A second wind for the cholinergic system in Alzheimer's therapy

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Abstract

Notwithstanding tremendous research efforts, the cause of Alzheimer's disease (AD) remains elusive and there is no curative treatment. The cholinergic hypothesis presented 35 years ago was the first major evidence-based hypothesis regarding AD etiology. It proposed that the depletion of brain acetylcholine was a primary cause of cognitive decline in advanced age and AD. It relied on a series of observations obtained in aged animals, elderly and AD patients which pointed to dysfunctions of cholinergic basal forebrain, similarities between cognitive impairments induced by anticholinergic drugs and those found in advanced age and AD, and beneficial effects of drugs stimulating cholinergic activity. This review comes back on these major results to show how this hypothesis provided the drive for the development of anticholinesterase inhibitor-based therapies of AD, the almost exclusive approved treatment in use despite transient and modest efficacy. New ideas for improving cholinergic therapies are also compared and discussed in light of the current revival of the cholinergic hypothesis based on two sets of evidence from new animal models and refined imagery techniques in humans. First, human and animal studies agree on detecting signs of cholinergic dysfunctions much earlier than initially thought. Second, alterations of the cholinergic system are deeply intertwined with its reactive responses providing the brain with efficient compensatory mechanisms to delay the conversion to AD. Active research in this field should give new insight to develop multi-therapies incorporating cholinergic manipulation, as well as early biomarkers of AD allowing earlier diagnostics. This is of prime importance to counteract a disease that is now recognized to start early in adult life.

Keywords: Alzheimer's disease; Acetylcholine; Animal Models; Cholinomimetics

1. Introduction

The etiology and early pathogenesis of Alzheimer's disease (AD) in its major sporadic form still remains highly mysterious mainly because causative pathways are very likely multifactorial. The greater known risk factor in the development of AD is aging and the major genetic risk factor is the apolipoprotein E gene allele *APOE4*. The most prominent clinical sign of AD is memory loss. Several forms of memory are affected at the early stage like episodic memory and working memory, both characterized by difficulties to recall information gathered during specific events (Almkvist, 1996; Salmon, 2011). Early deficits in spatial navigation tasks have also been reported (Kalová et al., 2005; Hort et al., 2007). As these forms of memory are also affected in normal aging (Moffat, 2009; Gazova et al., 2013), although to a much lesser extent, the frontiers between normal and pathological cognitive decline are difficult to set at the earliest stages of the disease. As the pathology progresses, the worsening of these deficits generates increasing perturbations of everyday life and compromises the quality of life of patients and their immediate entourage. Further aggravation of the pattern of cognitive deficits and emergence of other behavioral disturbance most often require institutional care, creating affective and financial burdens on families and heavy costs for the society. At the neuropathological level, brains of AD patients are expected to show the two main hallmarks of the disease, namely senile plaques and neurofibrillary tangles. Senile plaques are primarily constituted of the β-amyloid peptide (Aβ) which accumulates in the brain due to abnormal regulation of amyloidogenic proteolysis of the β-amyloid precursor protein (APP) and altered clearance of Aβ. Rare forms of early onset familial AD (FAD) are mainly due to gene mutations affecting APP metabolism in favor of Aβ accumulation. Neurofibrillary tangles are largely formed of abnormally phosphorylated tau protein. Among a constellation of other neuropathological signs, there is also a marked brain inflammatory reaction and a relatively specific pattern of cell loss which affects primarily the temporal lobe and the cholinergic basal forebrain. The present review focuses on the cholinergic alterations associated with AD and the development of the cholinergic hypothesis which prompted the

use of cholinomimetic drugs as the first therapeutic approach to AD. Our aim is to enlighten how complementary animal and human studies can be at each step of the development of cholinergic drug in the past and the future.

2. AD and the cholinergic hypothesis

2.1 The cholinergic system

Acetylcholine (ACh) is a neurotransmitter released by neurons from the peripheral and central nervous systems. The central cholinergic system is mainly organized in six nuclei (Ch1 to Ch6) which differ by their anatomical localization, functional characteristics and patterns of projection (Mesulam et al., 1983). Four of them form the basal forebrain cholinergic system: the medial septum (MS; Ch1), the diagonal band of Broca (Ch2, Ch3) and the nucleus basalis of Meynert (NBM; nucleus basalis magnocellularis in rodents; Ch4). Ch5 and Ch6 cholinergic neurons are located in the brain stem within the pedoculopontine nucleus and laterodorsal tegmental nucleus, respectively. A third main source of central ACh is provided by local projections of striatal cholinergic interneurons, but these cholinergic neurons, like those of ch5-6, are relatively preserved by AD (Jellinger, 1988; Geula et al., 1990). In contrast, the basal forebrain cholinergic system appears as the main locus of cholinergic dysfunctions associated with AD, as developed below. Cholinergic neurons of the MS (Ch1) and vertical limb of the diagonal band of Broca (Ch2) provide the main supply of ACh to the hippocampus and the entorhinal cortex, whereas those of the nucleus basalis of Meynert mainly innervate the cerebral cortex and the amygdala (Mesulam et al., 1992; Kitt et al., 1994).

