 40-fold lower than that needed with ICV infusions. Tests of spatial learning and memory conducted in the Morris water maze showed significant improvements in spatial navigation and acuity only in NGF-grafted rats. Although neocortical ChAT activity was not restored by NGF grafts, the increased size of NBM neurons and enhanced cholinergic fiber staining in the neocortex suggested that the functional recovery was linked to heightened activation of the basal-cortical system. In the second study aged impaired animals with NGF-producing fibroblasts grafted adjacent to the NBM

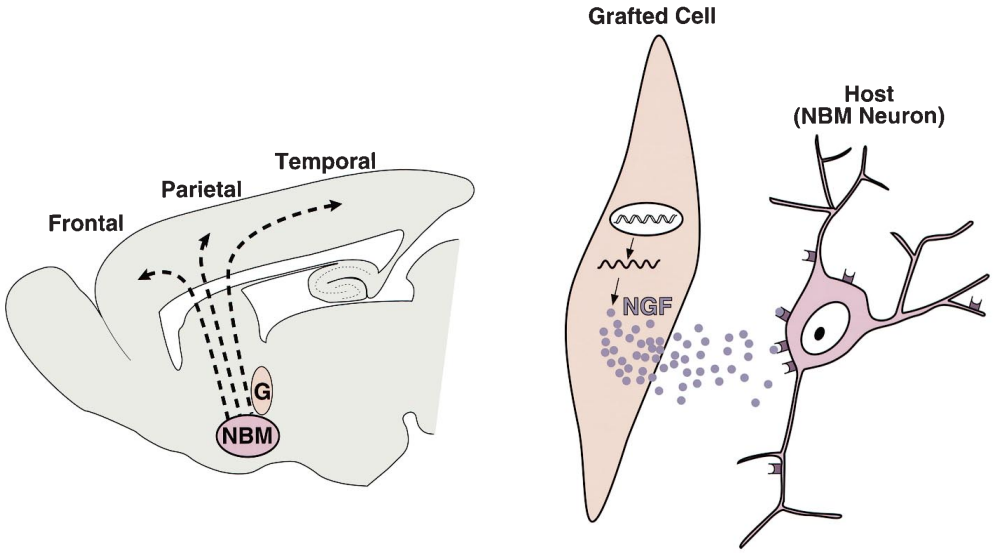

Fig. 1 NBM and NGF-secreting graft (*G*). *Left*, cells are implanted adjacent to the compromised NBM-cortical system (*dotted lines*): *right*, genetically engineered cells of the graft produce and secrete NGF (*purple dots*), which upon binding to NGF receptors induces hypertrophy of remaining neurons in the host NBM and increased cholinergic fiber staining in the neocortex

also showed significant improvements in acquisition and retention in the Morris water maze compared to aged-impaired animals implanted with control cells [123]. Again, a significant increase in size and number of p75NGFr-immunoreactive neurons in the NBM of the aged-impaired animals with NGF grafts suggested an anatomical correlate for the functional improvement.

To date, primary fibroblasts have been most widely used for somatic gene transfer into the brain. Fibroblasts, however, develop a clear boundary at the site of grafting and are not well connected or integrated into either the host parenchyma or neural circuitry. This prompted a search for neural-derived cells that are suitable for gene transfer to the CNS, and that would produce better integration of grafted cells into the host brain. Conditionally immortalized, temperature-sensitive neural progenitor cells are one population that has shown promise for ex vivo gene transfer. Cells have been transduced to express full-length cDNA for mouse NGF (962-bp *Sma*I-*Pst*I fragment) from the MLV 5′LTR [124, 125]. Cells delivering 50–100 ng/day to the septum of rats with fimbria-fornix lesions showed sufficient bioactivity in vivo to rescue over 90% of the cholinergic neurons [125]. Similar graft doses of NGF delivered to the NBM of aged rats induced an almost complete reversal of cognitive deficits in the Morris water maze task [124]. In contrast to fibroblasts, the neural progenitors migrated as far as 1.5 mm from the site of injection, providing a route for locally dispersing NGF in the host brain. Although these cells have the potential for differentiating into neurons, most displayed a glialike phenotype after grafting and integrated organotypically within the host glia. Transduced progenitors did not form tumors after grafting and appeared to be immunocompatible with the host brain. Notably, rats implanted with either NGF fibroblasts or NGF progenitors have not suffered the deleterious effects of NGF seen following ICV administration (e.g., weight loss), further supporting the safety and potency of local delivery of neurotrophic factors into the brain.

