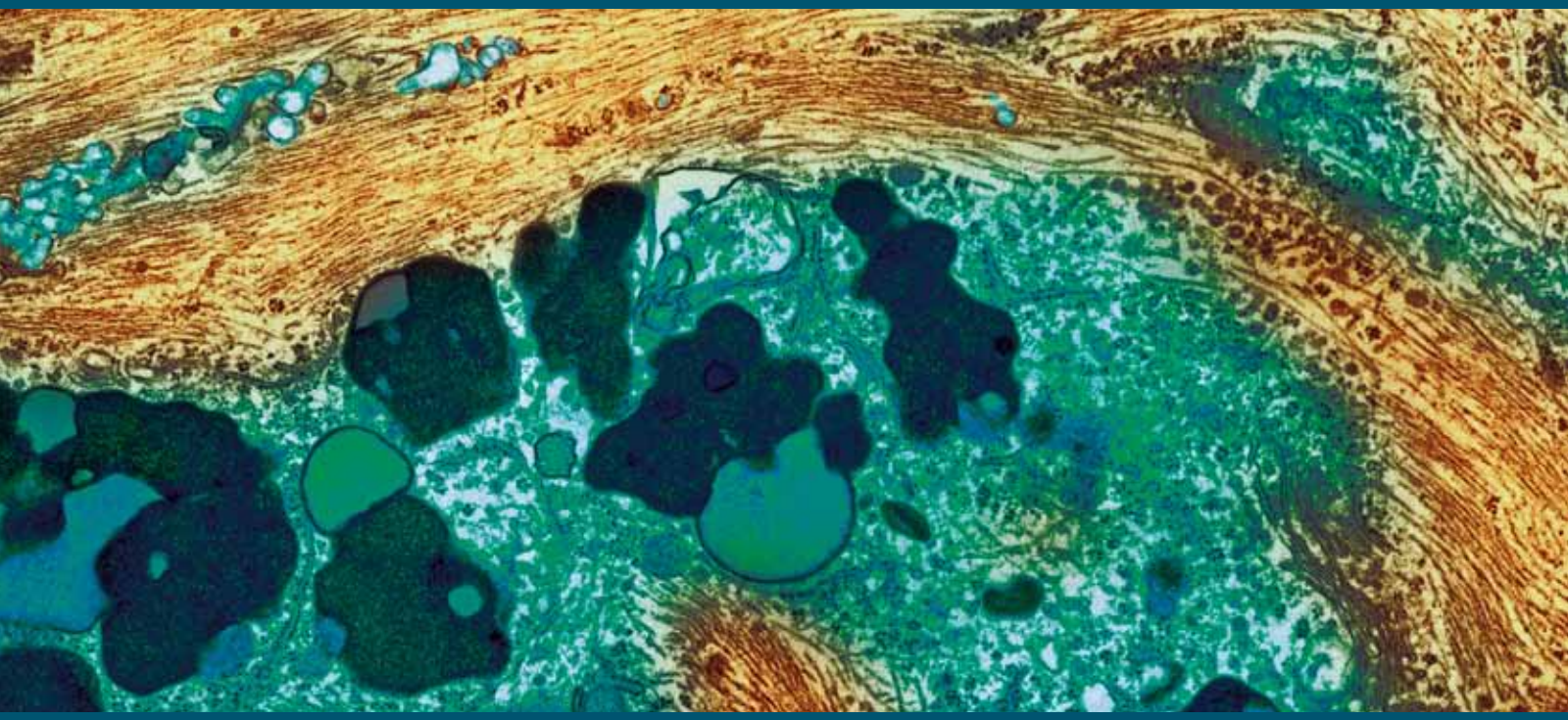
International Journal of Alzheimer's Disease

New Thinking on the Etiology and Pathogenesis of Late-Onset Alzheimer's Disease

Guest Editors: Alan P. Hudson, Brian J. Balin, Keith Crutcher, and Stephen Robinson



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Editorial Board

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Editorial

New Thinking on the Etiology and Pathogenesis of Late-Onset Alzheimer's Disease

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For many years, it has been accepted that the etiology of early-onset Alzheimer's disease (AD) is genetic in nature, with the relevant mutations involving genes encoding products that function in pathways dealing with processing of β-amyloid. The etiology of the far more prevalent late-onset AD is not primarily genetic in origin even though research over the last two decades has identified one or more genetic risk factors that predispose their bearers to development of the disease. Studies of late-onset AD have been strongly influenced by the amyloid cascade hypothesis. This influential hypothesis posits that plaques of β-amyloid (i.e., neuritic senile plaques, NSP) accumulate in the neuropil, where they eventually initiate a neuropathogenic process that engenders production of insoluble tangles of modified *tau* protein (i.e., neurofibrillar tangles, NFT), the ultimate result of which is progressive cognitive dysfunction. Firm and final diagnosis of the disease remains dependent on *postmortem* quantitation of the density and character of NSP and NFT in specific regions (e.g., hippocampus) of the affected brain. In addition, inflammation in the late-onset AD brain has been well documented, but this potentially important aspect of pathogenesis has not found a major place in the suite of factors thought to play primary roles in the neuropathogenic process.

Substantial evidence derived from clinical trials, animal model studies, and other sources indicates that late-onset AD cannot be explained solely or primarily by accumulating NSP and NFT despite the fact that these are the most obvious and consistent pathological features of this disease. Rather, the evidence strongly suggests that late-onset AD results from a complex interplay between genetic and environmental factors, most of which remain to be elucidated. In this special issue, we were interested particularly in papers that present alternative viewpoints concerning the complex etiology and pathogenic processes underlying late-onset AD. The solicitation targeted reports of primary research from different fields, including new genome sequence or structure data that shed light on the complex genetic background giving increased susceptibility to disease induction, new insights regarding relevant environmental influences that may contribute to that induction, and new studies focusing on the biochemistry and molecular genetics of AD. Further, we wanted review articles that summarized recent research developments that engender new views on the etiology and/or neuropathogenic mechanisms of AD as well as hypothesis-driven but evidence-based arguments regarding etiology and neuropathogenesis.

The first paper included in this special issue presents an interesting, and we think cogent, argument for a reconsideration of the amyloid cascade hypothesis. The authors of this review conclude that current evidence indicates that the production of NFT and NSP is independent of one another and that these are probably the products, not the cause, of the neurodegeneration that characterizes late-onset AD. The article presents a modified version of the hypothesis that provides a reasonable explanation of the pathogenesis of the disease.

Since firm diagnosis of late-onset AD depends on *postmortem* examination of the brain of affected individuals, a pressing need exists for identification of markers that can reliably indicate the presence of AD in individuals at much earlier stages of the disease induction process. The second paper included in this special issue presents new research indicating that delineation of certain aspects of the IL-10 genotype and expression of one membrane-located adenosine receptor might prove useful in identifying individuals with the early signs of dementia who are at high risk of progressing to incipient late-onset AD.

The final three papers accept the amyloid cascade hypothesis as a mechanistic explanation for the characteristic neuropathogenesis of late-onset AD, but each develops an interesting new idea concerning how the accumulation of β-amyloid at high levels might be initiated and maintained. The first of the three papers reviews the biochemical roles of the transglutaminase enzymes, the long-standing hypothesis that the activity of these enzymes contributes to oligomerization and accumulation of β-amyloid, and posits that specific inhibitors of the enzymes found in the CNS may prove to be effective therapeutic targets to ameliorate disease progression.

The next paper reports that when Aβ1-42 is infused into mice, the activity of a particular ABC transport system is impaired, leading to attenuated removal of β-amyloid from the brain. This intriguing observation suggests that the documented age-related decrease in the expression of that transporter may contribute to the accumulation of β-amyloid in the brain and thus of NSP formation. The final paper in this special issue is a study of the function(s) of a noncoding transcript from the BACE1-encoding gene, using a transgenic mouse model in which Aβ production is excessive. Previous work from this group had demonstrated that these noncoding transcripts stabilize the mRNA specifying BACE1, thereby increasing the production of insoluble β-amyloid. In the present study, knockdown-based inhibition of either the noncoding or BACE1-encoding transcript engendered decreased β-amyloid production and attenuated neuronal degeneration. These last three papers point to new ways of reducing the burden of NSPs in the brains of transgenic mice. The challenge now is to determine if these approaches can be applied to the brains of aging primates, and if so, whether reducing the burden of NSPs will be sufficient to slow the rate of cognitive decline.

AD and stroke together account for the bulk of cognitive impairment and mortality worldwide. As the risk factors and causes of stroke are becoming better understood, interventions have started to decrease the incidence of stroke (see, e.g., [1]). By contrast, despite three decades of intense research, the etiology of late-onset AD remains to be elucidated, and the incidence of this disease is steadily increasing (see, e.g., [2]). The present collection of papers illustrates that progress is being made on several fronts and indicates that many avenues of enquiry remain to be explored. We hope that these papers will stimulate new lines of thinking and experimentation, thereby hastening our understanding of this cruel and insidious disease.

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Research Article

The Pathogenesis of Alzheimer's Disease: A Reevaluation of the "Amyloid Cascade Hypothesis"

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The most influential theory to explain the pathogenesis of Alzheimer's disease (AD) has been the "Amyloid Cascade Hypothesis" (ACH) first formulated in 1992. The ACH proposes that the deposition of β-amyloid (Aβ) is the initial pathological event in AD leading to the formation of senile plaques (SPs) and then to neurofibrillary tangles (NFTs) death of neurons, and ultimately dementia. This paper examines two questions regarding the ACH: (1) is there a relationship between the pathogenesis of SPs and NFTs, and (2) what is the relationship of these lesions to disease pathogenesis? These questions are examined in relation to studies of the morphology and molecular determinants of SPs and NFTs, the effects of gene mutation, degeneration induced by head injury, the effects of experimentally induced brain lesions, transgenic studies, and the degeneration of anatomical pathways. It was concluded that SPs and NFTs develop independently and may be the products rather than the causes of neurodegeneration in AD. A modification to the ACH is proposed which may better explain the pathogenesis of AD, especially of late-onset cases of the disease.

1. Introduction

Ever since the first description of presenile dementia by Alzheimer in 1907 [1], senile plaques (SPs) and neurofibrillary tangles (NFTs) have been regarded as the "signature" pathological lesions of Alzheimer's disease (AD) [2–4]. AD became a nosological entity in 1910 and was named after Alzheimer by Kraepelin based on the clinical and pathological description of the original cases. Of the two original cases described by Alzheimer, however, both had numerous SPs but only one of the cases had significant numbers of NFTs [5], thus creating a controversy as to the relative significance of the two lesions that still persists today.

Studies of the molecular composition of the SPs played a critical role in the development of hypotheses as to the pathogenesis of AD. Hence, the discovery of β-amyloid (Aβ) as the most important molecular constituent of the SPs [6] resulted in the formulation of the "Amyloid Cascade Hypothesis" (ACH), the most important model of the molecular pathology of AD developed over the last 18 years [7]. Essentially, the ACH proposes that the deposition of Aβ (Figure 1) is the initial pathological event in the disease leading to the formation of NFTs, cell death, and ultimately dementia. Nevertheless, there are observations that are difficult to reconcile with the hypothesis. For example, in transgenic mice, genes overexpressing amyloid precursor protein (APP) do not produce the predicted cascade [8, 9]. Furthermore, SPs and NFTs appear to be separated in the brain both temporally [9, 10] and spatially [11]. The uncertainty as to the significance of SPs and NFTs in AD has led to alternative models being proposed, especially in late-onset cases, based on perturbation of vesicular trafficking at synapses, disruption of the cytoskeletal network, or the distribution of membrane cholesterol [12]. Some authors have even suggested that SPs/NFTs may be the reactive products of neurodegeneration, arising as a consequence of oxidative stress [13], and that the proteins involved in their formation are protective in function [14]. These observations suggest a more complex relationship between SPs and NFTs and the pathogenesis of AD and, therefore, that a reappraisal of the ACH may be necessary.

This paper examines two questions regarding the ACH: (1) is there a relationship between the pathogenesis of SPs and NFTs, and (2) what is the relationship of these lesions

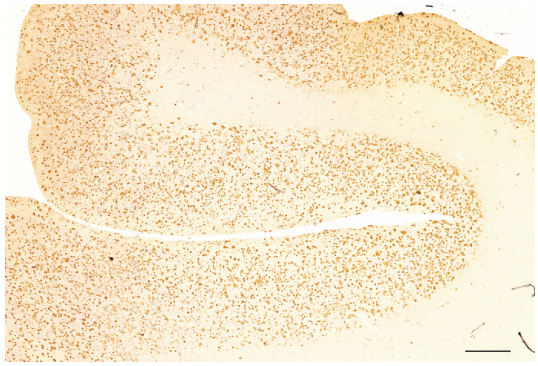

Figure 1: Extensive β-amyloid (Aβ) deposition in gyri of the temporal lobe in a case of Alzheimer's disease (AD) (Aβ immunohistochemistry, bar = 1 mm).

to disease pathogenesis? These questions are discussed with reference to (1) studies of the morphology and molecular composition of SPs and NFTs, (2) studies of the effects of gene mutation, (3) studies of head injury patients, (4) experimental studies involving brain lesions and transgenes, and (5) studies of the degeneration of anatomical pathways.

2. The "Amyloid Cascade Hypothesis" (ACH)

Two key observations resulted in the original formulation of the ACH [7] (Figure 2). First, the discovery of Aβ as the most important molecular constituent of the SPs [6] drew attention to the importance of these amyloid peptides in AD. Second, mutations of the *APP* gene [15, 16] and, subsequently, of the *presenilin* genes (*PSEN1/2*) [17, 18] were directly linked to cases of familial AD (FAD). Hence, the presence of Aβ within SPs was regarded as the residue of the effect of these pathogenic gene mutations and which, via the accumulation of toxic and insoluble Aβ peptides, led to cell death and dementia. Since the pathological phenotype of FAD is similar, apart from age of onset, to that of the more common late-onset, sporadic AD (SAD) [19–21], it was assumed that a similar mechanism, via genetic risk factors and/or environmental factors, could explain the pathogenesis of all cases of AD [22].

Evidence supporting the ACH comes from several sources. First, experiments using transgenic mice expressing high levels of APP result in Aβ deposition, synaptic loss, and gliosis [23]. Second, FAD caused by the substitution of valine by isoleucine at codon 717 of the *APP* gene also has significant numbers of NFTs thus supporting a link between APP and the cytoskeleton [24]. Third, cases linked to *PSEN1* have greater numbers of SPs and NFTs compared with cases of sporadic AD suggesting that *PSEN1* may increase tau deposition [25].

There have been several attempts to establish a mechanism by which the deposition of Aβ directly leads to the formation of NFTs but none have become universally accepted. First, Giasson et al. [26] concluded that Aβ promoted the formation of intracellular tau, although the mechanism of this interaction was uncertain. Second, attempts have been made to show that there is a synergistic

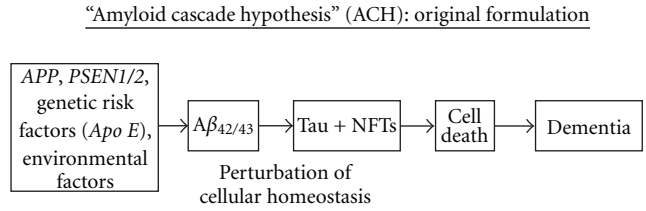

Figure 2: The original Amyloid Cascade Hypothesis (ACH) [7]. Aβ: β-amyloid, APOE: apolipoprotein E, APP: amyloid precursor protein, *PSEN1/2*: *presenilin genes 1 and 2*, NFTs: neurofibrillary tangles.

interaction between NFTs and Aβ [27, 28]. Third, when fetal rat hippocampal neurons and human cortical neurons were treated with Aβ, fibrillar forms of Aβ could apparently induce tau phosphorylation [29]. It was concluded that amyloid fibril formation might alter the phosphorylation state of tau resulting in the loss of microtubule-binding capacity. Fourth, Pérez et al. [30] showed that Aβ_{25-35} could induce the aggregation of tau proteins and that a decrease in aggregation of Aβ was induced by tau peptides. Hence, aggregation of tau may be associated with disassembly of Aβ which could explain the lack of spatial correlation of the SPs and NFTs [11].

3. Limitations of the ACH

There are two major objections regarding the ACH as originally formulated. First, SPs and NFTs may be reactive products resulting from neurodegeneration in AD rather than being its cause [31] and, second there is no generally accepted mechanism to explain how the deposition of Aβ leads to the formation of NFTs.

Is the Formation of Aβ and Tau a Reactive Process?. In survivors of head injury, APP is found in neuronal perikarya and in dystrophic neurites surrounding Aβ deposits, similar pathological features to AD [32]. The processing of APP into Aβ in these cases occurs within the synaptic terminal fold of the axons, the presence of glial cells not being necessary for the conversion. Hence, the production of APP may be a response of the brain to neuronal injury [32]. Subsequently, it was shown that specific neurons in the medial temporal lobe secreted large quantities of APP and that there were more APP-immunoreactive neurons in these areas in head injury patients [33]. Hence, increased expression of APP in head trauma cases may be an acute-phase response to neuronal injury [34], the overexpression of APP leading to the deposition of Aβ. This conclusion is supported by the observation that several acute-phase proteins are localised within the different morphological forms of Aβ deposit, including diffuse, primitive, and classic deposits (Table 1), for example, amyloid-P, complement factors, and α-antichymotrypsin [35]. Furthermore, Regland and Gottfries [36] proposed that, in AD, APP was involved in disease processes secondarily to help maintain cell function. Hence, APP may maintain neuronal growth and survival, and its putative neurotrophic action is supported by the

Table 1: Molecular composition of β-amyloid (Aβ) deposits in Alzheimer's disease (AD).

Deposit subtype	Molecular composition
Diffuse Aβ	APP (lacking C terminus), A$\beta_{42/43}$ apolipoprotein E, α_1-antichymotrypsin, HSPG, complement proteins (C1q, C_3, C_4), amyloid-P
Primitive Aβ	APP (N & C-terminal), A$\beta_{42/43}$, free and conjugated ubiquitin, PHF antigen, phosphorylated tau, chromogranin-A, bFGF, apolipoprotein E, interleukin-6
Classic Aβ	A$\beta_{42/43}$ "core", α-synuclein "ring", Aβ_{40}, actin, tubulin, phosphorylated tau, NF-protein, CAM, chromogranin-A "ring", α_2-macroglobulin, complement proteins "core", immunoglobulins "core", amyloid-P, α_1-antichymotrypsin, antitrypsin, antithrombin III, apolipoprotein E and D "core", bFGF, PrP, silicon/aluminium "core", interleukin-6 "ring"

Aβ: β-amyloid, APP: amyloid precursor protein, bFGF: basic fibroblast growth factor, CAM: cell adhesion molecule, HSPG: heparan suphate proteoglycan, NF-protein: neurofilament protein, PHF: paired helical filament, and PrP: prion protein.

observation that APP shares structural features with the precursor for epidermal growth factor [36]. Furthermore, NFTs may be part of the neurons response to injury [37]. Hence, studies of head injury patients support the hypothesis that Aβ deposition and NFTs formation could be reactive processes.