ACh is synthesized by the choline acetyl transferase (ChAT) from two immediate precursors, choline and acetyl-coenzyme A. It is then packed by the vesicular acetylcholine transporter inside presynaptic

vesicles of axonal terminals. Once released by the presynaptic cholinergic neurons, ACh binds to cholinergic receptors present on post-synaptic or pre-synaptic cell membranes. It is subsequently hydrolyzed in choline and acetate by the acethylcholinesterase enzyme (AChE) or by the less specific butyrylcholinesterase enzyme (BuChE) (Unzeta et al., 2016). Both are found in neuronal synapses, though at a much higher concentration for AChE. BuChE is also less selective for ACh and operates with different kinetics. These enzymes have two substrate binding sites: the catalytic anionic site responsible for the hydrolysis itself and the peripheral anionic site which concentrates the substrate towards the central site. Note that Aβ interacts with the peripheral site to trigger amyloid fibrillogenesis (Inestrosa et al., 1996). Choline resulting from ACh hydrolysis is then captured back to the presynaptic neuron where it is transformed into ACh by ChAT. Once released, ACh can bind onto two categories of cholinergic receptors: the G-protein coupled muscarinic receptors (mAChRs), divided in five subtypes (M1 to M5), and the pentameric ionotropic nicotinic receptors (nAChRs) constituted of α subunits (α2-10) and β subunits (β2-4). To sum up, several possible pharmacological means of enhancing cholinergic transmission are available. So far, investigation of AD therapies have focused primarily on the acetylcholinesterase inhibitors (AChEI), which increase the availability of brain ACh, and to a lesser extent on various compounds possessing agonistic effects on cholinergic receptors.

2.2 The cholinergic hypothesis

Formulated 35 years ago, the cholinergic hypothesis posits that cholinergic dysfunction contributes to cognitive deficits associated with aging and AD (Bartus et al., 1982). This hypothesis was based on three main streams of contemporary evidence emerging from both human and animal studies. First, cholinergic markers were affected in subjects with age-related cognitive decline. Second, alterations of the cholinergic system induced deficits similar to those of aged subjects and Alzheimer patients. Third, increasing central cholinergic activity had a beneficial effect on age-related cognitive deficits.

Hence, one of the most convincing evidence supporting the cholinergic hypothesis was provided by studies showing dramatic changes in the cholinergic system associated with cognitive decline. Strikingly, late stages of AD were accompanied by a severe loss of neurons from the NBM (Whitehouse et al., 1981). In addition, a reduction of ChAT activity was reported as stronger and more reliable in AD brains compared to age-matched ones and it correlated to the degree of memory impairment (Bowen et al., 1976; Davies and Maloney, 1976; Perry et al., 1978). This particular neurochemical change was thus considered highly specific to AD. Moderate cholinergic cell loss and reduction of cerebral ChAT activity associated with memory deficits were later confirmed in aged animals (Strong et al., 1980; Gilad et al., 1987). Perturbations of other cholinergic markers such as muscarinic receptor binding were subtle but reliable in aged animals and elderly while quite inconsistent in AD patients (reviewed in Bartus et al. 1982). It is important to note that, when the cholinergic hypothesis was formulated, there was no animal model of AD yet. Research on the cholinergic system and cognitive aging was limited to aged animals from species that do not develop spontaneously AD-like pathology. The idea of a specific role of the cholinergic system in cognitive aging was further supported by studies in humans showing that low doses of the muscarinic antagonist scopolamine induced cognitive deficits in young subjects resembling those of aged subject in tasks evaluating delayed recall of recent information (Drachman and Leavitt, 1974). This was also true in animals, from monkeys to rodents (Meyers and Domino 1964; Bartus 1979). As a logical correlate, several compounds stimulating the cholinergic system were thus considered in preclinical as well as clinical studies for their potential effects on memory impairments in normal and pathological aging. Most of them, such as ACh precursors (e.g., lecithin) or muscarinic agonists (e.g., arecoline), generally failed to improve performance in aged subjects and all of them led to disappointing clinical outcomes mainly due to poor pharmacokinetics and deleterious side effects. In fact, the best results came from anticholinesterase drugs, especially physostigmine which demonstrated facilitatory effects on cognitive performances in non-human primates (Bartus, 1979), young and old humans

(Drachman and Sahakian, 1980) and AD patients (Muramoto et al., 1979). The additional finding that deficits induced by scopolamine could be reliably relieved by physostigmine in rodent models made this compound the genuine ancestor of the cholinomimetics and opened a large avenue of preclinical and clinical research leading to the development of therapeutic drugs possessing anticholinesterase activity.