Acetylcholine-producing fibroblasts in the neocortex

The hypothesis that cholinergic function in the neocortex is essential for cognitive processes such as attention, learning, and memory suggests that restoration of cholinergic neurotransmission following NBM damage should be sufficient to improve some aspects of cognition. Cells genetically modified to produce ACh could thus provide an effective approach to alleviate cholinergic hypofunctioning in the neocortex. In studies conducted with primary fibroblasts obtained from adult Fischer 344 rats cells were genetically modified to express *Drosophila* ChAT (dChAT), the synthetic enzyme that converts choline and acetyl-coenzyme A to ACh [126]. Although fibroblasts do not normally contain ChAT, these cells are particularly appropriate for this application since they have a high-affinity uptake system for choline [126] and intrinsic mechanisms for the packaging and quantal release of ACh [127]. Cells were transduced using an MLV-derived vector that expressed dChAT from the 5′LTR, and the neomycin resistance gene from an internal Rous sarcoma virus promotor. In vitro characterizations of transduced cells revealed high levels of ChAT activity (240–987 nmol ACh/h per milligram of protein) and the production and release of ACh into the media [128]. In vivo microdialysis of dChAT fibroblasts implanted in the intact hippocampus showed good production of ACh, and these levels could be enhanced with localized administration of choline to the grafted cells [128]. Choline-induced changes in ACh production demonstrated one exogenous route for manipulating the “dose” of neurotransmitter delivered to the CNS, an important consideration if the basal release of ACh from transduced cells is functionally inadequate in the damaged brain.

In a recent study dChAT fibroblasts were implanted into the frontal and parietal neocortex of rats with bilateral IBO lesions of the NBM (Fig. 2) [129]. These animals were then behaviorally assessed in the Morris water

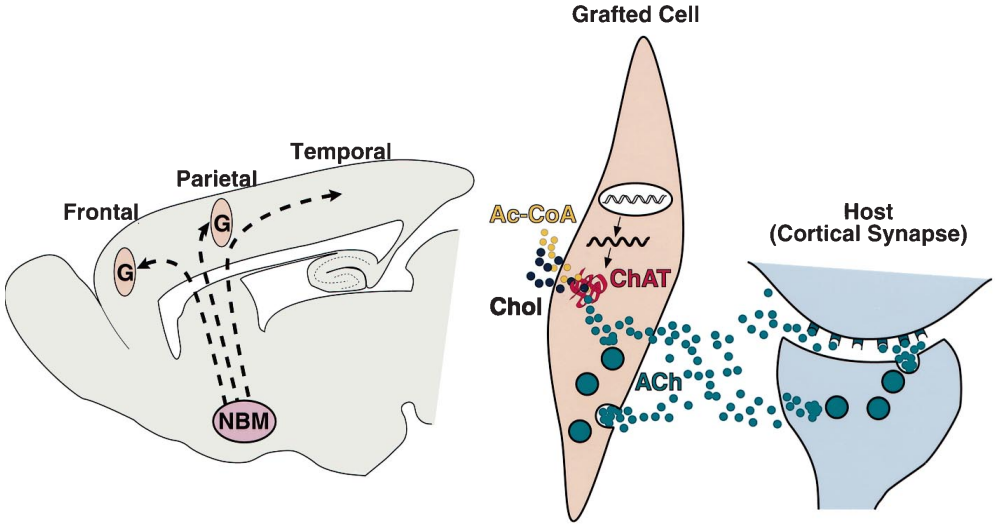

Fig. 2 Neocortex and ACh-producing grafts (*G*). *Left*, cells are implanted in the frontal and parietal cortices of the compromised NBM-cortical system (*dotted lines*); *right*, genetically engineered fibroblasts in the graft expressing the synthetic enzyme ChAT take up choline (*Chol*; *blue dots*) and acetyl-coenzyme A (*Ac-CoA*; *yellow dots*) from the extracellular milieu and synthesize acetylcholine (*ACh*; *green dots*). Fibroblasts show constitutive release of ACh but also have the capacity to package ACh in vesicles for quantal secretion. Released ACh could be utilized by remaining NBM-cortical fibers or directly stimulate postsynaptic ACh receptors in the neocortex

maze, open field activity, retention of a passive avoidance task, and startle response. During acquisition training in the Morris water maze dChAT-grafted animals found the hidden platform faster than either β-gal grafted or nongrafted lesioned controls. The most marked improvements were evident in tests of spatial acuity, where dChAT grafted rats performed at the level of unlesioned controls. No other behavioral measures were affected either by the lesion or by grafts, indicating a lesion- and graft-specific modulation of spatial acquisition and memory. Analysis of microdissected dChAT grafts confirmed activity of the transgene (presence of dChAT mRNA by reverse transcriptase polymerase chain reaction) and showed elevated levels of ACh in neocortical tissue. Since the presence of ACh in the neocortex was the only difference between the experimental groups, amelioration of the spatial memory deficit could be linked to enhanced cholinergic function within the denervated neocortex. This study confirmed the hypothesis that restoration of ACh in the damaged basal-neocortical system is sufficient for improving cognitive function. Interestingly, dChAT cells grafted into the neocortex of animals with excitotoxic NBM lesions resulted in the same pattern of functional recovery in spatial learning and memory that was observed with grafts of NGF-producing cells into the damaged NBM. These combined observations strongly support therapeutic strategies for the cognitively impaired brain that focus on site-specific manipulation of cholinergic function.