The results of animal experiments also suggest that the formation of Aβ may be a reactive process. Experimental lesions that damage the nucleus basalis in the brain of the rat elevate APP synthesis in the cerebral cortex suggesting that the production of APP could be a specific response to loss of functional innervation of the cortex [38]. Chemically induced lesions of the brain produce similar results. For example, lesions of the nucleus basalis using N-methyl D-aspartate (NMDA) elevate APP synthesis in cortical polysomes [38], and, in areas of brain damaged by kainite [39], APP695 was recorded in dystrophic neurites near to the lesion. In addition, intrathecal or intraparenchymal injections of a toxin induced APP in hippocampal neurons subsequent to neuronal damage [40].

Lesion experiments may also induce pathological changes implicated in the development of NFTs. Denervation of the dopamine pathways and septal lesions affecting both the cholinergic system and γ-aminobutyric acid (GABA) neurons projecting to the dentate gyrus results in a loss of dendritic microtubule-associated protein 2 (MAP2) and the appearance of tau-immunoreactive dentate gyrus granule cells [41]. It was concluded from this experiment that denervation causes transsynaptic changes in dentate gyrus neurons and that these changes may represent a precursor stage to NFTs formation.

Is the Formation of NFT Related to Aβ?. A number of studies have suggested that SPs and NFTs occur in distinct but independently distributed patterns in AD [11, 42]. Studies of the spatial patterns of SPs and NFTs show them to be clustered with, in a significant proportion of cortical areas, a regular distribution of the clusters parallel to the pia mater [43]. The clusters of SPs and NFTs, however, are distributed independently of each other, that is, neither in nor out of phase, which would not support a direct pathogenic link between them. In addition, SPs and NFTs appear to be separated in the brain temporally [10]. Indeed, in the entorhinal cortex, the NFTs may actually precede the appearance of SPs [9].

In transgenic experiments [44], the presence of *APP* mutations alone or in combination with *PSEN1* can induce Aβ deposits in normal brain, but apart from some evidence for hyperphosphorylated tau in neurites associated with the plaques, do not appear to induce tau pathology or a significant inflammatory response. Hence, the presence of tau transgenes in the form of a triple model appears to be necessary to replicate AD pathology.

4. Modification of the ACH

A modification of the original ACH which incorporates these concerns is presented in Figure 3. In this modified hypothesis, the essential trigger to the development of AD is ageing of the brain and associated risk factors such as head trauma, vascular disease, and systemic disease, collectively referred to as the "allostatic load" [45]. These factors exacerbate processes leading to cell death. As neurons degenerate, various proteins are upregulated leading to the formation of extracellular Aβ deposits and intracellular tau, the latter resulting in the development of NFTs. These reaction products may be toxic and initiate a further phase of secondary degeneration that accelerates the neuronal loss leading to dementia. In this modified hypothesis, genetic factors, rather than initiating disease, indirectly influence the formation and composition of peptides formed when neurons degenerate. Hence, the modified ACH incorporates information suggesting a more complex relationship between SPs and NFTs and proposes that the lesions are essentially reactive rather than causal.

5. Discussion

5.1. Predictions of the Modified ACH. The modified ACH suggests that it is ageing and the diseases associated with ageing that provide the "trigger" initiating the "cascade" of events leading to AD rather than the initial deposition of Aβ. The modified hypothesis makes a number of predictions. First, the hypothesis predicts that significant signs of neuronal degeneration in AD should precede those of Aβ deposition and the effect of Aβ is secondary rather than

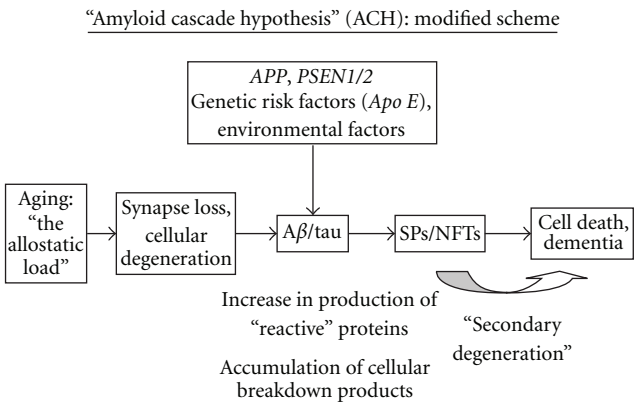

Figure 3: A modification of the Amyloid Cascade Hypothesis (ACH). Aβ: β-amyloid, APOE: apolipoprotein E, APP: amyloid precursor protein, *PSEN1/2*: *presenilin genes 1 and 2*, NFTs: neurofibrillary tangles, and SPs: senile plaques.

primary in causing neurodegeneration. Second, it predicts that the pathogeneses of SPs and NFTs are not directly linked and the two lesions essentially arise independently. Third, in transgenic experiments, the effect of the transgene will be age-dependent. In a model which incorporates an APP, V717I mutation, for example, there was an age-related loss of pyramidal neurons in the hippocampus CA sectors included at sites devoid of plaque deposition [46] consistent with this prediction.

5.2. Predictions of the Modified ACH. First, the modified hypothesis suggests that SAD is not a disease linked primarily to defective genes but a complex syndrome dependant on the rate of ageing and indirectly influenced by genetic risk factors and the environment. Second, the hypothesis questions whether the presence, distribution, and molecular determinants of SPs and/or NFTs (Table 1) should continue to play a primary role in the pathological diagnosis of AD. There are two problems that need to be considered. If SPs/NFTs are the products of brain degeneration and not its cause, then they may represent relatively late stages in pathogenesis. Hence, there may be cases of AD that are difficult to classify because they may have insufficient numbers of SPs and NFTs or exhibit early developmental stages of these pathologies. In addition, if SPs and NFTs represent the consequences of specific types of neurodegeneration rather than being characteristic of a particular disease, then there are likely to be many cases that show combinations of pathological features; that is, there will be a considerable degree of overlap between different disorders. Numerous examples of such cases have been reported in the literature, for example, dementia with Lewy bodies (DLB) with associated AD pathology, Creutzfeldt-Jakob disease (CJD) with AD, and Pick's disease (PkD) with AD, and these cases are often difficult to classify within the existing system [47]. Third, assuming that the role of SPs and NFTs in the pathogenesis of AD is at least controversial, should significant effort continue to be devoted to immunotherapy and other treatments designed to remove Aβ from the brain? Such treatments could be beneficial in limiting the degree of secondary degeneration induced by Aβ. Nevertheless, Aβ might be beneficial to the nervous system by promoting neurogenesis [48] and having a range of other protective functions [49]. Hence, excessive removal of Aβ could reduce chelation within the brain and result in enhanced oxidative stress [13].

6. Conclusions

Since 1992, the ACH has played an influential role in explaining the etiology and pathogenesis of Alzheimer's disease (AD). It proposes that the deposition of β-amyloid (Aβ) is the initial pathological event in AD leading to the formation of senile plaques (SPs), and then to neurofibrillary tangles (NFTs), death of neurons, and ultimately dementia. There are, however, two limitations of the ACH: (1) SP and NFT may develop independently, and (2) SPs and NFTs may be the products rather than the causes of neurodegeneration in AD. A modification to the ACH is proposed which may better explain the pathogenesis of AD, especially in late-onset cases of the disease. The modifications to the ACH make a number of predictions which could be usefully investigated.

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Review Article

Possible Role of the Transglutaminases in the Pathogenesis of Alzheimer's Disease and Other Neurodegenerative Diseases

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Transglutaminases are ubiquitous enzymes which catalyze posttranslational modifications of proteins. Recently, transglutaminase-catalyzed post-translational modification of proteins has been shown to be involved in the molecular mechanisms responsible for human diseases. Transglutaminase activity has been hypothesized to be involved also in the pathogenetic mechanisms responsible for several human neurodegenerative diseases. Alzheimer's disease and other neurodegenerative diseases, such as Parkinson's disease, supranuclear palsy, Huntington's disease, and other polyglutamine diseases, are characterized in part by aberrant cerebral transglutaminase activity and by increased cross-linked proteins in affected brains. This paper focuses on the possible molecular mechanisms by which transglutaminase activity could be involved in the pathogenesis of Alzheimer's disease and other neurodegenerative diseases, and on the possible therapeutic effects of selective transglutaminase inhibitors for the cure of patients with diseases characterized by aberrant transglutaminase activity.

1. Biochemistry of the Transglutaminases

Transglutaminases (TGs, E.C. 2.3.2.13) are a family of enzymes (Table 1) which catalyze irreversible posttranslational modifications of proteins. Examples of TG-catalyzed reactions include (I) acyl transfer between the γ-carboxamide group of a protein/polypeptide glutaminyl residue and the ε-amino group of a protein/polypeptide lysyl residue; (II) attachment of a polyamine to the γ-carboxamide of a glutaminyl residue; (III) deamidation of the γ-carboxamide group of a protein/polypeptide glutaminyl residue (Figure 1) [1, 2]. The reactions catalyzed by TGs occur by a two-step mechanism (Figure 2). The transamidating activity of TGs is activated by the binding of Ca^{2+}, which exposes an active-site cysteine residue. This cysteine residue reacts with the γ-carboxamide group of an incoming glutaminyl residue of a protein/peptide substrate to yield a thioacyl-enzyme intermediate and ammonia (Figure 2, Step 1). The thioacyl-enzyme intermediate then reacts with a nucleophilic primary amine substrate, resulting in the covalent attachment of the amine-containing donor to the substrate glutaminyl acceptor and regeneration of the cysteinyl residue at the active site (Figure 2, Step 2). If the primary amine is donated by the ε-amino group of a lysyl residue in a protein/polypeptide, an N^{ε}-(γ-L-glutamyl)-L-lysine (GGEL) isopeptide bond is formed (Figure 1, example (a)). On the other hand, if a polyamine or another primary amine (e.g., histamine) acts as the amine donor, a γ-glutamylpolyamine (or γ-glutamylamine) residue is formed (Figure 1, example (b)). It is also possible for a polyamine to act as an N,N-bis-(γ-L-glutamyl) polyamine bridge between two glutaminyl acceptor residues either on the same protein/polypeptide or between two proteins/polypeptides [3]. If there is no primary amine present, water may act as the attacking nucleophile, resulting in the deamidation of glutaminyl residues to glutamyl residues (Figure 1, example (c)). It is worthwhile noting that two of these reactions, in particular, the deamidation of peptides obtained from the digestion of the gliadin, a protein present in wheat, and the N^{ε}-(γ-L-glutamyl)-L-lysine (GGEL) isopeptide formation between these peptides and "tissue" transglutaminase (TG2 or tTG), have been recently shown to cause the formation of new antigenic epitopes which are responsible of immunological reactions during the celiac disease (CD), one of the most

Table 1: TG enzymes and their biological functions when known.

TG	Physiological role	Gene map location	Reference
Factor XIIIa	Blood clotting	6p24-25	[10]
TG 1 (Keratinocyte TG, kTG)	Skin differentiation	14q11.2	[11]
TG 2 (Tissue TG, tTG, cTG)	Apoptosis, cell adhesion, signal transduction	20q11-12	[12]
TG 3 (Epidermal TG, eTG)	Hair follicle differentiation	20p11.2	[13]
TG 4 (Prostate TG, pTG)	Suppression of sperm immunogenicity	3q21-2	[14]
TG 5 (TG X)	Epidermal differentiation	15q15.2	[15]
TG 6 (TG Y)	Unknown function	20p13	[15]
TG 7 (TG Z)	Unknown function	15q15.2	[15]

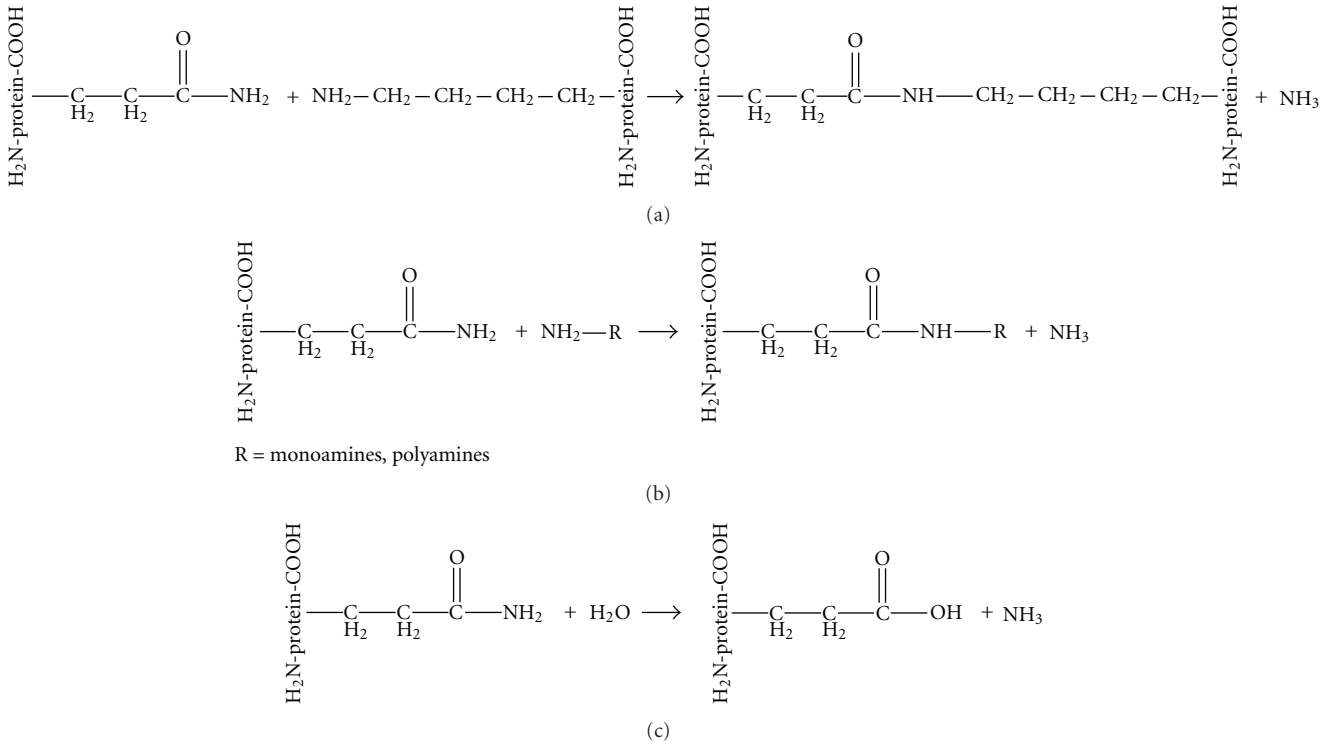

Figure 1: Transglutaminase-catalyzed reactions. Examples of TG-catalyzed reactions: (a) acyl transfer between the γ-carboxamide group of a protein/polypeptide glutaminyl residue and the ε-amino group of a protein/polypeptide lysyl residue; (b) attachment of a polyamine to the carboxamide group of a glutaminyl residue; (c) deamidation of the γ-carboxamide group of a protein/polypeptide glutaminyl residue.

common human autoimmune diseases [4, 5]. The reactions catalyzed by TGs occur with little change in free energy and hence should theoretically be reversible. However, under physiological conditions the cross linking reactions catalyzed by TGs are usually irreversible. This irreversibility partly results from the metabolic removal of ammonia from the system and from thermodynamic considerations resulting from altered protein conformation. Some scientific reports suggest that TGs may be able to catalyze the hydrolysis of N^{ε}-(γ-L-glutamyl)-L-lysine cross-links (GGEL) isopeptide bonds in some soluble cross-linked proteins. Furthermore, it is likely that TGs can catalyze the exchange of polyamines onto proteins [2]. In some TGs, other catalytic activities, such as the ability to hydrolyze GTP (or ATP) into GDP (or ADP) and inorganic phosphate, a protein disulfide isomerase activity, a serine/threonine kinase activity, and an esterification activity, are often present [6–9].

2. Multiple Biological Activities of the Transglutaminases

Experimental evidences indicate that some TGs are multifunctional proteins with distinct and regulated enzymatic activities. In fact, under physiological conditions, the transamidation activity of TGs is latent [16], while other activities, recently identified, could be present. For example, in some pathophysiological states, when the concentration of Ca^{2+} increases, the crosslinking activity of TGs may

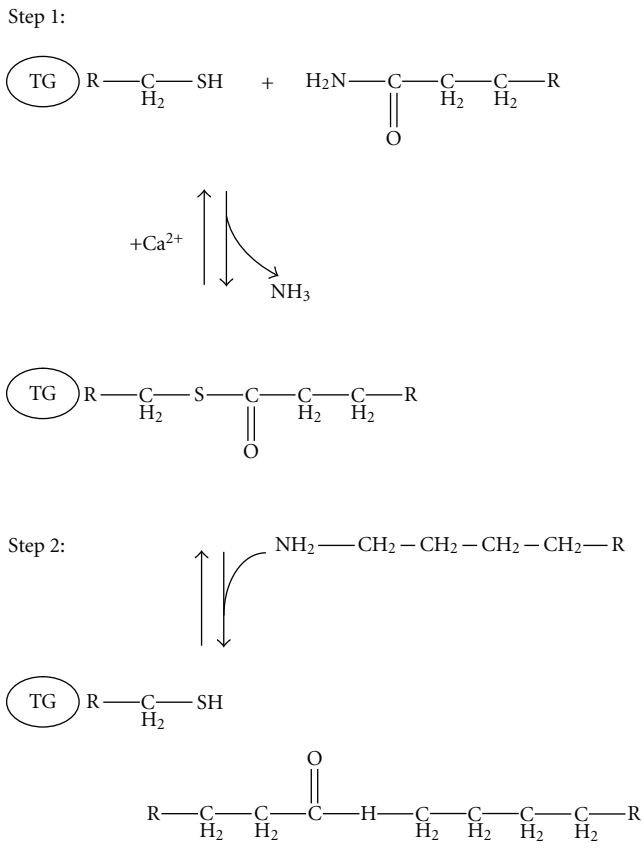

Figure 2: Schematic representation of a two-step transglutaminase reaction. Step 1: In the presence of Ca^{2+}, the active-site cysteine residue reacts with the γ-carboxamide group of an incoming glutaminyl residue of a protein/peptide substrate to yield a thioacyl-enzyme intermediate and ammonia. Step 2: The thioacyl-enzyme intermediate reacts with a nucleophilic primary amine substrate, resulting in the covalent attachment of the amine-containing donor to the substrate glutaminyl acceptor and regeneration of the cysteinyl residue at the active site. If the primary amine is donated by the ε-amino group of a lysyl residue in a protein/polypeptide, an N^{ε}-(γ-L-glutamyl)-L-lysine (GGEL) isopeptide bond is formed.

contribute to important biological processes. As previously described, one of the most intriguing properties of some TGs, such as TG2, is the ability to bind and hydrolyze GTP and, furthermore, to bind to GTP and Ca^{2+}. GTP and Ca^{2+} regulate its enzymatic activities, including protein cross-linking, in a reciprocal manner; the binding of Ca^{2+} inhibits GTP-binding and GTP-binding inhibits the transglutaminase cross-linking activity of the TG2 [6]. Interestingly, TG2 shows no sequence homology with heterotrimeric or low-molecular-weight G-proteins, but there is evidence that TG2 (TG2/Ghα) is involved in signal transduction, and, therefore, TG2/Ghα should also be classified as a large molecular weight G-protein. Other studies, along with ours, showed that TG2/Ghα can mediate the activation of phospholipase C (PLC) by the α_{1b}-adrenergic receptor [17] and can modulate adenylyl cyclase activity [18]. TG2/Ghα can also mediate the activation of the δ1 isoform of PLC and of maxi-K channels [19]. Interestingly, the signaling function of TG2/Ghα is preserved even with the mutagenic inactivation of its crosslinking activity by the mutation of the active site cysteine residue [20]. Evidence of a pathophysiological role of the TGs in cell signaling, in disulfide isomerase activity, and in other biological functions, is lacking to date.