Within less than 15 years after the publication of Bartus' seminal paper (1982), marketing authorizations were given to the first of four cholinomimetics approved for the treatment of AD, namely tacrine (Cognex®, abandoned due to side effects) followed by donepezil (Aricept®), galantamine (Reminyl®) and rivastigmine (Exelon®). These compounds are mainly prescribed at the early stages of AD when cognitive symptoms are light to moderate. Benefits reported are modest and last less than two years (Courtney et al. 2004): cognitive performances increase or at least stabilize, and there is a global improvement in daily life activities. However, these effects are limited to a subpopulation of responders (30-40 % of the patients) impossible to identify *a priori*. It is noteworthy to remind that these drugs are the only ones approved as AD directed treatments up to now, aside the *N*-methyl-D-aspartate (NMDA) antagonist memantine. Although these treatments are not disease-modifying, these symptomatic drugs should certainly be acknowledged as useful for the daily life of patients within the limits evoked above. As long as the root causes of AD remains obscure, there is no pharmacological alternative at this time. It must be noted here that the widely used aged animal model may have misled research strategies to develop anticholinergic drugs based on several fundamental differences between healthy elderly and AD patients concerning the dramatic neuronal loss even at early stages (nearly absent in normal aging; see as example Small et al., 2004), the pattern of cortical vulnerability more centered on the medial temporal lobe and the nature of cholinergic basal forebrain alterations in AD (Grothe et al., 2010; Grothe et al., 2012).

2.3 The preclinical models of cognitive aging used for cholinomimetics development

As shown above, animal studies contributed to a great degree to the genesis of the cholinergic hypothesis in parallel to human studies. Thereafter, animal models were instrumental to the development of cholinomimetics. Similarities in the pattern of memory deficits induced by scopolamine compared to those found in AD prompted the use of animal models based on muscarinic receptor blockade for many years. These models have benefited from a long-standing experience in modulating memory performances through pharmacological manipulations with cholinergic drugs (Deutsch, 1971). The loss of cholinergic neurons in AD has also led to the development of models based on lesions of basal forebrain cholinergic nuclei using various approaches, first electrolytic, then excitotoxic and more recently immunotoxic (e.g., 192 IgG-saporin in rats). However, as the immunotoxic lesion technique was targeting more specifically the cholinergic neurons, results ended up disappointing in regard to the limited extent of memory deficits obtained in these models (Gallagher and Colombo, 1995; Parent and Baxter, 2004). As a matter of fact, the use of lesion models in developing cholinomimetics has been quite limited (Riekkinen, Riekkinen, et al., 1991; Mulder et al., 2005). This relative lack of effect on memory performance was totally unexpected and it seriously questioned the cholinergic hypothesis. Moreover, the strong impact of less specific lesions was subsequently interpreted as resulting from the loss of non-cholinergic basal forebrain neurons (e.g., GABAergic, glutamatergic) which contributed evidently more to cognitive processing than initially thought (Parent and Baxter, 2004). However, it was later shown that specific cholinergic lesions did provoke massive spatial navigation impairments in both reference memory and working memory tasks when associated with mild neuronal loss within the temporal lobe (i.e., entorhinal cortex lesions; Traissard et al., 2007). This finding rehabilitated the concept of a critical role of cholinergic neuronal loss as a major aggravating factor for cognitive deficits when considered in the context of an already degenerating AD brain. In conclusion, preclinical behavioral studies that led to the successful development of cholinomimetic treatments were mainly restricted to pharmacological

(scopolamine) and lesion (NBM electrolytic and non-specific neurotoxin) models. Transgenic mouse model of AD, nowadays widely used for preclinical studies, was introduced later.

As AD is primarily characterized by memory deficits, the animal models of cholinergic dysfunction were often tested for cognitive deficits on two main forms of memory affected in early AD: short term memory for events and spatial memory. Short-term memory was mostly evaluated using discrete trials characterized by particular trial-specific information to be recalled in various types of tasks taxing working memory, recognition memory or episodic-like memory (Bartus and Dean, 1988; Rupniak et al., 1990; Dawson and Iversen, 1993; Luine et al., 2002; Prickaerts et al., 2005; Tronche et al., 2010). A particular attention was drawn to the rate of forgetting for recent event as immediate memory is typically not affected in aging and early AD, whereas delayed recall performance is expected to show accelerated decline. As for spatial navigation memory, it is usually tested in radial, Barnes or Morris water maze tasks (Hodges et al., 1990; Kwo-On-Yuen et al., 1990; Cheng et al., 1996), sometimes with an additional working memory component (Sweeney et al., 1988; Marighetto et al., 2008; but see Riekkinen, Aaltonen, et al., 1991). As evidently expected, both types of task are very sensitive to central cholinergic dysfunctions (Whishaw et al., 1985). Again, commonalties in the nature of memory impairments in early AD and healthy elderly, as well as in aged animals and models of AD, led to the use of aged animals to test the efficacy of cholinomimetic candidates (Bartus and Dean, 1988). Hence, although not in full agreement with the previously described task requirements, some of the most popular rodent memory tasks at that time, such as passive and active avoidance paradigms, were also used with some success in preclinical approaches mainly because testing parameters were set so that recent memory performance decayed rapidly. These tasks similarly highlighted a sensitivity of the memory performance to cholinergic modulation and aging in rodents (e.g., Flood et al., 1985). Finally, attentional paradigms were often included in the behavioral battery of tasks used to evaluate candidate drugs. Indeed, improvements of attention performance have been reported in AD patients treated with

cholinomimetics (e.g., Alhainen, Helkala, & Riekkinen, 1993). A similar enhancement of visuospatial attention by cholinergic drugs has been repeatedly shown in animal studies (Kirkby et al., 1996; Lindner et al., 2006), confirming a putative role of the basal forebrain cholinergic neurons in some forms of attentional functions critical to visuospatial tests (Robbins et al., 1989; Chiba et al., 1999).