Gene therapy

Safety is a major concern when considering ex vivo gene therapy for the treatment of neurodegenerative disorders in patients. Graft-induced tumor formation, inadvertent release of infectious viral particles into the host, and ineffective transgene levels are some of the potential risks associated with genetically engineered cells. Strategies are currently under investigation to address such issues. Encapsulating cells within polymer membranes is one technique for restricting cellular growth and isolating certain cellularly released factors from the host [130, 131]. This approach also provides a method for retrieving cells, if necessary, from the CNS. Exogenous manipulation of transduced cells is likely to be necessary for directly controlling cells and/or transgene expression in vivo. In one approach cells could be modified to express vectors that contain regulatable or conditionally lethal genes [132]. For example, expression of the herpes-derived thymidine kinase gene is normally innocuous but becomes deadly when exposed to ganciclovir, a widely used antiviral drug.

A second system that has been recently been described involves the use of a tetracycline (TET)-responsive element to control transgene expression [133, 134]. Fusion of the prokaryotic TET repressor (Tn10-derived TET-resistance operon of *Escherichia coli*) to the activating domain of herpes simplex virus VP16 protein forms an eukaryotic transactivator. This transactivator, working in concert with a TET operator sequence, is necessary for maintaining transgene expression. In the presence of TET, transgene expression can be shut off. An advantage of the TET system is that it can also be designed to have a reverse function (turning on gene expression). This involves the use of a mutant TET repressor that requires TET for transcription activation. Such manipulations not only help to ensure the safety of transduced cells but also make gene transfer strategies more effective both as a tool for exploring CNS function and as a potential therapeutic approach for neural damage or disease in the human CNS.

There is significant interest in the possible use of gene transfer as a therapeutic approach for CNS disorders. However, substantial technical and theoretical problems remain to be solved before this technology can be seriously considered for clinical application. Among the most relevant requirements when considering gene therapy in a clinical setting are: (a) nontoxic methods of gene delivery; (b) efficient and reliable methods of gene deliv-

ery; (c) localized and controllable gene delivery; (d) stable and predictable duration of gene expression; (e) functionally adequate levels of gene product; and (f) safety of ex vivo and in vivo systems. Significant progress has been made in addressing these concerns, particularly in respect to gene regulation and delivery systems, but many challenges still remain. Continuing advances in the development of in vitro and in vivo technology will be invaluable for solving remaining issues and will ultimately lead to successful implementation of this technique for patients.

Conclusion

Profound cholinergic dysfunction in the basal-cortical system associated with cognitive deficits in patients with AD has led to enormous efforts in developing rodent models that mimic cholinergic hypofunctioning in the NBM-neocortex system. Acute excitotoxic and more selective immunotoxic NBM lesions have brought new insights in understanding the role of the cholinergic system in cognitive processes such as attention, learning, and memory. In addition, significant progress in generating transgenic models that manipulate NGF or overexpress human mutant APP offer additional tools to study the underlying neurobiological cascade of AD-related cognitive deficits. Despite the limited replication of AD-specific neurochemical and neuropathological features in these different rodent models, it is evident that these models are useful for identifying and testing new potential therapeutic strategies.

To date, the majority of studies have focused on enhancing the cholinergic system to alleviate cognitive deficits. Cholinergic manipulations such as the systemic application of AChe inhibitors, the intracerebral administration of NGF, and ex vivo gene transfer approaches to locally deliver NGF or ACh in rodents with a compromised basal-cortical systems have proven to be consistently efficacious in reversing behavioral and biochemical sequelae of these lesions. The continuing efforts will provide new insights in utilizing these approaches to reverse some of the cholinergic deficits and cognitive impairments seen in AD patients.

Acknowledgements We thank H.G. Kuhn for designing the figures for the manuscript. J.W. is fellow of the Brookdale Foundation Group (New York). We gratefully acknowledge the support of the VA Research Service, NIH, NIA, and the Sam and Rose Stein Foundation for Research on Aging.

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