3. Molecular Biology of the Transglutaminases

At least eight different TGs, distributed in the human body, have been identified (Table 1). Complex mechanisms regulating the gene expression of TGs, both at transcriptional and translational levels, determine a complex but precise distribution of these enzymes in a cell and/or a tissue [21]. Such complex gene expression reflects the physiological roles that these enzymes play in both the intracellular and extracellular compartments. In the nervous system, for example, several forms of TGs are simultaneously expressed [15, 22, 23]. Moreover, several alternative splice variants of TGs, mostly in the 3′-end region, have been identified. Interestingly, some of them are differently expressed in human pathologies, such as Alzheimer's disease (AD) [24]. On the basis of their ubiquitous expression and their biological roles, we may speculate that the absence of these enzymes would be lethal. However, this does not always seem to be the case, since, for example, null mutants of the TG2 are usually phenotypically normal at birth [25]. This result may be explained by the multiple expressions of other TG genes that could be substituting the missing isoform.

Bioinformatic studies have shown that the primary structures of human TGs share some identities in only few regions, such as the active site and the calcium-binding regions. However, high sequence conservation and, therefore, a high degree of preservation of residue secondary structure among TG2, TG3, and FXIIIa indicate that these TGs all share four-domain tertiary structures which could be similar to those of other TGs [26].

4. Transglutaminases and Alzheimer's Disease

Numerous scientific reports suggest that TG activity is involved in the pathogenesis of Alzheimer's disease and other neurodegenerative diseases. To date, however, definitive experimental findings about the role of these enzymes in the development of these neurological diseases have not yet been obtained. Protein aggregates in affected brain regions are histopathological hallmarks of Alzheimer's disease and many other neurodegenerative diseases [27]. More than 20 years ago, Selkoe et al. [28] suggested that TG activity might contribute to the formation of protein aggregates in AD brain. In support of this hypothesis, tau protein has been shown to be an excellent in vitro substrate of TGs [29–32], and GGEL cross-links have been found in the neurofibrillary tangles and paired helical filaments of AD brains [33, 34]. In addition to these experimental findings, it has been shown that TGs and transglutaminase-catalyzed cross-links colocalize with pathological lesions in Alzheimer's disease brain [34–36]. Interestingly, a recent work showed the presence of bis γ-glutamyl putrescine in

human CSF, which was increased in Huntington's disease (HD) CSF [37]. These are important experimental data which demonstrate that protein/peptides cross-links and protein/peptides cross-linking by polyamines do indeed occur in brain, and that these transglutaminase-catalyzed reaction products are increased in AD and HD brains. More recently, TG activity has been shown to induce amyloid β-protein oligomerization and aggregation at physiologic levels in vitro [38, 39]. By these molecular mechanisms, TGs could contribute to AD symptoms and progression [39]. Moreover, there is evidence that TGs also contribute to the formation of proteinaceous deposits in Parkinson's disease (PD) [40, 41] and in supranuclear palsy [42, 43]. To support the role of the TG activity in the pathogenesis of neurodegenerative diseases, expanded polyglutamine domains, present in HD and other neurodegenerative diseases caused by a CAG expansion in the affected gene (Table 2) [44], have been reported to be substrates of TG2 in vitro [45–47]. Therefore, aberrant TG activity could contribute to the pathogenesis of neurodegenerative diseases, including Alzheimer's disease and other neurodegenerative diseases, by different molecular mechanisms, as described in Figure 3. However, although all these studies suggest the possible involvement of the TGs in the formation of deposits of protein aggregates in neurodegenerative diseases, they do not indicate whether aberrant TG activity per se directly determines the disease's progression. In support of the hypothesis of a pathophysiological role for protein aggregates in neurodegenerative diseases, it is worth noting that the aggregate formation has been shown to inhibit the proteasome degradation of expanded polyglutamine proteins [48].

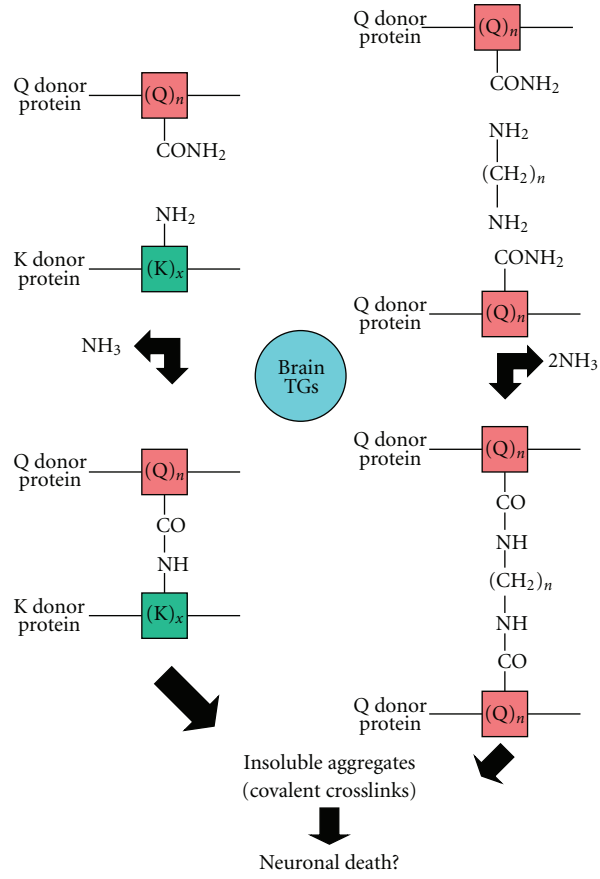

Figure 3: Possible mechanisms responsible for protein aggregate formation catalyzed by TGs.

5. Transglutaminases as Potential Therapeutic Targets of Neurodegenerative Diseases

Since up to now there have been no long-term effective treatments for human neurodegenerative diseases, then the possibility that selective TG inhibitors may be of clinical benefit has been seriously considered. In this respect, some encouraging results have been obtained with TG inhibitors in preliminary studies with different biological models of CAG-expansion diseases. For example, cystamine (Figure 4) is a potent in vitro inhibitor of enzymes that require an unmodified cysteine at the active site [59]. Inasmuch as TGs contain a crucial active-site cysteine, cystamine has the potential to inhibit these enzymes by disulfide interchange reactions. A disulfide interchange reaction results in the formation of cysteamine and a cysteamine-cysteine mixed disulfide residue at the active site. Recent studies have shown that cystamine decreases the number of protein inclusions in transfected cells expressing the atrophin protein containing a pathological-length polyglutamine domain, responsible for the Dentato-Rubro-Pallido-Luysian Atrophy (DRPLA) [60]. In other studies, cystamine administration to HD-transgenic mice resulted in an increase in life expectancy and amelioration of neurological symptoms [61, 62]. Neuronal inclusions were decreased in one of these studies [62]. Although all these scientific reports seem to support the

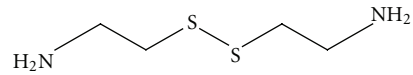

Figure 4: Chemical structure of cystamine.

hypothesis of a direct role of TG activity in the pathogenesis of the polyglutamine diseases, cystamine is also found to act in the HD-transgenic mice by mechanisms other than the inhibition of TGs, such as the inhibition of Caspases [63], suggesting that this compound can have an additive effect in the therapy of HD. The pharmacodynamics and the pharmacokinetics of cystamine, therefore, should be carefully investigated in order to confirm the same effectiveness in patients with neurodegenerative diseases. Another critical problem in the use of TG inhibitors in treating neurological diseases relates to the fact that, as previously reported, the human brain contains at least four TGs, including TG1, 2, 3 [23], and possibly TG6 [64], and a strong nonselective inhibitor of TGs might also inhibit plasma Factor XIIIa, causing a bleeding disorder. Therefore, from a number of standpoints, it would seem that a selective inhibitor, which discriminates between TGs, would be preferable to an indiscriminate TG inhibitor. In fact, although most of the TG activity in mouse brain, at least as assessed by

Table 2: List of polyglutamine (CAG-expansion) diseases.

Disease	Sites of neuropathology	CAG triplet number		Gene product (Intracellular localization of protein deposits)	Reference
		Normal	Disease		
Corea major or Huntington's disease (HD)	Striatum (medium spiny neurons) and cortex in late stage	6–35	36–121	Huntingtin(n, c)	[49]
Spinocerebellar Ataxia Type 1 (SCA1)	Cerebellar cortex (Purkinje cells), dentate nucleus, and brainstem	6–39	40–81	Ataxin-1 (n, c)	[50]
Spinocerebellar Ataxia Type 2 (SCA2)	Cerebellum, pontine nuclei, substantia nigra	15–29	35–64	Ataxin-2 (c)	[51]
Spinocerebellar Ataxia Type 3 (SCA3) or Machado-Joseph disease (MJD)	Substantia nigra, globus pallidus, pontine nucleus, cerebellar cortex	13–42	61–84	Ataxin-3 (c)	[52]
Spinocerebellar Ataxia Type 6 (SCA6)	Cerebellar and mild brainstem atrophy	4–18	21–30	Calcium channel subunit (α1A) (m)	[53]
Spinocerebellar Ataxia Type 7 (SCA7)	Photoreceptor and bipolar cells, cerebellar cortex, brainstem	7–17	37–130	Ataxin-7 (n)	[54]
Spinocerebellar Ataxia Type 12 (SCA12)	Cortical, cerebellar atrophy	7–32	41–78	Brain-specific regulatory subunit of protein phosphatase PP2A (?)	[55]
Spinocerebellar Ataxia Type 17 (SCA17)	Gliosis and neuronal loss in the Purkinje cell layer	29–42	46–63	TATA-binding protein (TBP) (n)	[56]
Spinobulbar Muscular Atrophy (SBMA) or Kennedy disease	Motor neurons (anterior horn cells, bulbar neurons) and dorsal root ganglia	11–34	40–62	Androgen receptor (n, c)	[57]
Dentatorubral-pallidoluysian atrophy (DRPLA)	Globus pallidus, dentatorubral and subthalamic nucleus	7–35	49–88	Atrophin (n, c)	[58]

Cellular localization: c, cytosolic; m, transmembrane; n, nuclear.

an assay that measures the incorporation of radioactive putrescine (amine donor) into N,N-dimethyl casein (amine acceptor), seems to be due to TG2 [65], no conclusive data has been obtained by TG2 gene knock-out experiments about the involvement of this TG in the development of the symptoms in HD-transgenic mice [66]. However, a recent scientific report showed that cystamine reduces aggregate formation in a mouse model of oculopharyngeal muscular dystrophy (OMPD), in which also the TG2 knockdown is capable to suppress the aggregation and the toxicity of the mutant protein PABPN1 [67], suggesting this compound as a possible therapeutic for OMPD.

6. Conclusions

Although many scientific reports have implicated aberrant TG activity in Alzheimer's disease and other neurodegenerative diseases, still today we are looking for data which could definitely confirm the direct involvement of TGs in the pathogenetic mechanisms responsible for these diseases. The use of inhibitors of TGs could be then useful for experimental approaches. To minimize the possible side effects, however, selective inhibitors of the TGs should be required in the future. Progress in this area of research may be achieved also through pharmacogenetic techniques.

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Research Article

Adenosine A_{2A} Receptor and IL-10 in Peripheral Blood Mononuclear Cells of Patients with Mild Cognitive Impairment

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Adenosine suppresses immune responses through the A_{2A} receptor ($A_{2A}R$). This study investigated the interleukin 10 (IL-10) genetic profile and the expression of $A_{2A}R$ in peripheral blood mononuclear cells (PBMCs) of patients with mild cognitive impairment (MCI), Alzheimer disease (AD), and age-matched controls to verify, if they may help distinguish different forms of cognitive decline. We analyzed the IL-10 genotype and the expression of $A_{2A}R$ in 41 subjects with AD, 10 with amnestic MCI (a-MCI), 49 with multiple cognitive domain MCI (mcd-MCI), and 46 controls. There was a significant linear increase in $A_{2A}R$ mRNA levels and $A_{2A}R$ density from mcd-MCI to a-MCI, with intermediate levels being found in AD. The IL-10 AA genotype frequency was 67% in a-MCI, 46% in AD, 35% in mcd-MCI, and 20% in controls. These data suggest that the assessment of the IL-10 genotype and the expression of $A_{2A}R$ in PBMCs may be a valuable means of differentiating between a-MCI and mcd-MCI.

1. Introduction

The purine ribonucleoside adenosine (Ado) is a naturally occurring metabolite that is ubiquitously distributed throughout the body as a metabolic intermediary. Ado accumulates in the extracellular space at the site of inflammation [1] in response to metabolic stress and cell damage, and there is evidence that it could play a key role in preserving homeostasis [2]. The physiological responses to Ado take place as a result of the binding and activation of different transmembrane receptors: high-affinity A_1 receptor (A_1R) and $A_{2A}R$, low-affinity $A_{2B}R$, or low-abundance A_3R. $A_{2A}R$ are found throughout the body, and an increasing wealth of evidence supports the idea that they represent the major immunoregulatory arm of the Ado receptor system, down-regulating inflammation [3–7] and also preventing beta amyloid ($A\beta$) induced synaptotoxicity [8].

A recent study showed that stimulation of $A_{2A}R$ represents a crucial second signal in the production of IL-10, the major anti-inflammatory cytokine [9].

Inflammation is accepted to be a feature of Alzheimer's disease (AD) [10, 11] and the pathogenesis of neurodegeneration has been at least in part attributed to the release of proinflammatory cytokines from brain resident cells [12, 13] and, although less consistently, from peripheral cells [14, 15]. Recently, specific risk sets of pro-inflammatory and anti-inflammatory alleles were found to be associated with AD at different ages of onset [16]. Such alleles also comprise the −1082 polymorphism (G/A substitution) in the IL-10 gene promoter region [17]. On the basis of these considerations, we investigated (1) the expression of the $A_{2A}R$ gene and $A_{2A}R$ protein levels in the peripheral blood mononuclear cells (PBMCs) of patients with mild cognitive impairment (MCI, the preclinical state of dementia), patients with outright AD and age-matched controls without cognitive impairment and (2) the relationship between the IL-10 genetic profile and the A_{2A} phenotype, since $A_{2A}R$ regulates IL-10 production.

2. Materials and Methods

2.1. Study Design. The study involved 41 AD patients (mean age 79.47 ± 6.30 years), 46 non demented age- and gender-matched healthy controls with similar educational levels (mean age 79.98 ± 6.36 years), and 59 subjects with MCI (mean age 78 ± 5.16 years). All of the patients were

Caucasians living in Northern Italy who were prospectively enrolled from a larger population of outpatients attending the Geriatric Unit of the Ospedale Maggiore IRCCS, University of Milan, Italy.

Probable AD was diagnosed using standard clinical procedures and DMS IV and NINCDS-ADRDA criteria [18]. Functional status was assessed by means of the Activities of Daily Living (ADL) and Instrumental Activities of Daily Living (IADL) scales and cognitive performance by means of the Mini-Mental State Examination (MMSE) and of an extensive neuropsychological evaluation. A computed tomography or magnetic resonance imaging scan corroborated the diagnosis of AD.

The criteria for the diagnosis of normal cognition were as follows: (1) no active neurological or psychiatric disorder; (2) no ongoing medical problems or related treatments interfering with cognitive function; (3) a normal neurological exam; (4) no psychoactive medications; (5) the ability to live and function independently in the community.

The MCI subjects were divided into two groups on the basis of their cognitive characteristics: amnestic MCI (a-MCI, 10 patients) and multiple cognitive domain MCI (mcd-MCI, 49 patients). The a-MCI subjects met the criteria described by Petersen [19]: only memory impairment (>1.5 SD above age- and education-specific norms) with no difficulties in any other area of cognitive functioning. The mcd-MCI subjects were impaired in at least two cognitive domains (>1 SD below the mean of an age- and education-matched population), their cognitive decline was self-reported or reported by a reliable informant, but they could not be diagnosed as having dementia. The cutoff score of 1 SD (less than the threshold used for a-MCI) was selected in order to ensure greater diagnostic sensitivity, albeit at the expense of diagnostic specificity. In fact the presence of more than one cognitive deficit and frequent initial IADL impairment may lead to mcd-MCI being mistaken for dementia, and the less restrictive criterion of >1 SD allows their better differentiation [20].

Blood from all patients and controls was collected in the morning between 8 and 9 AM, after a 6-hour fast.

None of the patients or controls was on treatment with dipyridamole or methylxanthines, and their caffeine consumption was about 80 mg/die (one cup of coffee) or less.