New animal models of AD were also created in light of the second major hypothesis of AD etiology. Ten years after the cholinergic hypothesis proposal and shortly before approval of the first AChEI, the amyloid cascade hypothesis indeed postulated that Aβ accumulation was the primary event in AD pathogenesis (Hardy and Higgins, 1992). This hypothesis has since drawn impressive effort in fundamental research as well as in the developments of drugs aimed at reducing Aβ accumulation. It prompted the emergence of models of AD based on intracerebral injection of Aβ, and other even more popular approaches that benefited from the transgenic mouse revolution. After several attempts based on APP knock-outs or normal human APP transgene, two mouse lines transgenic for APP FAD mutated genes finally developed the amyloid plaques which were the gold standard for an animal model at the time (Hsiao et al., 1995; Masliah et al., 1996). Since then, several other transgenic mouse and rat models have integrated human mutated tau and/or FAD mutations (Puzzo et al., 2014). Besides showing tau abnormalities, neuroinflammation and synaptic defects reminiscent from those found in AD, most of these models have displayed perturbations of the basal forebrain cholinergic system, although only very few models suffer from clear cholinergic neuronal loss (Cassel et al., 2008; Belarbi et al., 2011). The same is true for the temporal lobe in which only limited degeneration has been reported in these models. In general, the lack of massive AD-like neurodegeneration even at the oldest ages is one of the major limits that should be taken into account when interpreting preclinical approaches based on these models (Ashe and Zahs, 2010). The pattern of cognitive impairment is reminiscent of the one found in AD: most models show deficits in working memory, recognition memory, spatial navigation memory, and even in the newly developed episodic-like memory tasks (Webster et al., 2014). Surprisingly, no publication has yet

reported deficits in transgenic or other AD models in pattern separation tasks, despite their high sensitivity to cognitive decline in normal aging and early stages of AD in humans, and to cholinergic treatment in animals (Holden and Gilbert, 2012; Van Goethem et al., 2015). More importantly though, various AChEIs used in the primary AD models were successful in transgenic models (Dong et al., 2005), proving thereby the validity of these rodent models for the development of new cholinergic based therapies.

Besides, animal models should also be employed to resolve open questions on AD which appear critical for the future development of cholinomimetic drugs. The first fundamental issue relates to the existence of responders and non-responders to AChEI treatments. The origin of these individual differences might depend on general factors like estrogens or the genotype (Craig et al., 2011). Such inter-individual variability in response to these treatments has been known for a long time in humans as well as in animals (Bartus, 1979; Davis et al., 1979), but the underlying mechanisms are unknown. Animal studies should certainly help to clarify this issue by exploring its potential physiological basis. It has been suggested that the efficacy of AChEIs could be related to the level of impairment or neurodegeneration of the subject as shown in aged rats (Stemmelin et al., 1998; Dumas and Newhouse, 2011). The work of Connelly *et al* (2005) tends to confirm this interpretation as AChEI non-responders show higher atrophy of the medial temporal lobe. Based on a morphometric indicator, the extent of cholinergic degeneration of the NBM has been inversely correlated with the magnitude of response to treatment with AChEI in AD patients (Tanaka et al., 2003). These results suggest that AChEI responders benefit from the prolonged availability of ACh because cholinergic innervation within target structures decreases as basal forebrain atrophy progresses. Also, the genetic risk factor *APOE4* has been associated with both reduced hippocampal cholinergic markers and unresponsiveness to AChEI in AD patients (Farlow et al., 1996; but see Waring et al., 2015). Recently, we highlighted a potential mechanism by which *APOE4* could disrupt AChEI response. By mimicking the earliest stage of AD with a partial entorhinal cortex lesion in mice, we

have shown the extraordinary ability of the mammalian brain to normalize lesion-induced spatial memory deficit and hippocampal neuronal hyper-activity through the compensatory hippocampal cholinergic sprouting in response to entorhinal cell loss (Bott et al., *in press*). This cholinergic sprouting transiently increased the territory of septo-hippocampal cholinergic innervation in the whole hippocampus before glutamatergic reinnervation occurred and seemed to take over for functional recovery. Interestingly, the cholinergic sprouting was abolished in mice expressing the human *APOE4* allele, but not in those expressing the *APOE3* allele, which may explain several negative effects of *APOE4* such as marked hippocampal hyperactivity (Filippini et al., 2009) and reduced responsiveness to AChEI in AD patients (Farlow et al., 1996). Moreover, it is noteworthy that reactive cholinergic sprouting and glutamatergic reinnervation do exist within the hippocampus in response to entorhinal cortex pathology in early stages of the disease (Geddes et al., 1985; Ikonomovic et al., 2003). Another study in monkeys has recently shown that cholinergic innervation within the temporal lobe cortex facilitates functional recovery after structural lesions impairing episodic memory performances (Croxson et al., 2012). Taken together, these animal studies confirm that the cholinergic system plays an important role in brain compensatory mechanisms relevant to AD. These are worth being further explored experimentally with the aim of developing interventions favoring or mimicking natural defenses of the brain against the disease. Another example of cholinomimetic-relevant issue that should benefit from investigations in animals is the characterization of pharmacological activities, apart from AChE inhibition, of some clinical drugs which could play a role in their beneficial effects in AD (Wilkinson et al., 2004). For example, inhibition of BuChE (Cheng et al., 1996) as well as the stimulating effect on nicotinic receptors density or NMDA receptor activity in key structures for memory may contribute to these effects (Barnes et al., 2000). Altogether, these examples demonstrate how critical animal studies can be in the future to improve our understanding of the mechanisms involved in AChEI responsiveness and to characterize more precisely the role of the basal forebrain cholinergic neurons in the earliest stages of AD.

3. Renewed interest for the cholinergic system in the development of Alzheimer's disease

It is obvious these days that the ACh deficit associated to the degeneration of the cholinergic basal forebrain neurons cannot be the single cause of AD as proposed initially in the cholinergic hypothesis. This is mainly because treatments increasing the cholinergic drive do not halt the pathological process (Raschetti et al., 2007; Schneider et al., 2014) and other cerebral structures involved in memory, such as the entorhinal cortex, were found to degenerate at least as early as the basal forebrain (Kordower et al., 2001). In the meantime, the major AD features, namely soluble forms of Aβ and tau, have been put forward as responsible for the development of the disease. ACh-related deficits were progressively relegated to a mere consequence of these proteomic-related events. However, despite considerable research effort, evidence for Aβ- and/or tau-related abnormalities as being the cause of AD still remains inconclusive as corresponding treatments have failed so far. Due to this dramatic difficulty to identify the causes of AD, the cholinergic hypothesis is now going through a renewal period inscribed within the concept of AD being a more complex and multifactorial disease in which cholinergic deficits represent only one aspect of the pathogenesis. For example, a recent view postulates that AD is primarily a hippocampal dementia resulting from a combination of factors not necessarily identical in all patients (Craig et al., 2011). In this context, cholinergic depletion, already present in the elderly, is seen as a risk factor of AD interacting with other risk factors like stress or injury. As the occurrence of such circumstances becomes more frequent with age, an increasingly large combination of them would ultimately disrupt cognitive and structural compensatory mechanisms which are normally engaged to cope with brain dysfunctions and damages. This would favor the conversion to AD.

Cholinergic deficits, not the sole cause of AD anymore, could nonetheless heavily contribute to the disease progression. Recent findings indicate that atrophy of the cholinergic basal forebrain begins during normal aging and is aggravated in patients with mild cognitive impairment (MCI, a prodromal stage of AD) (Grothe et al., 2012). It thus appears at an earlier stage than initially thought and progresses in parallel to cortical atrophy (Kilimann et al., 2016). Indeed, early atrophies of both the NBM and of cortical structures of the temporal lobe were shown to be associated with impaired delayed recall in MCI patients (Grothe et al., 2010). Reduction in basal forebrain volume also contributes to spatial navigation deficits in AD patients (Kerbler et al., 2015). Interestingly, hippocampal atrophy in amnestic MCI patients (i.e., likely to become AD) was slowed down by a one-year donepezil treatment (Dubois et al., 2015). Note however that no cognitive improvement ensued, similarly to clinical trials testing the preventive effects of AChEIs on MCI patients' cognition (Schneider et al., 2014). This highlights that early cholinergic deficits cannot entirely elucidate the AD pathogenesis. Long-term cholinergic depletion has nonetheless recently been shown to alter gene expression of some transcripts related to the AD pathology in the hippocampus of aged mice with a knockout of hippocampal vesicular acetylcholine transporter (Kolisnyk et al., 2016). These mice displayed age-related changes in APP processing, tau hyperphosphorylation, hippocampal neuronal loss and synaptic abnormalities, as well as cognitive deficits.

Thus, early cholinergic deficits appear to have long-term consequences on the integrity of innervated systems. This could be explained by the neuroprotective action of the cholinergic system against several AD neuropathological events such as neuro-inflammation, Aβ accumulation and abnormal tau phosphorylation (Ovsepian et al., 2015; Echeverria et al., 2016). For instance, cholinergic receptors possess a high affinity for Aβ or tau protein whose binding induce some intracellular mechanisms controlling further production of these ligands (Ovsepian et al., 2015). M1 receptors activation promotes non-amyloidogenic cleavage of APP via the modulation of major APP-cleaving proteases (Davis et al., 2010). Tau phosphorylation is also regulated by cholinergic receptors, decreased and increased by

mAChRs and nAChRs activation respectively (Caccamo et al., 2006; Buckingham et al., 2009). Basal forebrain cholinergic terminals expressing p75 neurotrophic receptors would also play a special role in clearing Aβ via its degradation after endocytosis (Ovsepian et al., 2015).