The risk of possible inflammatory processes was minimized by the fact that none of the subjects selected showed any clinical signs of inflammation (their body temperature was normal, and none of them had a concomitant inflammatory condition) and that they all had normal plasma albumin, transferring, and C-reactive protein levels. All of the subjects and their relatives gave their informed consent, and the study protocol was approved by the University Hospital's Ethics Committee.

2.2. Apolipoprotein E (ApoE) Genotyping. Whole blood was collected by means of a venipuncture into Vacutainers (Becton Dickinson Co., Rutherford, NJ). Genomic DNA was extracted using a previously described salting-out method [21], and its concentration and purity were determined by means of spectrophotometric analysis. The ApoE genotypes were determined by means of the PCR amplification of a 234 base-pair fragment of exon 4 of the ApoE gene, followed by digestion using Cfo1, as previously described [22]. Restriction patterns were revealed by means of 4% agarose gel electrophoresis.

2.3. Cytokine Genotyping. A polymerase chain reaction-sequence-specific primers (PCR-SSP) method was employed to assess IL-10 genotypes. The sequence in the promoter region of the IL-10 (polymorphism −1082) gene was amplified using the cytokine genotyping tray method (One Lambda, Canoga Park, CA, USA); the human β-globin gene was amplified as an internal control for the genomic DNA preparation. PCR conditions were indicated by the One Lambda PCR program (OLI-1), and the PCR products were visualized by electrophoresis in 2.5% agarose gel.

2.4. $A_{2A}R$ mRNA Expression. PBMCs were separated by density gradient using the Lympholyte-H kit (Cedarlane Laboratories Limited, Canada), and total RNA was isolated from 15×10^6 frozen cells using Chomczynski and Sacchi's modified method [23]. The concentration of total RNA was quantified by means of spectrophotometry, and 2 ug of RNA were reverse-transcribed using the M-MLV Reverse Transcriptase System and oligo (dT) (Clontech, Italy). The resulting cDNA was real-time amplified in a final volume of 50 μL. The mix contained 25 μL of 2x iQ SYBR Green Supermix (dNTPs, iTaq, and $MgCl_2$; Bio-Rad, Italy) and 300 mM of each primer ($A_{2A}R$: Forward 5′-GGCTGCCCCTACACATCATC-3′, Reverse 5′-GCCAGGTACATGAGCCAGAGA-3′). PCR was performed using a Chromo 4 instrument and analyzed using Opticon Monitor 2 (Celbio, Italy). All of the reactions were performed in duplicate, with thermal cycling conditions of 10 min at 95°C followed by 40 cycles at 95°C for 15 s, 56°C for 40 s, and 72C° for 30 s, with a ramp of 5C°/s. Real-time PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed simultaneously (GAPDH: Forward 5′-ATTCCACCCATGGCAAATTC3′, Reverse 5′-TGGGATTTCCATTGATGACAAG-3′).

The relative quantification of A_{2A} mRNA expression was carried out by using the comparative cycle threshold (Ct) method and the formula normalization ratio (NR) = $2^{-\Delta\Delta Ct}$. The ΔCt value of each sample was calculated as the Ct of the target gene minus the Ct of GAPDH, and then the ΔΔCt value was obtained as the difference between the ΔCt of the sample and the ΔCt of the calibrator [24]. According to this formula, the normalization ratio of the calibrator in each run is 1. The calibrator in each sample run was the same RNA extracted from a single healthy control and stored at −80°C.

2.5. A_{2A} Receptor Densities. PBMCs from 10 a-MCI, 27 AD, 20 mcd-MCI, and 20 controls subjects were available for protein expression. The cells were lyzed in Triton X-114 Tris buffer with a protease inhibitors cocktail (Sigma, Italy). Briefly, after centrifugation (12,000 rpm at 4°C), the supernatant was loaded onto a sucrose cushion buffer and

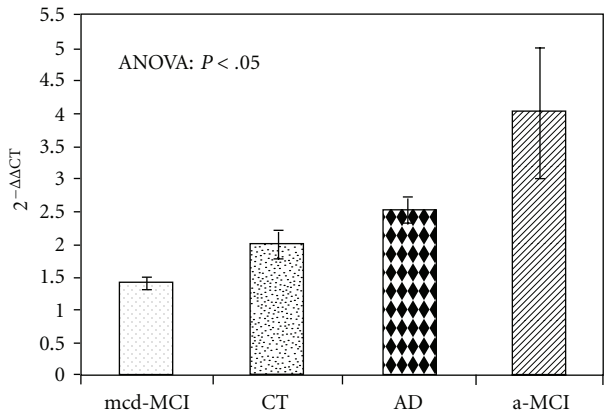

FIGURE 1: $A_{2A}R$ mRNA levels (mean values ± standard error) in PBMCs from 41 AD, 10 a-MCI, 49 mcd-MCI, and 46 control (CT) subjects quantified by the $2^{-\Delta\Delta Ct}$ method. Bonferroni post hoc: a-MCI versus controls and mcd-MCI patients ($P < .05$).

incubated at 37°C for 3 min. The samples were then centrifuged again in order to obtain two phases, and 10 μg of the total protein extracts of the aqueous phase were denatured by boiling for 10 min at 100°C in Laemmli SDS loading buffer, reduced with 5% mercaptoethanol and separated by 10% SDS polyacrylamide gel [25]. Electrophoresis was performed at 20–30 mA for 110 min, and the proteins were blotted onto a 0.45 μm polyvinylidene fluoride membrane (Immobilon, Millipore, Italy) at 90 V for 90 min at 4°C; after transfer, nonspecific binding was blocked for 120 min using 5% milk in phosphate buffer saline with 0.1% Tween 20 (PBST). The membranes were incubated overnight with polyclonal rabbit antihuman $A_{2A}R$ antibody (1 : 2000) (Calbiochem, Germany) and goat antirabbit IgG HRP (1 : 2000) (Bio-Sciences, Italy) and chemiluminescent substrate (Bio-Sciences, Italy). Purified $A_{2A}R$ (Chemicon, Italy) was loaded as positive control (one major band of about 45 kDa) and to evaluate the interassay variability (less than 10% in all experiments). To account for protein loading variation [26], the membranes were stripped and then reprobed with monoclonal antihuman GAPDH (1 : 8000) (Chemicon International, USA) and rabbit antimouse IgG-HRP (1 : 8000) (Sigma, Italy) and chemiluminescent substrate. All experiments were run in duplicate. Membranes were scanned, and the density of the protein bands was estimated by IM1D software (Bio-Sciences, Italy). The results were expressed as Arbitrary Units ($A_{2A}R$ /GAPDH ratio).

2.6. Statistical Analysis. The statistical analysis was performed using the SPSS statistical package (SPSS version 17, Chicago, IL). An outlier analysis was conducted before the comparisons. The differences in mRNA and protein levels, expressed as mean values ± standard error (SE), were calculated using the one-way analysis of variance followed by Bonferroni's post hoc test in the case of multiple comparisons. The genotype and allele distributions were evaluated using Pearson's χ^2. A P value <.05 was considered statistically significant.

3. Results

3.1. Distribution of the ApoE Genotype. The frequency of ApoE ε4 was 50% in a-MCI patients, 44% in AD patients, 38.8% in mcd-MCI patients, and 11% in control subjects. These frequencies are similar to those already reported [27].

3.2. Distribution of the IL-10 Genotype. The distribution of the −1082 (G/A) polymorphism was similar to that previously described [22]. In particular, the frequency of the AA genotype, associated with a higher risk of AD, was 67% in a-MCI, 46% in AD, 35% in mcd-MCI, and 20% in controls, respectively ($P = .014$).

3.3. A_{2A} Gene Expression and Receptor Densities. The qPCR analysis (Figure 1) revealed significantly higher ($P < .05$) $A_{2A}R$ mRNA levels in patients with a-MCI (3.77 ± 1.02) than in controls (2.12 ± 0.21) and in patients with mcd-MCI (1.42 ± 0.12). It is interesting to note that also $A_{2A}R$ density (Figures 2 and 3), expressed as $A_{2A}R$/GAPDH ratio, was significantly higher in patients with a-MCI (0.71 ± 0.17) than in controls (0.62 ± 0.05) and in patients with mcd-MCI (0.43 ± 0.05). Both gene expression (2.50 ± 0.22) and density (0.50 ± 0.06) of the $A_{2A}R$ in PBMCs of AD patients were not significantly different from those of the controls (Figures 1 and 3). When the data were stratified according to the presence or absence of the ApoE ε4 allele, $A_{2A}R$ gene expression and receptor density were similar both in the ε4 carriers and noncarriers.

Interestingly, the carriers of the G allele of the −1082 IL-10 polymorphism (GG and GA) showed the lowest level of $A_{2A}R$ mRNA, independently of cognitive status (1.7 ± 0.13 and 2.7 ± 0.36, in carriers versus noncarriers, resp.; $P = .002$).

4. Discussion

The results of this observational study show that the level of $A_{2A}R$ expression is higher in PBMCs from a-MCI than from mcd-MCI. When the data were stratified according to the presence or absence of the ApoE ε4 allele, $A_{2A}R$ gene expression and receptor density were similar both in the ε4 carriers and noncarriers. This indicates that the ApoE ε4 allele, a major genetic risk factor for late onset sporadic AD [28–30], does not appear to participate in the modulation of this gene.

The small number of patients and the lack of $A_{2A}R$ measurements in the cerebrospinal fluid do not allow us to draw any conclusion on the relationship between peripheral $A_{2A}R$ modulation and Ado metabolism in the brain. Yet the finding of an upregulation of $A_{2A}R$ in the PBMCs of subjects with a-MCI but not with AD seems to fit with the results of a previous study [31] demonstrating that in the brain cortex the increased expression of both $A_{2A}R$ and A_1R is mainly an early event in the pathogenesis of AD [32]. One possible explanation of our finding is that a second event, which is required for the development of AD in a-MCI subjects, subsequently intervenes to downregulate $A_{2A}R$ expression

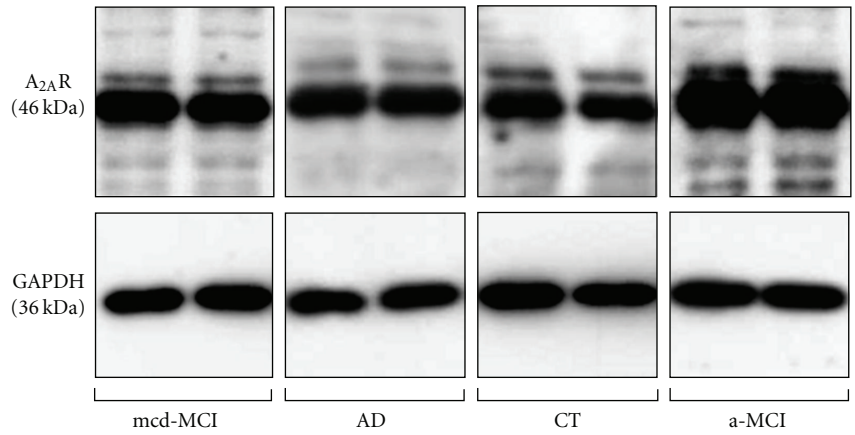

Figure 2: Paradigmatic example of the Western blot analysis of the $A_{2A}R$ densities in PBMC extract from one subject belonging to the AD, a-MCI, mcd-MCI, or control (CT) group, run in duplicate.

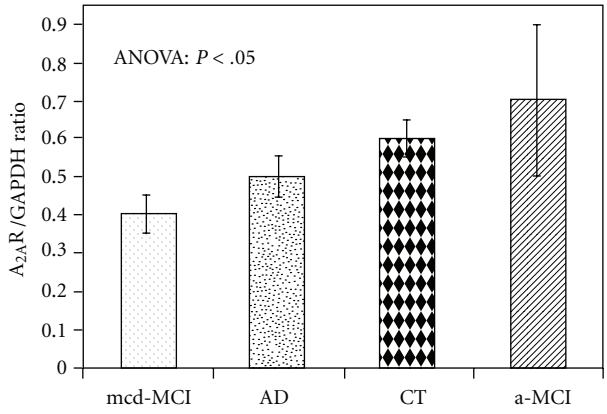

Figure 3: $A_{2A}R$ densities (mean values ± standard error) in PBMCs from 27 AD, 10 a-MCI, 20 mcd-MCI, and 20 control (CT) subjects expressed as $A_{2A}R$/GAPDH ratio. Bonferroni post hoc: a-MCI versus controls and mcd-MCI patients ($P < .05$).

in PBMCs by a negative feedback mechanism and thus counteracts the anti-inflammatory effect of Ado. Therefore, within the multifactorial pathogenesis of AD, there may be a specific time-window for the involvement of the Ado system.

It is still unclear whether and how peripheral inflammation acts on the AD brain; there are, however, several lines of evidence in favour of its contribution to the disease process. First, AD patients who also have short-term peripheral infections undergo a sudden decline in cognitive status [33]; second, plasma levels of alpha1-antichymotrypsin, interleukin-6, and C-reactive protein are increased before the clinical onset of various types of dementia, including AD [34]; third, the signs of inflammation observed in the brain of AD patients are comparable to those seen in peripheral inflammatory reactions and are likely to have a strong cytotoxic effect on neurons [35]; fourth, individuals who have levels of Aβ and tau aggregates similar to those of AD patients, but yet do not develop dementia, exhibit lesser signs of inflammation [36].

The lack of a significant difference in $A_{2A}R$ expression between AD subjects and controls is consistent with the data of former studies showing that also cytokine production is increased in PBMCs of MCI but not AD subjects [15, 37]. Thus, a peripheral inflammatory response seems to be involved only in the early stage of the disease and appears to be lost in overt AD.

In our patients the IL-10 low-producer AA genotype, which also seemed to be associated with the highest mRNA levels of $A_{2A}R$, was more frequent in the a-MCI group. IL-10, a powerful anti-inflammatory cytokine, maps to chromosome 1 between 1q31 and 1q32, is highly polymorphic, and its production is correlated to biallelic polymorphisms at position −1082 (G to A). In previous studies, we not only described a significantly greater prevalence of the IL-10 −1082 AA low-producer genotype in subjects with overt AD as well as in subjects with a-MCI who eventually progressed to AD [37], but we also found a reduced IL-10 generation in PBMCs from these patients after Aβ stimulation [22]. Interestingly a report on centenarians, in whom an exceptionally long lifespan is held to reflect the combined influence of lifestyle choices and genetic factors, demonstrated that longevity is significantly associated with the IL-10 high-producer GG genotype [38].

Limitations of this observational study are the small number of enrolled subjects, and that it was possible to evaluate the correlation between the IL-10 genetic profile and gene expression only for $A_{2A}R$. However, if the findings of this study are confirmed by a prospective study with a larger number of subjects, they can have a potential clinical application and provide further insight into the pathogenesis of AD. The evaluation of $A_{2A}R$ expression in the periphery could be a valuable means to differentiate mcd-MCI and a-MCI subjects. Also, it could be hypothesized that the overall

risk of developing AD is governed by a multifactorial "susceptibility profile" related not only to gene variants but also to alterations in the signalling pathway of molecules, such as adenosine, that play a key role in neuroinflammation.

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Research Article

Beta-Amyloid Downregulates MDR1-P-Glycoprotein (Abcb1) Expression at the Blood-Brain Barrier in Mice

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Neurovascular dysfunction is an important component of Alzheimer's disease, leading to reduced clearance across the blood-brain barrier and accumulation of neurotoxic β-amyloid (Aβ) peptides in the brain. It has been shown that the ABC transport protein P-glycoprotein (P-gp, ABCB1) is involved in the export of Aβ from the brain into the blood. To determine whether Aβ influences the expression of key Aβ transporters, we studied the effects of 1-day subcutaneous Aβ1-40 and Aβ1-42 administration via Alzet mini-osmotic pumps on P-gp, BCRP, LRP1, and RAGE expression in the brain of 90-day-old male FVB mice. Our results demonstrate significantly reduced P-gp, LRP1, and RAGE mRNA expression in mice treated with Aβ1-42 compared to controls, while BCRP expression was not affected. The expression of the four proteins was unchanged in mice treated with Aβ1-40 or reverse-sequence peptides. These findings indicate that, in addition to the age-related decrease of P-gp expression, Aβ1-42 itself downregulates the expression of P-gp and other Aβ-transporters, which could exacerbate the intracerebral accumulation of Aβ and thereby accelerate neurodegeneration in Alzheimer's disease and cerebral β-amyloid angiopathy.

1. Introduction

Burgeoning evidence implicates cerebrovascular dysfunction in the cognitive decline and neurodegeneration that characterize Alzheimer's disease (AD) [1, 2]. Impaired clearance of β-amyloid (Aβ) across the blood-brain barrier (BBB) and the senescence of the cerebrovascular system are thought to initiate processes that contribute to neurodegeneration [1, 3]. The transport of Aβ across the BBB comprises a two-step process involving transport through the abluminal (brain side) and then through the luminal (blood side) plasma membranes of the endothelial cells. The low-density lipoprotein receptor-related protein-1 (LRP1) mediates the incorporation of brain-derived Aβ peptides into the endothelial cells at the abluminal membrane [4, 5]. On the other hand, the receptor for advanced glycation end products (RAGE) is considered to be a major influx transporter of peripherally derived (blood-borne) Aβ into the endothelial cells of the cerebral vasculature [6, 7]. The ABC transporter P-glycoprotein (P-gp, ABCB1) is highly expressed at the luminal side of the endothelial cells that form the BBB, and may play a key role in the transport of Aβ peptides (either brain or blood derived) into the blood [8]. *In vitro* and *in vivo* experiments have shown that P-gp is an active transport protein for Aβ1-40 and Aβ1-42 [9–14]. These observations suggest that diminished P-gp expression due to increasing age, genetic, or environmental factors may lead to impaired Aβ clearance, followed by the accelerated accumulation of intracerebral Aβ and eventually the development of AD.

An autopsy study of nondemented elderly humans found that vessels with Aβ accumulation within their walls (cerebral amyloid angiopathy, CAA) showed little or no endothelial P-gp expression whereas unaffected capillaries had high P-gp expression, leading to the hypothesis that Aβ leads to a downregulation of P-gp expression whereas P-gp might be upregulated in the endothelium of capillaries lacking Aβ accumulation in an attempt to compensate for the loss of P-gp in amyloid-bearing vessels [11]. Thus, the aim of

the present study is to investigate the effect of Aβ administration on the expression of P-gp and other Aβ transporters in endothelial cells of the cerebral vasculature.

2. Material and Methods

2.1. Laboratory Animals. 90 day-old male FVB wildtype mice (purchased from Taconic, Hudson, NY, USA) weighing approximately 25 g were used. Animal care and experimental conditions were conducted in compliance with protocols approved by the local Animal Care and Use Committee.

2.2. Preparation and Subcutaneous Implantation of ALZET Mini-Osmotic Pumps. 10–12 animals per group were used for the systemic administration of Aβ1-40 (DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV), Aβ1-42 (DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA), or reverse Aβ40-1 (VVGGVMLGIIAGKNSGVDEAFFVLKQHHVEYGSDHRFEAD) or reverse Aβ42-1 (AIVVGGVMLGIIAGKNSGVDEAFFVLKQHHVEYGSDHRFEAD). All peptides were purchased from AnaSpec Inc. (Fremont, CA, USA).

1 mg of each peptide was dissolved in 4 μL DMSO and mixed with 2 mL isotonic NaCl solution, yielding a final Aβ concentration of 0.5 μg/μL. 200 μL (=100 μg peptide) of this solution were used to fill the Alzet mini-osmotic pumps. 200 μL of isotonic NaCl solution served as vehicle control.

ALZET mini-osmotic pumps (model 2001D; DURECT Corporation, Cupertino, USA) were implanted subcutaneously using a midscapular incision. Pumps delivered synthetic, human-type sequence Aβ1-40, Aβ1-42, reverse Aβ40-1, Aβ42-1 peptide, or isotonic NaCl solution, respectively. After a 2-hour start-up transient, the pumps infuse at a constant pumping rate of 8 μLh^{-1}.

After 26 h mice were sacrificed by cervical dislocation and the brains were immediately removed. One hemisphere was shock-frozen in liquid nitrogen and stored at −80°C until use. The other hemisphere was fixed with neutral-buffered 4.5% formalin and was embedded in paraffin.

2.3. Real-Time PCR Analyses. For real-time PCR analysis, RNA was isolated from deep-frozen brain tissue using the *RNeasy Mini* kit form Qiagen according to the manufacturer's instructions (Qiagen, Hilden, Germany). Reverse transcription of total-RNA was performed by the *TaqMan reverse transcription* kit using random hexamer primers (Applied Biosystems, Darmstadt, Germany). Quantitation of RNA (cDNA) levels for Abcb1a, Abcg2 (Mm00496364_m1), LRP1 (Mm00464608_m1), as well as RAGE (Mm00545815_m1) and 18SrRNA (part # 4319413E) for normalization was performed by real-time PCR on a 7900HT TaqMan system (Applied Biosystems) using predeveloped TaqMan assays (Assays on Demand, Applied Biosystems). All PCR reactions were performed in 10 μL reactions using a PCR master mix (2x concentrated) containing 45 mmol/L Tris-HCl (pH 8.4), 115 mmol/L KCl, 7 mmol/L $MgCl_2$, 460-μmol/L dNTPs, 9% glycerol, 2.3% ROX reference dye (Invitrogen, Paisley, Scotland) and 0.035 U/mL Platinum Taq DNA polymerase (Invitrogen). Thermal cycler conditions were as follows: (50°C, 2 min; 95°C, 10 min followed by 40 cycles of 95°C, 15 s and 60°C, 1 min).

Expression analysis was performed by the comparative $\Delta\Delta$Ct-method using the expression of 18SrRNA as reference gene.

2.4. Immunohistochemistry. For immunohistochemistry, brain tissues were fixed in neutral-buffered, isotonic 4.5% formalin and embedded in paraffin. 1 μm-thick tissue sections were cut, mounted on slides and dried overnight at 60°C. For staining, the biotin-streptavidin immunoperoxidase method with polymer detection system-HRP was used. Automated immunohistochemical staining was performed using the BOND-MAX (Leica) staining systems using pre-diluted antibodies to murine P-gp (clone C219, dilution 1 : 25, pH 9.0, Enzo Life Sciences) and BCRP (clone BxP53, dilution 1 : 50, pH 9.0, Enzo Life Sciences).

Negative controls for each treatment were processed without primary antibody.

2.5. Image Acquisition and Analysis. Digital images were captured using the Zeiss Mirax Scan Desk slide scanner (Carl Zeiss MicroImaging GmbH, Göttingen, Germany; Plan-Apochromat 20x/0.8, pixel resolution: 0.37 μm). To quantitate P-gp expression, image analysis algorithms were developed using ImageJ (version 1.43 q, Research Services Branch, National Institute of Mental Health/National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih.gov/ij/), which allowed the identification and analysis of immunolabeled cerebral capillaries in digital images. The amount of P-gp-specific labelling in each animal was determined in ten high-power fields (x40) of the brain cortex, the staining intensity for cortical P-gp of a single animal was the mean of all measurements.

2.6. Statistical Analysis. Statistical analysis was performed using Microsoft Excel and GraphPad Prism 5.01 (GraphPad, San Diego, USA) software using the ΔCt expression values. The statistical tests employed are indicated in the respective sections/figure legends. If the data are represented as boxplots, the whiskers indicate the 10th and 90th percentiles.

3. Results

First, mRNA expression for P-gp (Abcb1a), BCRP (Abcg2), LRP1, and RAGE was measured by real-time PCR in mice treated with Aβ1-42, vehicle control, or the reverse protein (Aβ42-1) for 24 h using subcutaneously transplanted ALZET pumps. Transporter expression was detected in all samples for Abcb1a and Abcb2 while analysis of LRP1, and RAGE failed in one Aβ1-42-treated mouse. Significant changes in the expression of Abcb1a, LRP1 and RAGE were detected in mice given Aβ1-42. While transporter expression was not significantly altered between the control perfusion group and the mice treated with equal amounts of the reverse Aβ protein, animals treated with Aβ1-42 had significantly reduced Abcb1a levels in brain (reduced by 63 ± 24% [mean ± SD]) compared to mice given the vehicle control, and

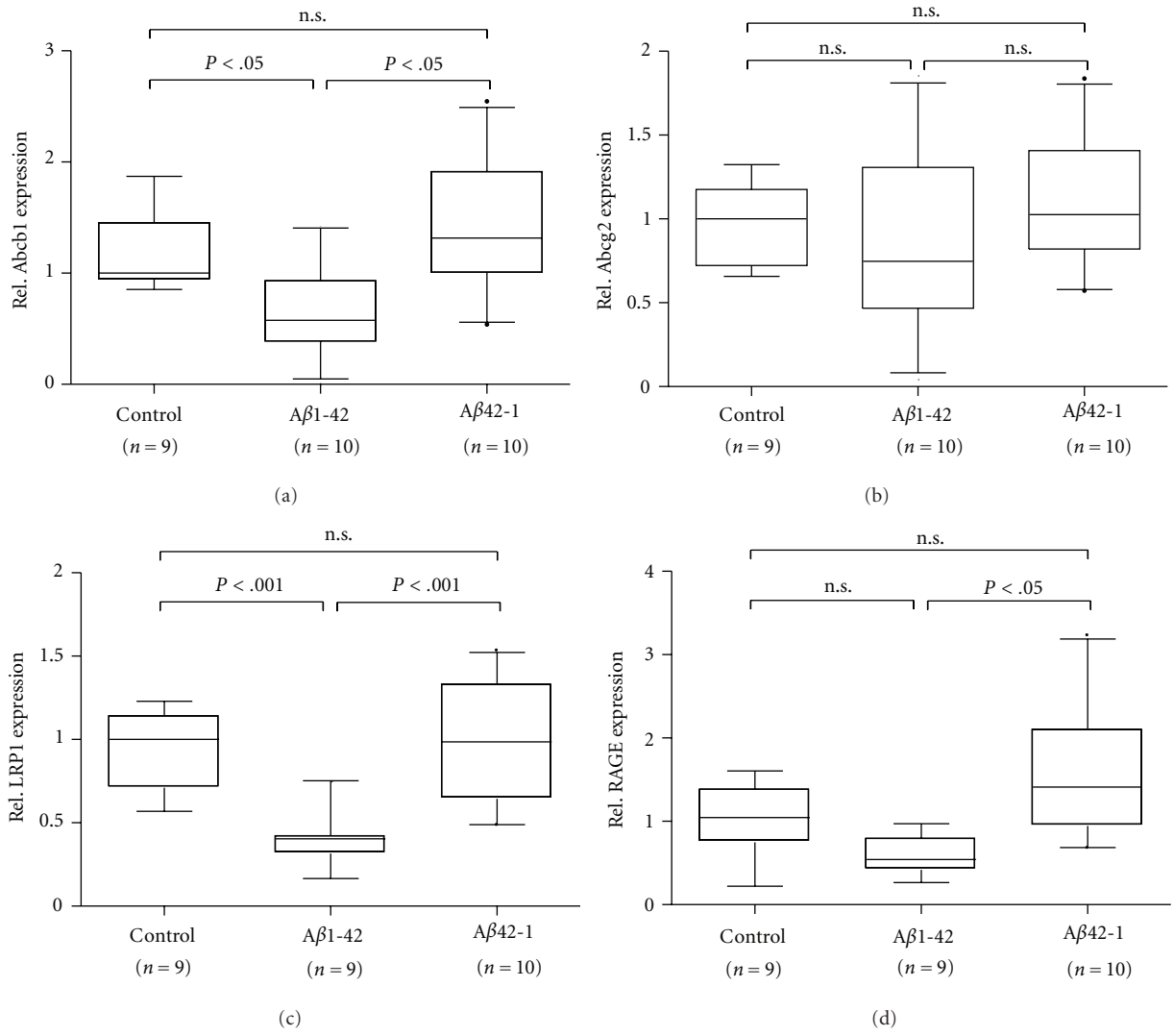


Figure 1: mRNA expression of Abcb1a (a), Abcg2 (b), LRP1 (c), and RAGE (d) in murine brain. Mice were treated with β-amyloid 1-42 (Aβ1-42), reverse β-amyloid 42-1 (Aβ42-1) and vehicle control (control) using ALZET pumps for 24 h (4 μg/h). mRNA expression of each transporter was normalized to 18S rRNA expression and values are depicted in relation to the median expression in vehicle perfused mice. Statistical analysis was performed by one-way ANOVA followed by Newmann-Keuls Multiple Comparison test.

by 54 ± 30% compared to mice given the reverse-sequence protein), LRP1 levels (reduced by 57 ± 17%) compared to vehicle controls and by 60 ± 17% compared to the reverse protein controls); and RAGE levels (reduced by 65 ± 15% compared to the reverse protein controls, no significant difference relative to vehicle controls Figures 1(a), 1(c), and 1(d)). In contrast, expression of Abcg2 remained unchanged in these samples (Figure 1(b)).

In a second experiment, mice were treated with Aβ1-40 in a similar manner. Here, no significant changes were detected in any of the four proteins; however, the expression of RAGE tended to be lower compared to the vehicle control samples (Figures 2(a)–2(d)).

At the protein level, P-gp and BCRP were detectable in endothelial cells of brain vessels in all animals using immunohistochemistry (Figures 3(a) and 3(b)). However, quantitatively no changes of P-gp or BCRP could be detected after administration of Aβ1-42 or Aβ1-40 in comparison to the reverse Aβ peptides or vehicle control, respectively (data not shown).

4. Discussion

There is growing evidence that an alteration of the transport of Aβ peptides across the BBB plays a crucial role in the pathogenesis of AD and CAA. In addition to other transport proteins such as LRP1 and RAGE, the multidrug resistance transport protein P-gp is known to eliminate Aβ1-40 and Aβ1-42 from the brain [12–14]. In nondemented elderly humans, Aβ deposition is inversely correlated with brain capillary P-gp expression [10]. Interestingly, investigations of the correlation between CAA and P-gp expression indicate a loss of P-gp in vessels with abundant cerebrovascular Aβ accumulation. In this study, CAA was

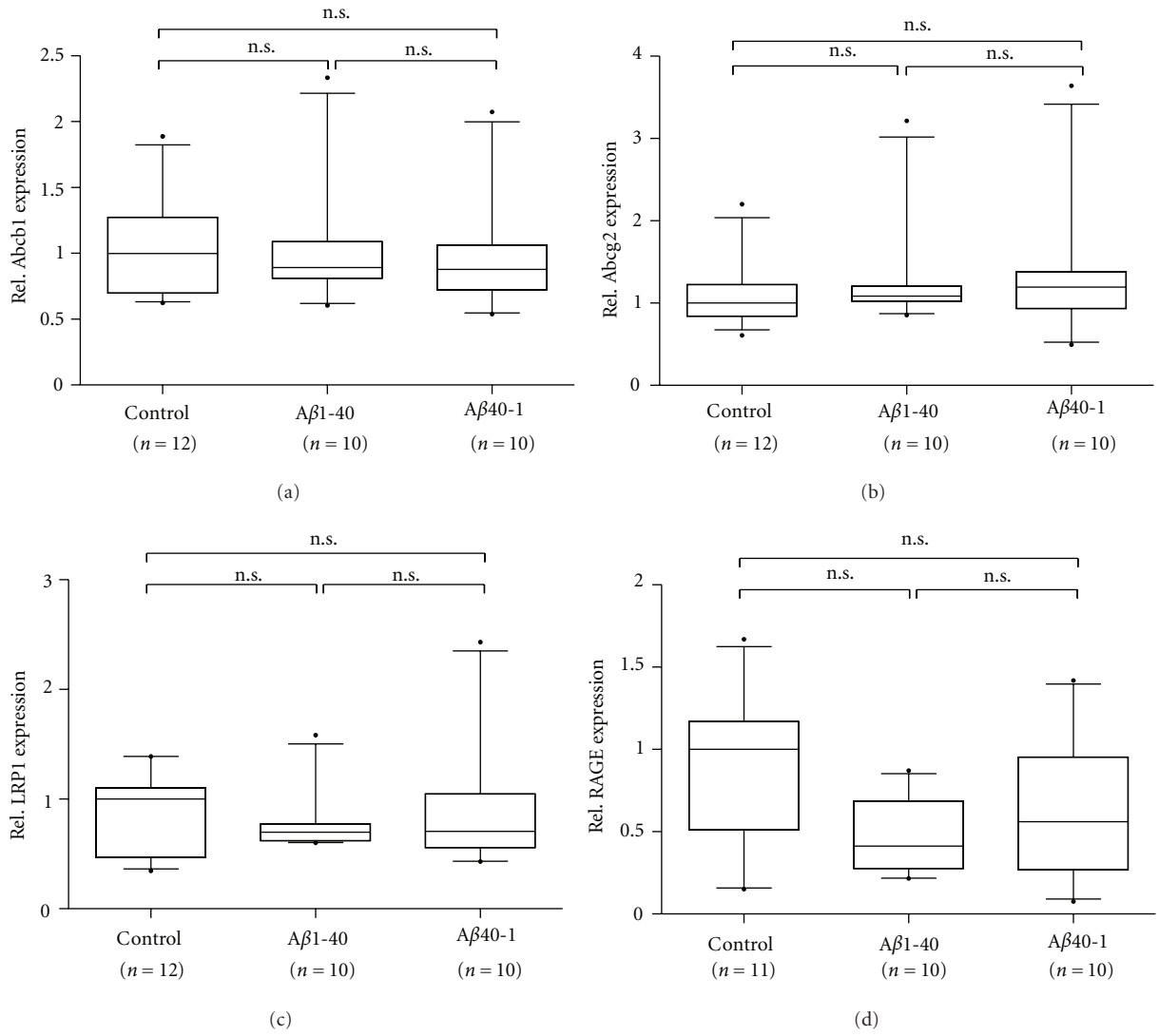


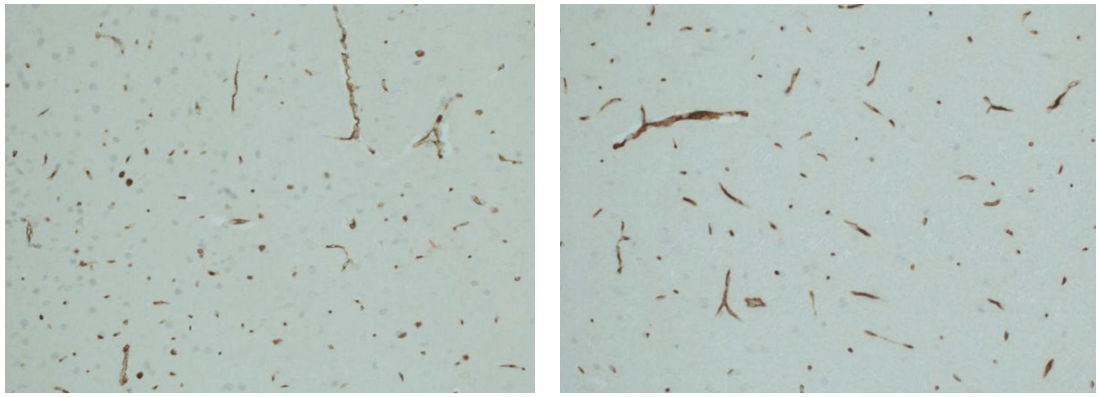
Figure 2: mRNA expression of Abcb1a (a), Abcg2 (b), LRP1 (c) and RAGE (d) in murine brain. Mice were treated with β-amyloid 1-40 (Aβ1-40), reverse-sequence β-amyloid 40-1 (Aβ40-1) and vehicle control (control) using ALZET pumps for 24 h (4 μg/h). mRNA expression of each transporter was normalized to 18S rRNA expression, and values are depicted in relation to the median expression in vehicle-perfused mice. Statistical analysis was performed by one-way ANOVA followed by Newmann-Keuls Multiple Comparison test.

primarily found in small arteries, but in the advanced stages of CAA, the capillaries also were affected [11], leading to the question of how Aβ itself affects P-gp expression in the endothelial cells that are a key component of the BBB.

The present study found that peripherally administered Aβ1-42 leads to a decrease of P-gp expression in vivo in mouse brains at the mRNA level. This finding is in concordance with the results of Hartz et al., who reported that P-gp expression and transport activity are compromised in brain capillaries isolated from hAPP transgenic mice that exhibit accumulation of human-type Aβ within the brain [14]. Thus, it can be hypothesized that the compromising effect of Aβ on P-gp expression leads to a further increase of Aβ accumulation, thus initiating a vicious circle that exacerbates the progression of the disease.

LRP1 is localized at the brain-side membrane of capillaries; as a receptor for Aβ, LRP1 mediates the transport of the peptide from the brain into the endothelial cells. LRP1 expression was reported to be reduced during normal aging in animals and in AD patients associated with accumulation of Aβ1-40 and Aβ1-42 within the cerebral vessels [4, 15, 16]. Our results revealed significantly reduced levels of LRP1 mRNA in mice treated with Aβ1-42, confirming the lowering effect of Aβ on LRP1 expression. However, 12-week-old hAPP mice revealed slightly increased LRP1 expression whereas RAGE was unchanged, suggesting the critical limiting step in Aβ brain clearance could be P-gp-mediated Aβ transport across the luminal endothelial membrane into the blood [14].