In addition to this neuroprotective action, there are several indications that the cholinergic system undergoes adaptive changes in cognitively important target structures like the hippocampus, as previously evoked (Bott et al., *in press*; Mufson et al., 2016), or the frontal cortex (DeKosky et al., 2002; Ikonomovic et al., 2003). In human studies, these changes were mainly represented by increased ChAT activity, which was interpreted as resulting from a transient compensatory sprouting by the remaining cholinergic terminals in the hippocampus following entorhinal disconnection, and more probably an up-regulation of the enzymatic activity in the frontal cortex (Mufson et al., 2016). Another example of cholinergic activity adaptability can be found in the rapidly increased production of an AChE variant following low levels of Aβ (Li et al., 2013). This variant is more frequent under stress conditions, helping neuroprotection, neural development and possibly ACh release. Besides, ACh itself can favor various forms of compensatory neuronal plasticity outside the sprouting described above: for example, dendritic branching (Mufson et al., 2016), neurogenesis (Kotani et al., 2006) and synaptic plasticity (e.g., Rasmusson, 2000). At a more functional level, the cholinergic system appears to be involved in the capacity of the brain to shift on alternative neuronal networks to offset the fornix degeneration and maintain visual recall memory performances (Ray et al., 2015). Indeed ACh is known to promote attention, memory and cognitive flexibility by supporting the compensatory engagement of frontal regions following AD-related degeneration of more posterior structures (Dumas and Newhouse, 2011; Hasselmo and Sarter, 2011). Moreover, increased reorganization of glutamatergic terminals has been shown in regions similar to those sustaining cholinergic plasticity, and in MCI more than in AD (Mufson et al., 2016). This may explain why AChEI/memantine combined therapies have some beneficial effects (Dantoine et al., 2006). In any case, there is clearly a need to better understand mechanisms and factors

involved in cholinergic and glutamatergic reactive neuroplasticity as they may efficiently delay a critical shift to heavier cognitive impairments associated with a serious degradation of the quality of life of the patients and their entourage. Better knowing the delicate equilibrium preserving cognitive performance and developing means to prolong this “compensated” state would open a larger time-window for symptomatic and possibly disease-modifying therapies.

4. The future of cholinergic drugs

Establishing the time line of the various subtle cholinergic dysfunctions should further suggest more specific therapies than the AChEIs used so far, possibly adapted to each stage of the disease. It is thus not surprising that, despite the modest (but consistent; Schneider et al., 2014) symptomatic benefits of AChEIs and the rise of alternative hypotheses about AD pathogenesis and etiology, cholinergic therapies are still relevant and actively researched. Presenting the numerous new cholinergic and non-cholinergic molecules currently tested *in vivo*, *in vitro* or even *in silico* is beyond the scope of this review. The following sections aim only at providing a brief perspective on the main cholinergic AD therapies in preparation, starting with the AChEI drug category.

4.1. Future of AChEIs

The most straightforward option for treating AD is to improve the AChEIs. Many compounds with more potent AChEI properties are currently being developed, either extracted from natural products (e.g., coumarins, flavonoid derivatives) or computationally designed (Anand et al., 2014; Kumar et al., 2016).

Research for newer drugs in AD therapy is however a complex process in which it is certainly difficult to take the multitude of factors involved in the disease. In terms of the AChEI action, at least four aspects should be considered.

First, the inhibition of the AChE could be more efficient by blocking both the catalytic and the peripheral sites of AChE. Except donepezil, the AChEI drugs used in therapies bind only to the catalytic site (Ismaili et al., 2016). This pharmacological aspect has been explored actively for the last decade and more recent molecules like donepezil-tacrine hybrids, coumarins and huperzine A often act as dual binding site inhibitors (Ismaili et al., 2016; Kumar et al., 2016). Interestingly, positive activity of the peripheral anionic site on the prevention of Aβ aggregation should be taken into consideration in the development of new AChEIs (Inestrosa et al., 1996).

Second, various forms of cholinesterase enzymes exist, with different locations and functions (Zimmermann, 2013). For example, AChE and BuChE differ mostly by their location and their affinity for ACh (Unzeta et al., 2016) but BuChE might compensate for the loss of AChE in AD (Greig et al., 2005). In severe cases of the disease, AChE expression is indeed decreased while that of BuChE is increased (Reid et al., 2013). Although no differences in clinical outcomes have yet been observed between AChE-selective (e.g., donepezil) and less selective drugs (e.g., rivastigmine inhibits both AChE and BuChE; Hogan, 2014), developing drugs inhibiting both enzymes might thus prove useful (Zimmermann, 2013). Overall, the existence of various forms of cholinesterase enzymes should be considered in the design of more efficient drugs.