RAGE is normally expressed at the luminal side of the endothelial cells, and mediates the transport of Aβ1-40 and



(a) (b)

Figure 3: Immunohistochemical expression of P-gp (a) and BCRP (b) in endothelial cells of mice brain capillaries (original magnification ×200).

Aβ1-42 from blood into the endothelium [6]. In AD and AD models, cerebrovascular RAGE was shown to be increased [17]. Our results show that mRNA levels were significantly reduced in mice treated with Aβ1-42 in comparison to the reverse-sequence peptide. Relative to the vehicle control, RAGE mRNA expression showed a trend to be reduced by Aβ1-42, but did not reach statistical significance. However, our findings are in concordance with the observation that the expression of RAGE in Brown-Norway/Fischer rats decreases in early stages of Aβ accumulation and increases with advanced age [18]. This suggests that an increase of RAGE expression plays a role in the later stages but not in the initiation of AD.

Our results show that Aβ1-42, but not Aβ1-40, inhibits P-gp, LRP1 and RAGE expression at the BBB. Under physiological conditions, the ratio of Aβ1-42 to Aβ1-40 in the brain is about 1 : 10 [19]. Thus, the amount of Aβ1-40 in our pumps might have been too low to achieve sufficient effects on P-gp expression at the capillary endothelial cells. However, since the aggregative ability and the neurotoxicity of Aβ1-42 are much greater than those of Aβ1-40, Aβ1-42 plays a more important role in the pathogenesis of AD [19, 20]. In this regard, Aβ1-42 was reported to induce free radical-related oxidative stress and neurodegeneration in AD brains [21]. Interestingly, in contrast to Aβ1-40, the concentration of free Aβ1-42 in cerebrospinal fluid is lower in patients with AD, suggesting that Aβ1-42 might be preferentially incorporated into growing plaques [20].

In our study, the decrease of P-gp expression was observed at the mRNA level but not at the protein level using immunohistochemistry. This might be due to the short period during which Aβ is present within the blood, leading to acute effects of Aβ on transcription processes that were not reflected in changes of protein expression within this timeframe. Possibly, a longer treatment period is necessary to address this question.

Like P-gp, BCRP is localized at the luminal site of vascular endothelial cells and contributes to the integrity of the blood brain barrier (BBB), thus protecting the brain parenchyma from the damaging effects of xenobiotics [22]. P-gp and BCRP share extensive similarities regarding their substrate specificity, and the list of substrates of BCRP has been steadily expanding since its discovery [23]. Using a human brain endothelial cell line (hCMEC/D3) Aβ1-40 has been shown to be a substrate of BCRP [24]. Xiong et al. reported that BCRP mediates the transport of Aβ in BCRP-overexpressing cell lines, and that Aβ accumulates in the brains of BCRP-knockout mice. On the other hand, BCRP expression was increased in brain tissue of AD cases [25]. However, in the present study we found BCRP expression to be unchanged by circulating Aβ. This is in agreement with the findings on young hAPP mice, where no evidence was found for BCRP-mediated Aβ transport in brain capillaries [14]. Thus, it can be hypothesised that BCRP, like RAGE, might not be involved in the early stages of AD, but rather may play a role in more advanced stages of the disease.

5. Conclusions

These findings lend further support for the involvement of the ABC transport protein P-gp in the transport of Aβ across the BBB. Specifically, circulating Aβ itself lowers P-gp expression at the BBB, leading to a circulus vitiosus that triggers further Aβ accumulation. The activity of P-gp is well known to be modulated pharmacologically by a range of commonly used drugs [26–28]. Thus, restoring P-gp function could be a novel therapeutic strategy to protect the brain from Aβ accumulation, and thus could be a tool to prevent and/or slow the progression of CNS disorders such as Alzheimer's disease.

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Research Article

Knockdown of BACE1-AS Nonprotein-Coding Transcript Modulates Beta-Amyloid-Related Hippocampal Neurogenesis

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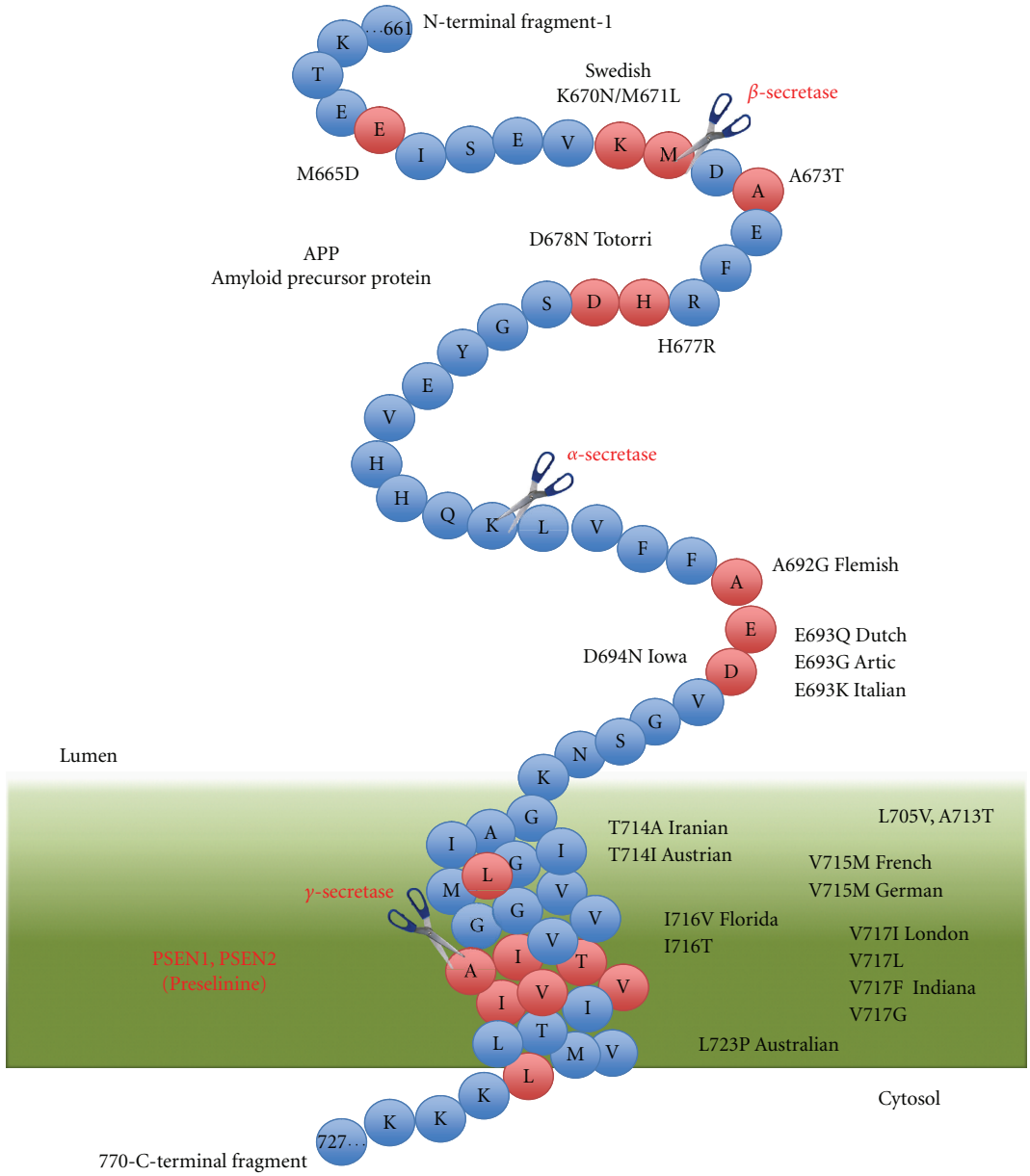
Background. Alzheimer's disease (AD) is a devastating neurological disorder and the main cause of dementia in the elderly population worldwide. Adult neurogenesis appears to be upregulated very early in AD pathogenesis in response to some specific aggregates of beta-amyloid (Aβ) peptides, exhausting the neuronal stem cell pools in the brain. Previously, we characterized a conserved nonprotein-coding antisense transcript for β-secretase-1 (BACE1), a critical enzyme in AD pathophysiology. We showed that the BACE1-antisense transcript (BACE1-AS) is markedly upregulated in brain samples from AD patients and promotes the stability of the (sense) BACE1 transcript. In the current paper, we examine the relationship between BACE1, BACE1-AS, adult neurogenesis markers, and amyloid plaque formation in amyloid precursor protein (APP) transgenic mice (Tg-19959) of various ages. *Results*. Consistent with previous publications in other APP overexpressing mouse models, we found adult neurogenesis markers to be noticeably upregulated in Tg-19959 mice very early in the development of the disease. Knockdown of either one of BACE1 or BACE1-AS transcripts by continuous infusion of locked nucleic acid- (LNA-) modified siRNAs into the third ventricle over the period of two weeks caused concordant downregulation of both transcripts in Tg-19959 mice. Downregulation of BACE1 mRNA was followed by reduction of BACE1 protein and insoluble Aβ. Modulation of BACE1 and BACE1-AS transcripts also altered oligomeric Aβ aggregation pattern, which was in turn associated with an increase in neurogenesis markers at the RNA and protein level. *Conclusion*. We found alterations in the RNA and protein concentrations of several adult neurogenesis markers, as well as non-protein-coding BACE1-AS transcripts, in parallel with the course of β-amyloid synthesis and aggregation in the brain of Tg15999 mice. In addition, by knocking down BACE1 or BACE1-AS (thereby reducing Aβ production and plaque deposition), we were able to modulate expression of these neurogenesis markers. Our findings suggest a distortion of adult neurogenesis that is associated with Aβ production very early in amyloid pathogenesis. We believe that these alterations, at the molecular level, could prove useful as novel therapeutic targets and/or as early biomarkers of AD.

1. Background

Alzheimer's disease is the most common form of dementia characterized by progressive impairment of cognition and short-term memory loss. The deposition of Aβ 1–42 in senile plaques is an established feature of AD neuropathology. Different species of Aβ have been shown to have toxic effects *in vitro* and *in vivo*; however, controversy still exists about whether the amyloidogenic pathway is the initiating mechanism for AD pathogenesis, or a mere consequence of other pathogenic events. Several human amyloid precursor protein (APP) mutations, as well as mutations in Presenilin-1 and Presenilin-2 were identified as genetic causes of familial AD [1] (Figure 1). These mutations related to familial and



A: alanine; D: aspartic acid; E: glutamic acid; F: phenylalanine; G: glycine; H: histidine; I: isoleucine; K: lysine; L: leucine; M: methionine; N: asparagine; P: proline; Q: glutamine; R: arginine; S: serine; T: threonine; V: valine; Y: tyrosine.
Aβ 1–42 sequence: DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
Aβ 1–40 sequence: DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA

Figure 1: Amyloid precursor protein (APP) cleavage and mutation. The present diagram represent APP, a transmembrane protein precursor of Aβ peptide, with the three principal cleavage site (β-secretase or BACE, α-secretase, and γ-secretase) and several known mutations.

early onset AD formed the basis for the amyloid cascade hypothesis to explain the pathogenesis of AD. β-secretase (BACE1) is the rate-limiting enzyme responsible for the production of Aβ peptides. Oligomeric forms of Aβ 1–42 can induce other components of AD pathophysiology, such as mitochondrial dysfunction [2, 3], Tau hyperphosphorylation (Tau hypothesis) [4], NMDAR endocytosis (channel hypothesis), excessive calcium influx (calcium hypothesis) [5, 6], synaptic dysfunction [7], neuronal stress [8], apoptosis (neurovascular hypothesis) [9], and aberrant neurogenesis [10, 11]. Therefore, BACE1 seems to be a critical component of many pathologic pathways, as its dysregulation can lead to excessive production of Aβ 1–42, formation of oligomeric Aβ species, and development of amyloid plaques.

Pluripotent neural stem cells (NSC) are present in the embryonic as well as adult brain [12–16]. Neural stem cells can be isolated from various parts of the brain and expanded *in vitro*, in the presence of mitogenic factors such

as epidermal growth factor (EGF) or basic fibroblast growth factor (bFGF) [17]. However, NSC remain quiescent, or are able to differentiate only to glial cells, in most parts of the adult nervous system with the exception of two regions, defined as neurogenic niches, where new neurons are actively generated, *in vivo*. Neurogenic niches, comprising the subventricular zone (SVZ) and subgranular zone of the hippocampus, contain active stem cells that differentiate towards neurons in the postnatal mammalian brain throughout their life under normal physiologic conditions [18, 19]. In the neurogenic niches, NSC are able to self-renew, proliferate, differentiate towards neurons, and incorporate into the neuronal circuitry [20]. Some reports indicate age-related decreases in adult neurogenesis that might be associated with a decline in hippocampus-dependent learning [18, 19, 21–23]. As memory loss and impaired learning are two of the most pronounced behavioural symptoms of AD, it seems likely that changes in neurogenesis could play a role in the progression of the disease. Increased neurogenesis has been described in neuronal stem cells treated with Aβ peptides *in vitro* [10], in animal models of AD [11] and in AD patients [24]. Interestingly, in the J20 APP transgenic mouse model of AD the observed *in vivo* effect of Aβ peptides on neurogenesis is mainly age dependent. In J20 mice, hippocampal neurogenesis is greatly enhanced at a young age, long before manifestation of any other AD-related symptoms, and dramatically reduced in older animals, when amyloid plaques are present and cognitive impairment is apparent [11]. Therefore, adult neurogenesis seems to correlate with the production of Aβ peptides and the accumulation of specific aggregates of Aβ in the brain. We sought to determine whether another mouse model of AD (Tg-19959) exhibits alterations in adult hippocampal neurogenesis markers and if so, to relate these changes with the levels of Aβ production and aggregation. Lastly, sought to determine whether we could modify these changes by targeting non-protein-coding RNAs.

Non-protein-coding RNAs (ncRNAs) appear to play an instrumental, but largely abandoned, role in human complex disorders [25]. Natural antisense transcripts are a group of regulatory RNAs, transcribed from the opposite strand of other (sense) genes [26]. Antisense transcripts are mainly ncRNAs and have been reported to regulate gene expression, either positively or negatively, through various mechanisms [27]. The discovery of BACE1 as the rate-limiting enzyme in the production of Aβ peptides has made it a prime therapeutic target for AD. Recently, we have identified and characterized one sense-antisense pair, BACE1 and its antisense partner BACE1-AS, and demonstrated a critical role of this non-protein-coding natural antisense transcript in AD [28]. BACE1-AS has the potential to participate in an ncRNA network that serves to fine-tune BACE1 protein output in the nervous system.

In the current study, we investigated mechanisms behind Aβ-induced hippocampal neurogenesis in the Tg-19959 model of AD. In particular, we investigated the extended effects of BACE1-AS transcripts on amyloid production, aggregation and plaque formation and their effects in adult neurogenesis *in vivo*.

2. Results and Discussion

2.1. Neurogenesis over Time in Tg-19959 Mice. We assessed adult neurogenesis in hippocampus at different time points throughout the life of Tg-19959 mice. We measured cell proliferation (by Ki67 staining) in the dentate gyrus of wildtype and Tg-19959 mice at different ages and observed a significant increase in NSC proliferation in young Tg-19959 animals (5 weeks old), as compared to age-matched wildtype mice (Figures 2(a) and 2(b)). This enhanced proliferation of NSC persisted over time as the animals aged (Figure 2(c)). We also quantified neural stem cell and progenitor markers in these mice over time, both at the RNA and protein level by RT-PCR and Western blot, respectively. Nestin is an intermediate filament protein used as a marker of pluripotent neural progenitors. It is expressed in type 1 and 2 cells in the adult hippocampus and it would represent the amount of neural progenitors and early neuronal progenitors in adult neurogenesis. Doublecortin (DCX) is a microtubule-associated protein expressed in neuronal committed precursor cells and immature neurons (type 2 and 3 cells) in the adult hippocampus [29]. The quantification of nestin and doublecortin levels in the hippocampus indicated a significant increase of both markers in Tg-19959, as compared to wild type mice both at the RNA (Figure 2(d)) and protein level (Figure 2(e)). Increased levels of neurogenesis markers were maintained over time as the animals aged. These data are consistent with the increased proliferation observed by Ki67 staining in the dentate gyrus and indicate that there is an early and persistent enhancement of hippocampal neurogenesis in Tg-19959 mice versus age-matched wild type mice.

2.2. Aβ Accumulation over Time in Tg-19959 Mice. Tg-19959 mice exhibit detectable Aβ plaques as early as 2 months of age and soluble Aβ peptides are detectable by 4 weeks of age (Figure 3). Five regions of Tg-19959 mouse brain (prefrontal cortex (PFC), hippocampus anterior (HPA), hippocampus posterior (HPP), striatum (ST), and cerebellum (CB)) were isolated at different ages and the levels of Aβ 1–42 and Aβ 1–40 quantified by AlphaLISA. Transgenic mice showed an age-dependent accumulation of soluble Aβ 1–40 and Aβ 1–42 in all regions examined (Figures 3(a) and 3(b)). Western blot analysis of hippocampal lysates from these mice showed an age-dependent accumulation of soluble Aβ aggregates (Figure 3(c)). Immunohistochemistry using 48G antibody, show a time-dependent increase in insoluble deposits of Aβ in hippocampus (Figures 3(d)–3(h)).