Third, the spatial selectivity of AChEI action should be better controlled. The systemic administration of the current AChEIs makes it difficult to target their action to the brain and, more importantly, to areas affected by cholinergic depletion. One of the main risks is to trigger excessive upregulation of the cholinergic tone in relatively preserved areas like the striatum. This could have potentially undesirable

consequences such as basal ganglia-related motor disorders that are rarely induced by AChEIs alone but may occur more frequently when combined with antipsychotic drugs in some AD prescriptions (Shimizu et al., 2015). In order to boost their spatial efficacy, AChEI treatment could be combined with a localized electrical stimulation of the NBM neurons (Gratwicke et al., 2013). Such therapeutic tool could also ultimately answer to another issue of AChEI drugs: their temporal dynamics.

Indeed, a fourth area of improvement relates to the poor temporal resolution of AChEI treatment. The fundamental mechanism of action of these drugs consists in extending the availability of released ACh over longer periods than normal. On top of maybe transiently reducing the probability of further ACh release due to a higher likelihood of presynaptic autoreceptors activation, this could mask incoming phasic cholinergic signal onto postsynaptic neurons (Dumas and Newhouse, 2011; Hasselmo and Sarter, 2011). This is especially important because ACh function would differ depending on the time scale of its release, phasic or tonic (Hasselmo and Sarter, 2011). Indeed, these two modes of ACh release are each known to contribute in a specific way to learning and memory processes. As suggested above, brain stimulation could help mastering temporal dynamics of ACh availability but the step to the clinical trials is far from now. It is noteworthy that such considerations on cholinergic dynamics appear more adapted for symptomatic therapies but of limited interest for disease modifying therapies.

The above considerations suggest ways of improving AChEI drugs, with some being already actively explored. The most notable development in the domain of AChEI drugs is nonetheless the combination of their AChEI action with other beneficial effects from non-cholinergic drugs (e.g., anti-amyloid, antioxidant, anti-inflammatory). Given the multifactorial nature of the AD pathology, the concurrent use of an AChEI and another drug has already showed interesting results. Indeed, memantine, a drug targeting primarily NMDA receptors, is now sometimes successfully administered alongside an AChEI drug (Dantoine et al., 2006). More recently it has been shown that the serotoninergic antagonist

idalopirdine can potentiate the pro-cognitive effect of donepezil in moderate AD patients (Wilkinson et al., 2014). Several preclinical investigations also explored the benefits of various combinations of existing therapeutics in mouse models of AD (Jacobsen et al., 2014; Chumakov et al., 2015). Nowadays, drug combination is taken further by creating single molecules possessing the effects of several drugs acting simultaneously on different targets. This should lead to a lower risk of drug interactions, an easier control of the pharmacokinetics and an easier treatment compliance given a simpler drug schedule. Unsurprisingly, several of such 'multi-target directed ligands' are based on classic AChEI molecules like donepezil (Agis-Torres et al., 2014; Ismaili et al., 2016; Unzeta et al., 2016). For example, donecopride is a promising AChEI-serotoninergic antagonist hybrid molecule that can counteract scopolamine-induced amnesia in a working memory task (Rochais et al., 2015) and enhance object recognition memory (Lecoutey et al., 2014) in mice.

In conclusion, as the main option for AD treatment, drugs offering an AChEI action are still actively developed. However, increasing their potency is not simply a question of improving the level and duration of ACh availability. On top of combining complementary neuroprotective influences, either intrinsic to AChE or by combining compounds acting on different systems, future AChEI drugs should ideally display an adequate targeting of the cholinesterase enzymes hopefully alongside a refined spatio-temporal dynamics.

4.2. An old alternative: targeting cholinergic receptors

Alternatively to reducing the degradation rate of ACh, the activity elicited by the cholinergic system can be modulated via its target receptors. The nicotinic cholinergic receptors (nAChRs) are ionotropic receptors displaying a fast activation time adapted for mediating phasic release-associated cholinergic functions. As muscarinic cholinergic receptors (mAChRs; five subtypes: M1-5) are metabotropic, being

coupled with a G protein, they show a slower action but also longer-lasting, which is maybe more adapted to cholinergic functions mediated through tonic release.

Muscarinic receptors

M1 receptors are the most promising cholinergic targets for AD (Foster et al., 2014). M2, M3 and M4 receptors should not be targeted to avoid psychotic or peripheral side effects (Bymaster et al., 2003; Foster et al., 2014). M1 receptors are involved in cognition and underlying mechanisms (e.g., Anagnostaras et al., 2003; Dennis et al., 2016). Highly selective M1 agonists have been available only recently and their efficacy is still being assessed. Overall, they induce pro-cognitive effects in rodents (Ma et al., 2009; Lebois et al., 2010; Digby et al., 2012; Melancon et al., 2013), AD-model mice (Shirey et al., 2009) or humans (Nathan et al., 2013; Schneider et al., 2014). As M1 receptor density is also relatively preserved in AD (Mulugeta et al., 2003), drugs activating M1 receptors are overall less dependent on the current state of cholinergic neurodegeneration. It could be hypothesized that direct agonists, by binding to the same site than ACh (i.e., orthosteric site), could be more helpful late in the pathology when ACh is in short supply (Jiang et al., 2014). Alternatively, positive allosteric modulators offer several advantages thanks to their binding to a different site (i.e., allosteric) of M1 receptors than ACh (Melancon et al., 2013; Foster et al., 2014). First, at earlier stages of AD, these molecules might be better suited because instead of replacing ACh, they will potentiate the effect of its natural release (Jiang et al., 2014; Kruse et al., 2014). Second, these drugs would also respect the dynamics of the cholinergic signaling, which partly answers to above considerations for improving the temporal selectivity of the AChEIs. In addition to their symptomatic action, M1 receptor agonists are very promising because they have a neuroprotective, disease-modifying potential by reducing tau phosphorylation and production of Aβ (e.g., Beach et al., 2001; Caccamo et al., 2006). As Aβ can disrupt the M1 receptor function, M1-