We hypothesized that specific soluble oligomeric Aβ aggregates might be involved in enhancement of neurogenesis over time, as we explained in a previous review [30]. Numerous reports have shown the important role of oligomeric Aβ species in AD pathologic processes [31–34]. In our studies, we show that Tg-19959 mice display an age-dependent enrichment of oligomeric Aβ species (Figure 3(c)). Our data show that compared to age-matched wild type mice, neurogenesis is greatly increased in Tg-19959 mice as early as 5 weeks of age, and at all subsequent ages examined. This increase in neurogenesis occurs in very early ages, correlates with detectable levels of Aβ aggregates in

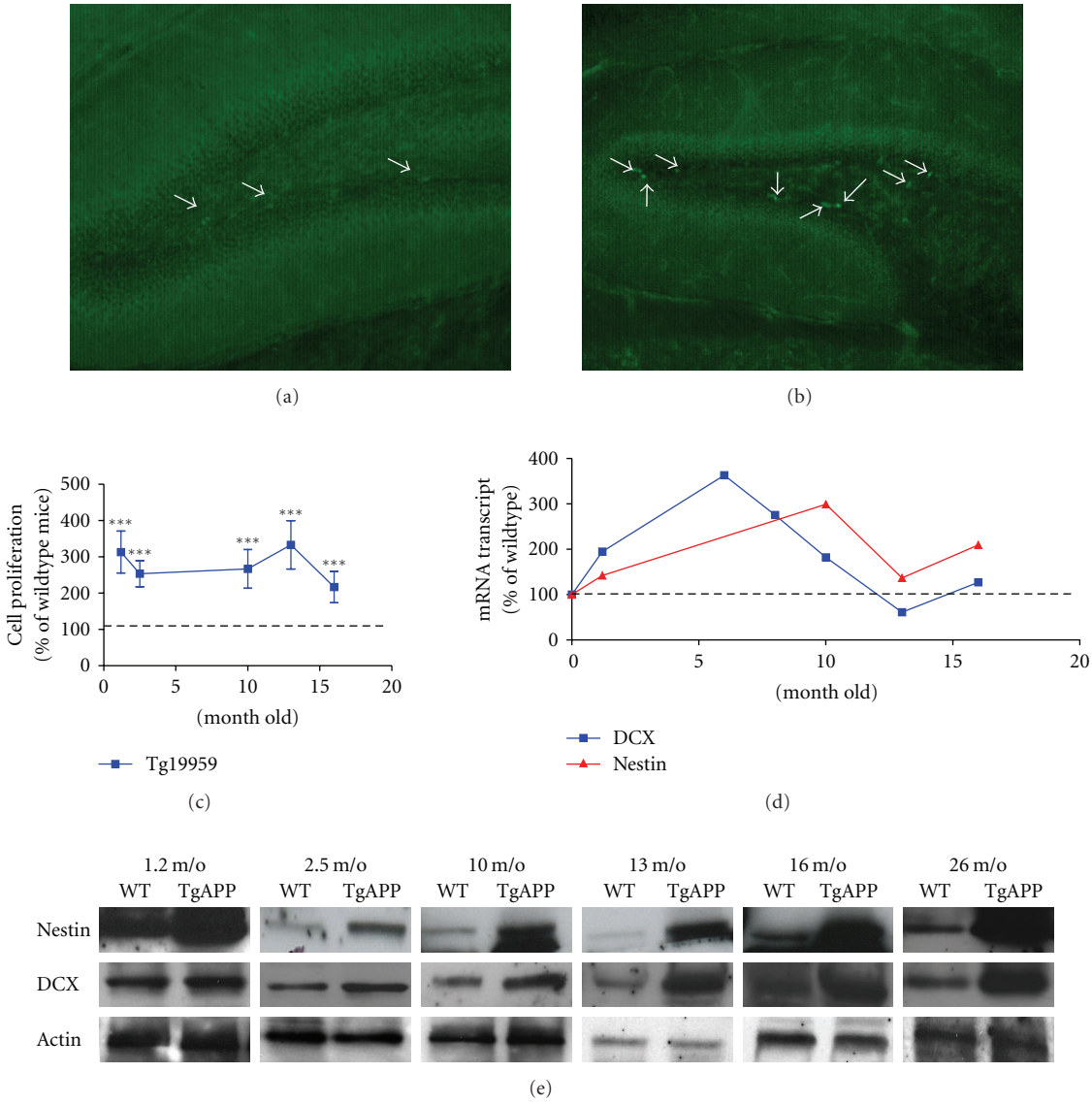


Figure 2: Tg-19959 mice exhibit increased proliferation in the dentate gyrus at all ages. We measured Ki67, a marker of cell proliferation in the dentate gyrus of wild type and Tg-19959 mice at different ages. There is significant increase in NSC proliferation in 5-week-old Tg-19959 mice (b) versus littermates wild type (a) that is maintained over time as animals aged (c). The markers of nestin (neural progenitor marker) and doublecortin (DCX, neuronal progenitor marker) were also significantly increased versus wild type in both mRNA (d) and protein (e) levels in hippocampus at all ages.

the brain, and it precedes any cognitive deficits in the mice, suggesting a relationship between Aβ and neurogenesis. These results also agree with our previous findings, where we described that specific oligomeric Aβ species are able to induce neurogenesis *in vitro* [10].

Consistently with our present results, in J20 APP overexpressing mice, there is a significant NSC proliferation in 3-month-old animals versus wild type animals. However, the neurogenesis rate in J20 mice decreases below wild type levels in older mice [11]. In Tg-19959 mice, we observed that this increase in neurogenesis in younger animals is more persistent and it extends to the aged animals. It has been reported that different aggregation states can play an important role in the effects of Aβ in cell death and/or neurogenesis [10, 32]. In addition, we showed an increased neurogenesis in Tg-19959 mice that is potentially related to enrichment of particular oligomeric assemblies [30]. We hypothesize that the differences in Aβ production and subsequent aggregation between both animal models could explain these differences. For this reason, the possibility to regulate Aβ production by manipulating BACE1 activity could open many possibilities in the neurogenesis-AD field.

2.3. Knock-down of BACE1-AS and BACE1 Transcript In Vitro. The modulation of BACE1 by ncRNAs could be a good tool to modify Aβ production, its further aggregation, and

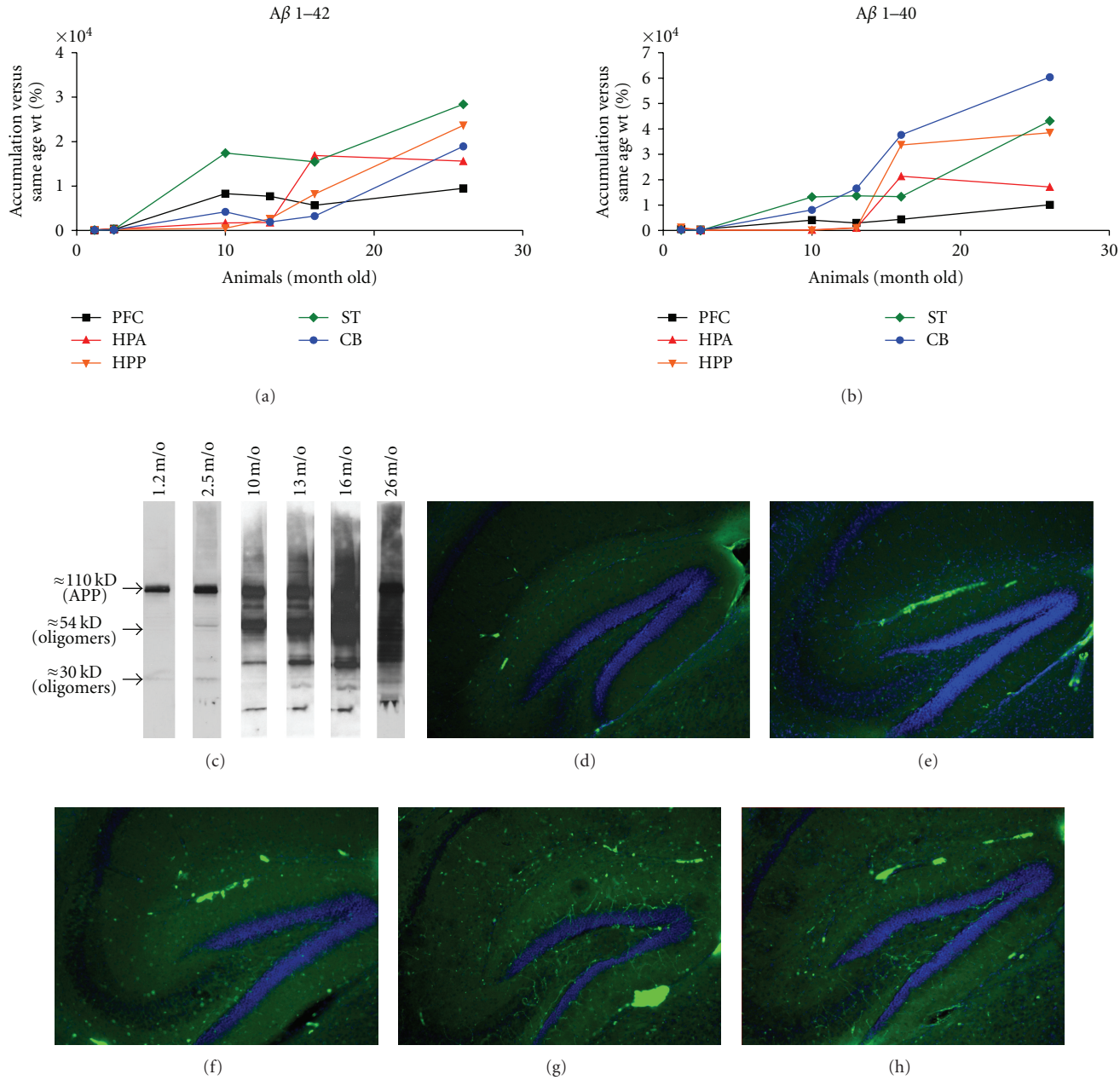


Figure 3: β-Amyloid peptide accumulates with aging in Tg-19959 APP transgenic mice. The level of soluble Aβ 1–42 and Aβ 1–40 amyloid peptides in prefrontal cortex (PFC), hippocampus anterior (HPA), hippocampus posterior (HPP), striatum (ST), and cerebellum (CB) at different ages, compared to wild type controls were quantified by AlphaLISA. Both Aβ 1–42 (a) and Aβ 1–40 (b) were gradually increased as animals aged, in all regions studied. The Western blot of hippocampal samples at different ages using the 48G antibody shows an increase over time in specific aggregation of Aβ (c). Immunohistochemistry with the 4G8 antibody against Aβ at different ages shows insoluble Aβ deposits in Tg-19959 hippocampi as early as in 2-month-old animals. (d): 2 m/o, (e); 6 m/o, (f): 10 m/o, (g): 13 m/o, and (h): 26 m/o. Green: 4G8. Blue: Hoechst.

its effect in hippocampal adult neurogenesis. To assess the regulatory effects of BACE1-AS on BACE1 transcripts, we designed LNA-modified oligos for knockdown of BACE1 and BACE1-AS transcripts. We also designed LNA-modified oligos simultaneously targeting both BACE1 and BACE1-AS transcripts, with equal nucleotide composition on both sides. We learned from previously published work that the two strands of an siRNA duplex are not equally eligible for assembly into RNA-induced silencing complex (RISC) [35]. Asymmetric stability of the 5′ end of an siRNA duplex determines the degree to which each strand participates in the RNAi pathway. Therefore, we designed our siRNA targeting the overlapping region between BACE1 and BACE1-AS transcripts, with symmetric stability at both ends to

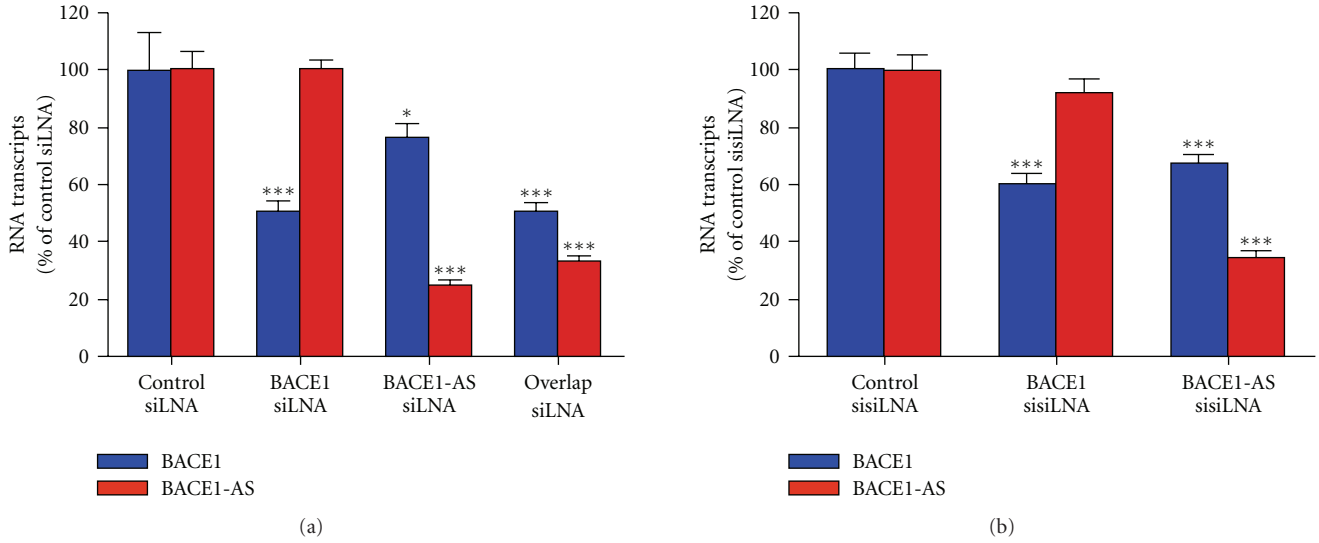


Figure 4: Effects of siLNA and sisiLNA in BACE1 and BACE1-AS RNA levels *in vitro*. To test the ability of siLNAs (a) and sisiLNAs (b) on BACE1 and BACE1-AS, we performed a study in mouse neuroblastoma N2a cells *in vitro*. Selective targets against BACE1-AS transcript were able to significantly knockdown not only BACE1-AS but also BACE1, supporting the proposed concordant regulation of BACE1 mRNA by BACE1-AS.

facilitate incorporation of both strands into RISC, resulting in simultaneous knockdown of both BACE1 and BACE1-AS transcripts. Additionally, we designed small internally segmented interfering RNAs (sisiRNAs) [36] for both BACE1 and BACE1-AS transcripts to be able to assess strand-specific effects of siRNA molecules. We examined LNA-modified oligos in mouse neuroblastoma N2a cells. In line with our previous findings [28], we found that selective targeting of the BACE1-AS transcript resulted in a statistically significant knockdown of not only the targeted BACE1-AS transcript, but also BACE1 mRNA (Figures 4(a) and 4(b)). Overlapping LNA modified siRNA reduced BACE1 and BACE1-AS transcripts, simultaneously. Strand-selective knockdown of BACE1-AS transcript, using sisiRNAs reduced the level of both BACE1 and BACE1-AS transcript (Figure 4(b)). These data confirm the proposed concordant regulation of BACE1 mRNA by non-protein-coding BACE1-AS transcript, in which the antisense transcripts change the level of the sense RNA or the corresponding protein abundance in a positive way.

2.4. Knockdown of BACE1-AS Reduces BACE1 Levels In Vivo. Having already demonstrated a role for BACE1-AS in regulating BACE1 function *in vitro*, we next assessed whether LNA-modified siRNAs show similar regulatory effects *in vivo* in mouse brain. We surgically implanted osmotic minipumps in the third ventricle of Tg-19959 mice and infused LNA-modified siRNA (1 mM) targeted to BACE1, BACE1-AS, the overlapping region between BACE1 and BACE1-AS transcripts, or an inert LNA-modified siRNA, as control (five animals per group). Continuous infusion of LNA modified siRNAs, over 14 days, resulted in reduced BACE1 mRNA levels across forebrain regions located adjacent to the third ventricle (Figure 5). Of the three siRNAs used, the overlapping siRNAs were most potently able to reduce BACE1 mRNA levels (approximately 60% knockdown of BACE1 in each of the brain regions examined), suggesting simultaneous targeting of both transcripts as the most potent therapeutic approach for reduction of BACE1, *in vivo* (Figures 5(a)–5(c)). Similar effect was found in BACE1 protein (Figure 5(d)). Surprisingly, we observed only a minimal knockdown of BACE1 after BACE1 siRNA infusion. This may be due to either a blockage of the osmotic mini-pump tubing, or due to an insufficient dose of siRNA since we have used a relatively low concentration of siRNA for these experiments (1 mM).

2.5. Assessment of Aβ Aggregation Species after BACE1 and BACE1-AS Knockdown. The ability to modulate Aβ production and its further aggregation could open new possibilities for AD therapeutics in relation to neurogenesis. We attempted to investigate whether we could alter Aβ accumulation by using siLNAs to BACE1, BACE1-AS, and the overlap region. Our initial *in vitro* experiments showed that siLNA targeting the BACE1 sense/antisense overlap region gave the greatest knockdown of both BACE1 and BACE1-AS mRNA, supporting our previous publication showing concordant regulation of BACE1 and BACE1-AS (Figure 4) [28]. Following 14 days of *in vivo* treatment with BACE1 sense/antisense overlap siLNA on Tg-19959 mice, we observed knockdown of BACE1 sense and antisense transcripts in all brain regions examined, confirming our *in vitro* data (Figure 5).

In order to determine whether the knockdown of BACE1 or BACE1-AS can alter the accumulation of particular oligomeric aggregates of Aβ, we examined Aβ aggregation in the hippocampus of mice after LNA-modified siRNA treatments. We homogenized mouse hippocampi after 14 days

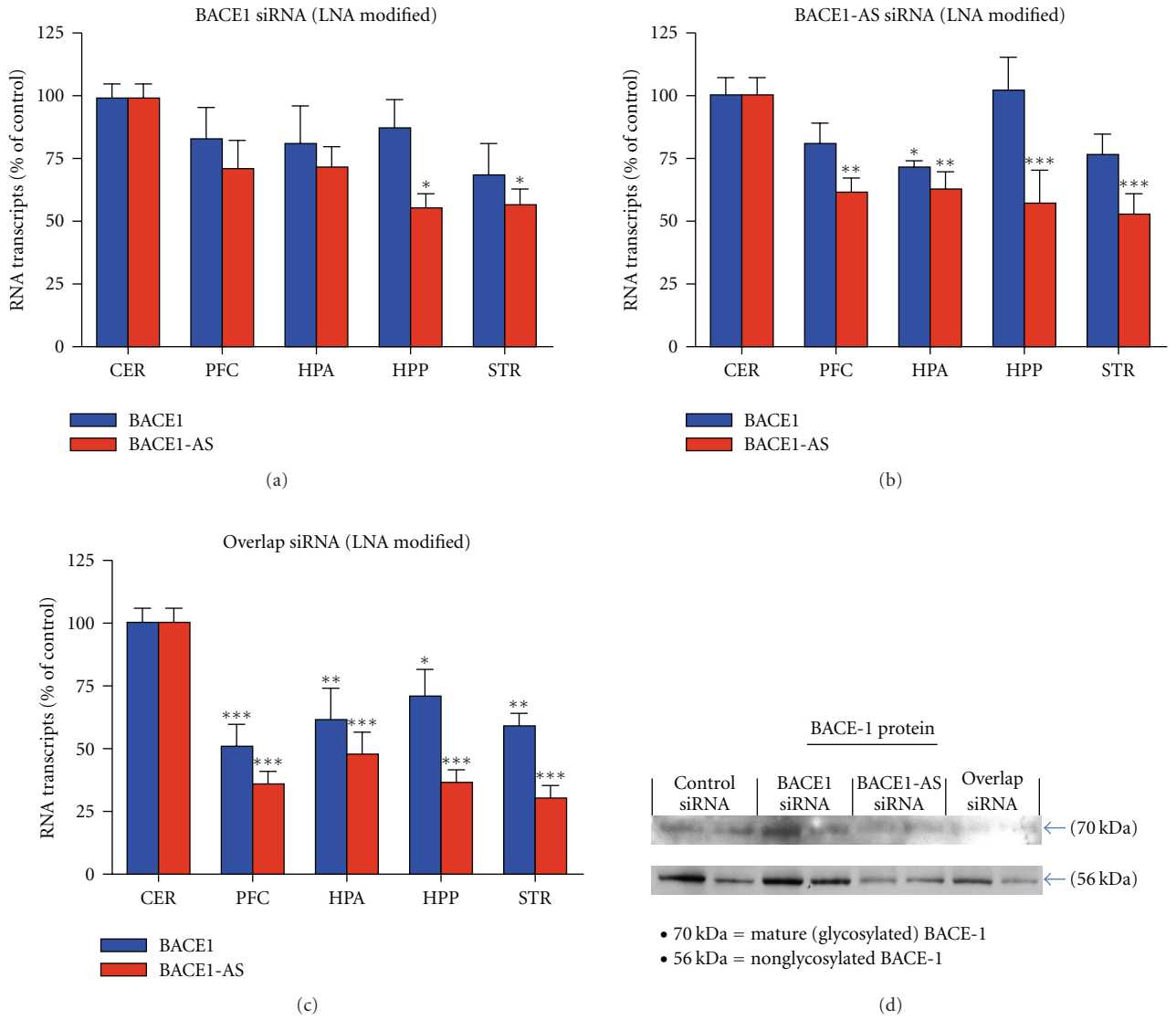


Figure 5: Effects of siLNA in BACE1 and BACE1-AS RNAs levels *in vivo*. To test the ability of siLNAs to affect BACE1 and BACE1-AS *in vivo*, we inserted a minipump as explained with overlap siLNA (a), BACE1-AS siLNA (b), and BACE1 siLNA (c) during two weeks. LNA-modified siRNAs reduced BACE1 mRNA levels in approximately 60% in each of the brain regions examined. In hippocampus, the protein levels of BACE1 were also clearly reduced (d, duplicates).

of LNA-modified siRNA infusion and measured Aβ 1–40 and 1–42, by alphaLISA. We found small in non-significant changes in the total amount of soluble Aβ (Figures 6(a) and 6(b)); however, we observed a significant reduction of insoluble (guanidine extracted) Aβ 1–40 and Aβ 1–42 levels after BACE-1-AS infusion (Figures 6(c) and 6(d)). Separation of Aβ species by Western blot revealed that all three LNA-modified siRNA treatments (BACE1 siLNA, BACE1-AS siLNA, and overlap siLNA) resulted in a significant change in Aβ aggregation pattern in mouse brain (Figure 6(e)). Several reports show the relation and equilibrium between soluble and insoluble levels of Aβ [37]. We did not detect significant changes in the total amount of soluble Aβ with the siRNA treatments; however, we found reduced levels of insoluble Aβ 1–40 and Aβ 1–42 (an indicator of plaque formation) in the brain regions examined (Figures 6(c) and 6(d)). Our data support our hypothesis that the modification of Aβ production is able to change the total amount of Aβ production by changing the pattern of aggregation. We hypothesize that a slower pace in Aβ production determines a slower aggregation that results in less accumulation in insoluble plaques.

Next, we assessed the effects of BACE1 and BACE1-AS reduction on DCX mRNA levels. We measured this marker of neuronal progenitors in the hippocampus after LNA-modified siRNA treatments. We found increased DCX mRNA levels in hippocampus (Figure 7(a)). We next measured the level of DCX protein in these same mice and

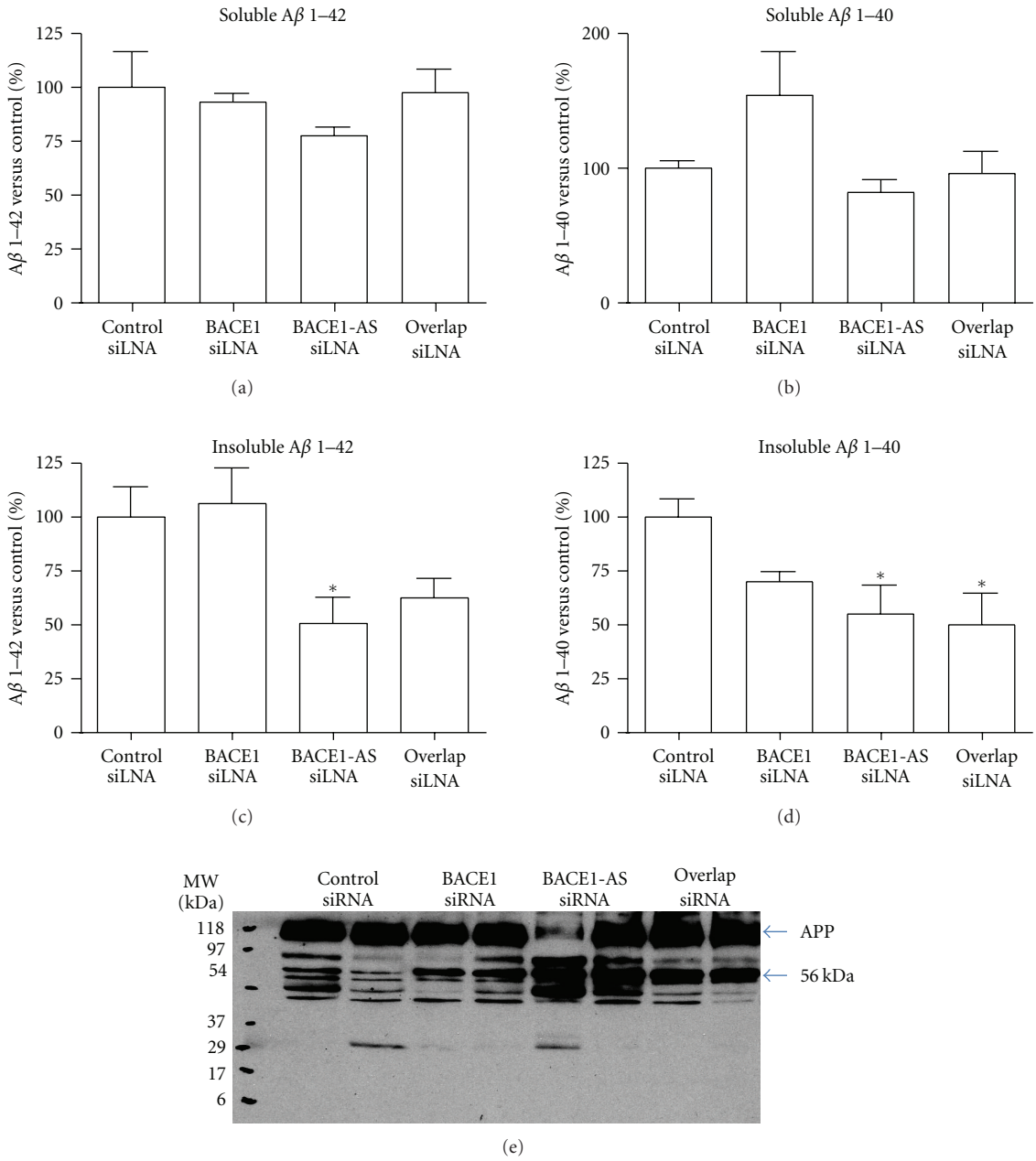


Figure 6: Effects of siLNA in for BACE1, BACE1-AS and overlap region in Aβ formation and aggregation. The infusion of siLNA for two weeks did not change the total amount of soluble Aβ in hippocampus (a), however, it did change the aggregation pattern (b). The measurement of insoluble Aβ (plaque aggregates) by guanidine extraction showed a significant decrease with BACE1-AS treatment (c-d).

observed an enhancement of DCX protein expression (Figure 7(b)). These data provide further evidence that particular Aβ assemblies may be responsible for driving neurogenesis in Tg-19959 mice, consistently with our previous reports in J20 mice [11]. We propose that the alteration of BACE1 protein levels changes the pace of Aβ production and ulterior aggregation state in APP overexpressing mice. This phenomenon would enrich the hippocampus in the soluble oligomers of Aβ responsible for the increased neurogenesis [10], inducing an increase in DCX. The final outcome would be the possibility of modulating adult neurogenesis in the AD brain by controlling BACE1 activity.

It is safe to predict that the observed increase in cell proliferation and neuronal differentiation in these AD animal models should result in an excess of neurons in the hippocampus. However, we did not detect morphological changes in cell number or tissue architecture in these mice. We postulate that there is an increase of newborn neurons in Tg-19959 mice; however, probably due to the lack of trophic factors or not reaching proper targets, most of these

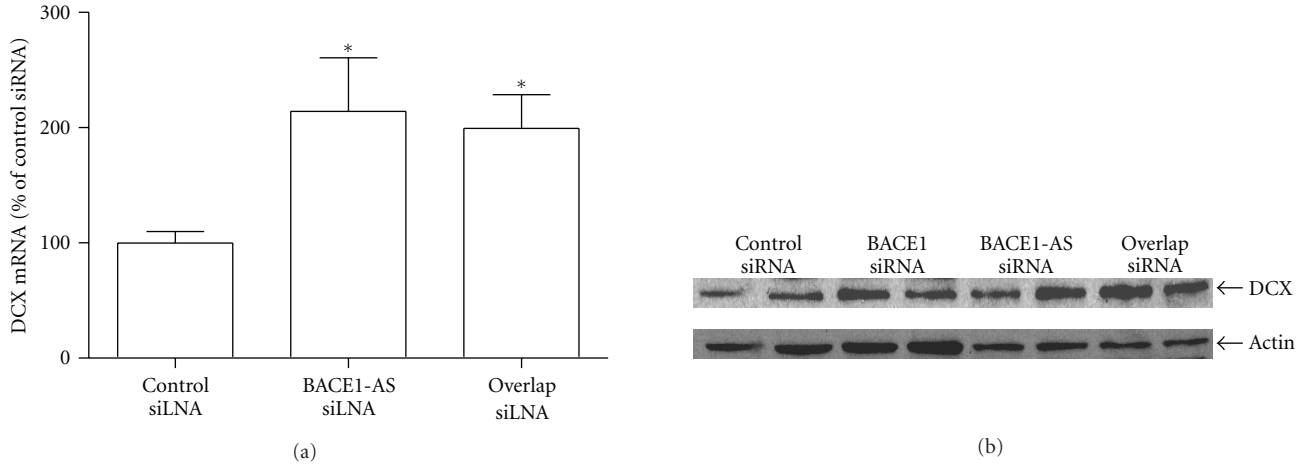


Figure 7: Effects of siLNA for BACE1, BACE1-AS, and overlap region in neurogenesis markers *in vivo*. The infusion of siLNA during two weeks *in vivo* increased the levels of the neuronal progenitor marker doublecortin (DCX) in both the mRNA (a) and protein (b, duplicates) level.

newborn neurons will probably die before reaching maturity. In our previous publication using J20 mice [11] we showed an increased neurogenesis (measured by Ki67 and BrdU) in younger animals and a decrease in older mice. We also describe that most of the newborn neurons died in one month, before reaching full maturity. We also speculate that it is possible that the overstimulation of NSC in early ages could have a negative effect in the NSC pool and neuronal turnover at older ages, contributing to a higher memory decline in older animals, as we suggested in a previous review [30]. The reason why we did not detect an increase in cell death in the mice is because the amount of newborn cells proliferation/death is very low versus the total number of cells, and a moderate increase in cell death could remain undetected. The final outcome of the Aβ-driven neurogenesis would not be a higher number of working neurons with Aβ production, quite the contrary, but we believe that the neurogenic response to Aβ and its manipulation can have a very important diagnostic value, because we could consider it the first symptom of AD, before cognitive impairment, cell death, and plaque formation, and the study of its mechanisms could be very useful in the development of new approaches in AD diagnostic and prevention.

3. Materials and Methods

3.1. Animal Surgeries. siLNAs were designed for mouse Bace1, Bace1-antisense, and overlapping region between two transcripts.

We selected 20 mice 8–12 weeks old, 5 animals per group for surgeries. We inserted cannula into the lateral ventricle of mice brain and connected the exit port to an Alzet micro osmotic pump model 1002. We prepared 100 nM of each siLNA, filled the micro pump, and infused it to the lateral ventricle of mouse brain, at rate of 0.25 μL per hour over a period of two weeks. Animals were sacrificed afterward and brain was extracted. Five brain regions were separated from each animal including cerebellum, prefrontal cortex, striatum, ventral hippocampus, and dorsal hippocampus. RNAs were isolated by TRIzol, and passed through RNeasy minicolumn (Qiagen) for on-column DNAse treatment. We made cDNA, using 400 ng of total RNA and random hexamers, followed by realtime PCR to assess the expression levels of target RNAs. Relative quantities were measured by normalizing ct values to β-Actin ct value.

3.2. Animal Studies. Experiments were performed following National Institute of Health guidelines and approved by The Animal Care and Use Committee (IACUC) at The Scripps Research Institute, Scripps, Florida. We are grateful to Dr. Leissring (Mayo Clinic) for providing APP transgenic (Tg-19959) mice, a model of AD that overexpresses a doubly mutated human APP [28]. We used Tg-19959 plus age- and sex-matched wild type littermates, at different ages for the *in vivo* experiments. The mice were sacrificed in CO_2 chamber followed by decapitation, and the brains were removed. Five tissues were dissected from each hemibrain; dorsal hippocampus, ventral hippocampus, cortex, dorsal striatum, and cerebellum and the other hemibrain were dissected for RNA and protein studies. The first hemibrain was fixed by immersion in 4% paraformaldehyde for 24 hours and washed several times in PBS for histology studies.

3.3. RNA Extraction and RT-PCR of the Mouse Brain Samples. We extracted RNA from dorsal hippocampus, ventral hippocampus, cortex, dorsal striatum, and cerebellum from each mouse brain, using Trizol reagent (Invitrogen), according to the manufacturer's protocol. RNA samples were passed through Qiagen RNeasy columns and were subjected to on-column DNAse treatment for removal of DNA contamination. We used 800 ng of each RNA sample for the first strand cDNA synthesis and carried out RT-PCR measurements. We plotted the percentile changes in RNA levels, for individual tissues compared to control mice, in each graph.

3.4. Realtime PCR (RT-PCR). We carried out RT-PCR with the GeneAmp 7900 machine (Applied Biosystems). The PCR reactions contained 20–40 ng cDNA, Universal Mastermix, 300 nM of forward and reverse primers, and 200 nM of probe in a final reaction volume of 15 μL. We designed the primers and probe using FileBuilder software (Applied Biosystems). They were strand-specific for sense-antisense pairs and the sense probes covered exon boundaries to eliminate the chance of genomic DNA amplification. The PCR conditions for all genes were as follows: 50°C for 2 min then 95°C for 10 min then 40 cycles of 95°C for 15 s and 60°C for 1 min. The results are based on cycle threshold (Ct) values. We calculated differences between the Ct values for experimental and reference genes (18 s RNA) as ΔΔCt and graphed as a percent of each RNA to the calibrator sample.

3.5. Western Blotting and ELISA. Brain tissues were homogenized in Tissue Extraction Reagent I (Biosource) containing protease inhibitor cocktail complete Mini (Roche) by 20 up-and-down passes in a glass Dounce homogenizer. Samples were centrifuged at 14,000 rpm for 30 min. Supernatants were used for ELISA quantification using an Aβ 1–40 and 1–42 AlphaLISA amyloid kit (PerkinElmer) and quantified with ENVISION 2104 Multilabel reader (PerkinElmer). Same supernatants were used for Western blot detection using mouse 4G8 antibody anti-Aβ (Signet), mouse Rat-401 anti-nestin (Developmental Studies Hybridoma Bank), and Rabbit anti-doublecortin (cell signaling).

3.6. Guanidine Extraction of Insoluble A-Beta Species. 200 μL of 6.25 M Guanidine-HCl (Sigma-Aldrich, MO) in 50 mM Tris at pH 8 was added to the pellet. The mixture was vortexed vigorously for 5 minutes until the pellet was fully dissolved. The solution was incubated overnight at room temperature. The following day, the mixture was centrifuged at 14,000 g for 10 minutes, and the supernatant collected into fresh tubes and labeled as Guanidine extracted (insoluble) fraction. Protein quantification and Aβ ELISA was performed as for the soluble fraction.

3.7. Immunohistochemistry. One hemibrain was fixed in phosphate-buffered 4% paraformaldehyde, pH 7.4, at 4°C for 24 hr, washed in phosphate buffer three times and embedded in 3% agarose for vibratome sectioning (Micron HM 650 V. Thermo Scientific). 50 μm sections of hemibrain were made in the sagittal axis. Sections were permeabilized in PBS with 1% TX-100 and maintained in this solution with 10% FCS with primary antibodies for two days with agitation. As a negative control, adjacent brain slices were similarly processed, except that the primary antibody was omitted. Rabbit anti Ki67 (Vector) and mouse 4G8 antibody anti-Aβ (Signet). Immunofluorescence secondary goat anti mouse or goat anti-rabbit Alexa Fluor 568 and 488 antibodies were used for detection.

4. Conclusions

We examined the pattern of neurogenesis in a transgenic mouse model of AD (Tg-19959) and found increased neurogenesis compared to age-matched wild type mice at all ages examined. This increase in neurogenesis occurs very early in the development of the disease, and is paralleled by an accumulation of Aβ oligomeric species. LNA-modified siRNAs targeting BACE1, BACE1-AS, or both transcripts reduced BACE1 at the mRNA and protein level, reduced the levels of insoluble Aβ peptides, and changed the aggregation pattern of soluble Aβ in the brain of Tg-19959 mice. Our data suggest that the increased neurogenesis observed in a mouse model of AD parallels the accumulation of oligomeric Aβ species and that we can manipulate it by using with LNA-modified siRNAs against BACE1 and BACE1-AS. We believe that the study of Aβ-driven neurogenesis could be a powerful tool in early detection of AD, and the use of ncRNAs could help to manipulate the development of the disease before the appearance of adverse symptoms.

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