mediated reduction of Aβ levels may initiate an interesting beneficial positive feedback loop (Fisher, 2012).

Nicotinic receptors

Along the development of AD, the cortical and hippocampal expression of several types of nAChRs is reduced (Guan et al., 2000; Sabbagh et al., 2006). Among those, α7 receptors are of particular interest because they participate in attentional and mnesic functions, as well as in synaptic plasticity (Fisher, 2012; Lombardo and Maskos, 2015; Echeverria et al., 2016). Furthermore, neurons expressing these receptors are especially vulnerable to AD (D'Andrea and Nagele, 2006) presumably via a "switch position" depending on Aβ levels (Buckingham et al., 2009; Ovsepian et al., 2015; Echeverria et al., 2016). At low dose, Aβ would activate α7 receptors and trigger neuroprotective intracellular mechanisms, while a higher concentration would prompt alternative intracellular pathways leading to neurotoxicity. Unlike M1 receptors though, α7 receptors might not protect against, but actually enhance, tau phosphorylation (Fisher, 2012). Therefore, despite some pro-cognitive effects observed in animals and humans (Hilt et al., 2009; Echeverria et al., 2011), the net effect of both beneficial and detrimental actions of α7 agonists is unclear (Anand et al., 2014). Several molecules are currently under test but α7 receptor-based therapies might yield less promising results than their M1 counterparts. In general, it must be recognized that recent clinical trials with cholinergic compounds have been disappointing at the level of efficiency on cognitive symptoms as well as undesirable side effects (Lewis et al., 2017; McArthur et al., 2010). This certainly emphasizes that we need a better understanding of the translational gap for therapeutics targeting nicotinic and muscarinic receptors.

5. As a conclusion, specific progresses are needed on early stages of AD

A convincing and exhaustive story of the etiology of AD has not been reached yet, supporting a multifactorial view of this disease. Cholinergic cells loss was first relegated to a late-phase consequence of the condition, whereas current research has revealed a multitude of plastic changes taking place much before the first overt cognitive symptoms. Clearly, several of these changes are of a cholinergic nature and seem to underlie compensatory mechanisms that efficiently delay conversion to AD. Unfortunately, advancing degeneration of the cholinergic basal forebrain progressively dismantles the compensatory mechanisms, mainly in the hippocampus and the cortex. Based on research in MCI and AD patients, it is however difficult to unravel the diversity and the exact role of cholinergic changes engaged in the maintenance of cognitive functions given the concomitance of amyloid and tau pathologies and their complex mutual interactions. Animal studies will certainly offer some valuable insight on this issue using, this time as a clear advantage, the large diversity of rodent models mimicking only limited aspects of early phases of this complex disease (Ashe and Zahs, 2010). Besides the study of cholinergic responses to the disease, there is also a crucial need to uncover the timeline and the nature of the 'precocious' deficits of the cholinergic system during normal aging (e.g., Schliebs and Arendt, 2011) as it is undeniably implicated in some aspects of AD etiology (disease superimposed on cholinergic decline) and the progression of the disease. In this regard, it would also be of great interest to study the cholinergic system in light of concepts such as brain resilience and cognitive reserve (e.g., Stern, 2012) focusing on factors leading to constitutively higher neuronal and/or synaptic density or increased ability to recruit alternative brain circuits. Both properties should alleviate the cognitive symptoms in AD and delay the conversion of MCI to AD (Mufson et al., 2016). Studies in rodents raised in enriched environments have already provided some interesting data showing a preventive effect of life enrichment on the cholinergic basal forebrain (Harati et al., 2013). Finally, characterizations of early clinical subgroups should be associated with the development of corresponding biological and cognitive biomarkers of easy use in

clinical settings. Tremendous technical progress has been made in this domain during the last few years regarding the detection of Aβ or cholinergic nuclei atrophy levels via structural imagery in humans, but non-invasive markers of the cholinergic activity, possibly more important than purely structural markers, are lacking. The battery of cognitive tasks used to categorize the patients as NCI, MCI or AD could be refined by the inclusion of new paradigms like pattern separation tasks (Stark et al., 2013). Functional markers of the cholinergic state could be further highlighted when combining these tasks with functional imagery techniques. Electroencephalography, especially, holds some interesting potential by allowing detection of frontal and hippocampal theta oscillations abnormalities during early phases of cognitive decline (Hamm et al., 2015).

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